NUCLEASES AND RELATED ENZYMES

IN

HUMAN TISSUES AND BODY FLUIDS

by

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ABBREVIATIONS

The following abbreviations and symbols will be used. An explanation of the nomenclature employed for designation of nucleotide structure will be provided in Figure 1.

RNA: Ribonucleic Acid.

DNA: Deoxyribonucleic Acid.

DNA-P: Deoxyribonucleic Acid Phosphorus.

RNAase: Ribonuclease. The terms "alk. RNAase" and "acid RNAase" will be used to denote ribonuclease activity measured at alkaline and at acid pH values ; the presumption is made that these activities are due to enzymes with pH optima in the alkaline and acid ranges, but this presumption may not necessarily be true.

Pu : Purine residue .

Py : Pyrimidine residue.

EDTA: Ethylenediaminetetra-acetic acid, disodium salt.

Km : Michaelis Constant.

DAB : Dimethylaminoazobenzene.

X-radiation: Gamma-radiation.

r : Rads.

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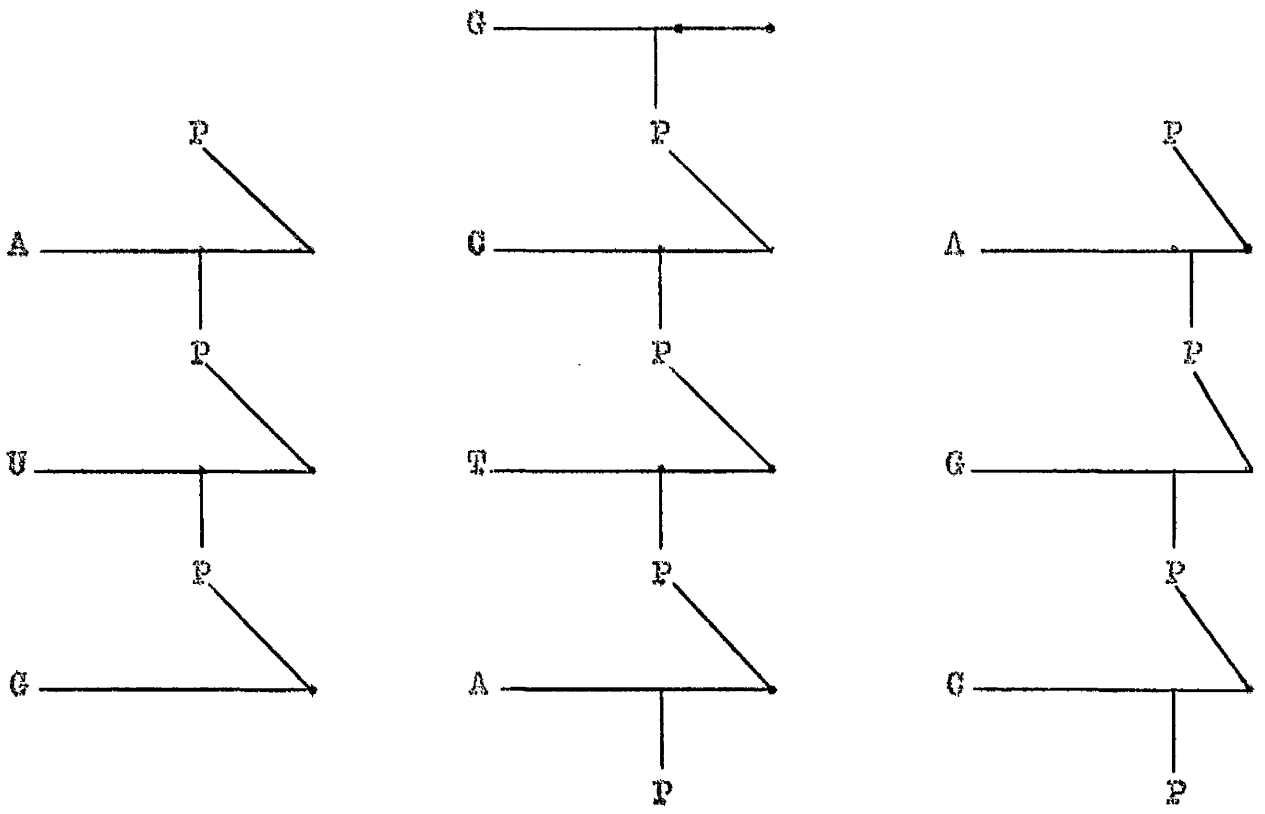
INTRODUCTION

The enzymic degradation of nucleic acids, in all biological systems in which this phenomenon has been so far demonstrated, commences with cleavage of the phosphoric ester linkage by means of which adjacent nucleotides are joined to each other in the polynucleotide chain (Schmidt, 1955). A host of enzymes capable of carrying out this step have been identified in living organisms. Many of these have been purified to a point where it is possible to make definite statements regarding their mode of behaviour and their mechanism of action. From these studies, it has become clear that these enzymes - to which the generic names "nucleases" and "nucleodepolymerases" have been given - display considerable variety; yet a sufficient number of properties are shared by a large enough number of enzymes to make classification into a number of different groups worth while.

The system most widely accepted was first suggested by Laskowski (1959), who considered four cardinal criteria as the basis for classification. These were

1) The nature of the substrate specificity.
2) The type of attack employed, by which he distinguished between those enzymes acting sequentially from one end of the nucleotide chain (exonucleases) and those acting in a manner that is random with respect to position within the chain (endonucleases).
3) The position of the monoesterified phosphoryl radical in the products formed .
4) The nature of any preference shown by the enzyme towards specific



$pApUpG$ $GpCpTpAp$ $pApGpCp$

A phosphate group is denoted by the letter P. When placed to the right of the nucleoside symbol, the phosphate is esterified at C_3' of the ribose moiety. When placed to the left of the nucleoside symbol, the phosphate is esterified at C_5' of the ribose moiety. The letters A, G, C, U, and T represent adenosine, guanosine, cytosine, uridine and thymidine respectively. The prefix d (e.g. dA) may be used to indicate a deoxyribonucleoside.

FIGURE 1

An Explanation of the Abbreviations Used to Indicate the Structure of Nucleotides (Davidson, 1960).

nucleoside phosphoryl groups and the 5'-hydroxyl groups of the adjacent purine or pyrimidine nucleotide (Schmidt, Cubiles, Zollner, Hecht, Strickler, Seraidarian, Seraidarian and Thannhauser, 1951). More recently, 2 enzymes were isolated from takadiastase which exhibit yet narrower specificities. One will hydrolyse only those links between the 3'- phosphoryl group of guanosine and adjacent residues, while the action of the other is restricted to the bond between the 3'- phosphoryl group of adenosine and adjacent residues (Sato-Asano and Egami, 1960).

Earlier statements regarding the preferential hydrolysis by pancreatic DNAase of the linkage between the 5'-pyrimidine nucleoside phosphoryl group and the 3'- hydroxyl group of the adjacent purine residue ($d\text{-}_{p}Pu\text{-}_{p}Py$)* made by Laskowski (1961) had to be abandoned when further work demonstrated that this was not so; moreover, it became clear that the preference of the enzyme for certain linkages changed in the course of digestion (Vanecko and Laskowski, 1962).

In what follows, a survey of nucleases will be presented. More prominence will be given to those of animal origin. In addition to their characteristics envisaged in the scheme of Laskowski (1959), information on their behaviour with respect to certain ions will be given, studies on their intracellular location will be summarised, the existence of specific inhibitors will be indicated, and current knowledge of their biological function will be presented.

* See Figure 1.

RIBONUCLEASES

PANCREATIC RIBONUCLEASE

/Polyribonucleotide 2-oligonucleotidotransferase (cyclizing)/ EC 2.7.7.16

Although the presence of a heat-stable factor in pancreatic extracts capable of degrading RNA had been recognised since the beginning of this century, it was with the investigations of Dubos and Thompson (1938) that its enzymic nature was conclusively established. With the crystallisation of the enzyme by ammonium sulphate fractionation (Kunitz, 1940), systematic examination of the chemistry of the enzyme protein and the nature of the catalytic reaction became possible. The material so obtained had a molecular weight calculated from osmotic pressure data to be in the region of 15,000. Fractionation of the crystallised enzyme by column chromatography revealed the presence of two components, called RNAase A and RNAase B (Hirs, Moore and Stein, 1953). The concerted efforts of several laboratories culminated in the presentation of the complete amino acid sequence of bovine RNAase A (Hirs, 1960; Hirs, Moore and Stein, 1960), while the establishment of the precise situation of disulphide bonds in the molecule permitted its two-dimensional structure to be visualised (Spackman, Stein and Moore, 1960). Analysis of sheep pancrease RNAase has revealed differences in 3 of the amino-acid residues compared with those of the bovine enzyme, so that the iso-electric point of the former is at a higher pH value, but none of these differences concern residues implicated in the catalytic mechanism (Anfinsen, Aqvist, Cooke and Jonsson, 1959). No antigenic

differences between RNAase A and RNAase B could be detected in earlier work (Ginader and Pearce, 1956), and more recently it has been shown that the amino acid composition of the two are identical, but the latter has a carbohydrate moiety containing glucosamine and mannose associated with part of the molecule involving an asparagine-leucine dipeptide sequence (Plummer and Hirs, 1964). Reports that these two major components of crystalline pancreatic RNAase had different substrate specificities (Hakim, 1957a; Hakim, 1957b) have not been confirmed (Dekker, 1960). Knowledge of the covalent structure of RNAase A has permitted the elucidation of those residues essential for enzymatic activity, and therefore concerned either in the catalytic process itself, in the binding of substrate, or in the maintenance of the correct configuration at the active centre (White and Anfinsen, 1959; Scherage and Rupley, 1962; Richards, 1964). The available evidence strongly suggests the participation of histidine residues at positions 12 and 119 in the catalytic process; lysine at position 41 and glutamine at position 11 are also important in this process though they may not be directly involved; four methionyl residues, and 3 of the 6 tyrosine residues participate in intramolecular binding essential for maintaining the shape of the protein in an enzymatically active form.

Mechanism of Action

It has now been fully established that RNAase catalyzes the cleavage of only those internucleotide bonds of RNA involving a 3'-pyrimidine nucleoside phosphoryl group and the 5'-hydroxy group of the adjacent purine or pyrimidine nucleotide group (Schmidt et al., 1951; Schmidt,

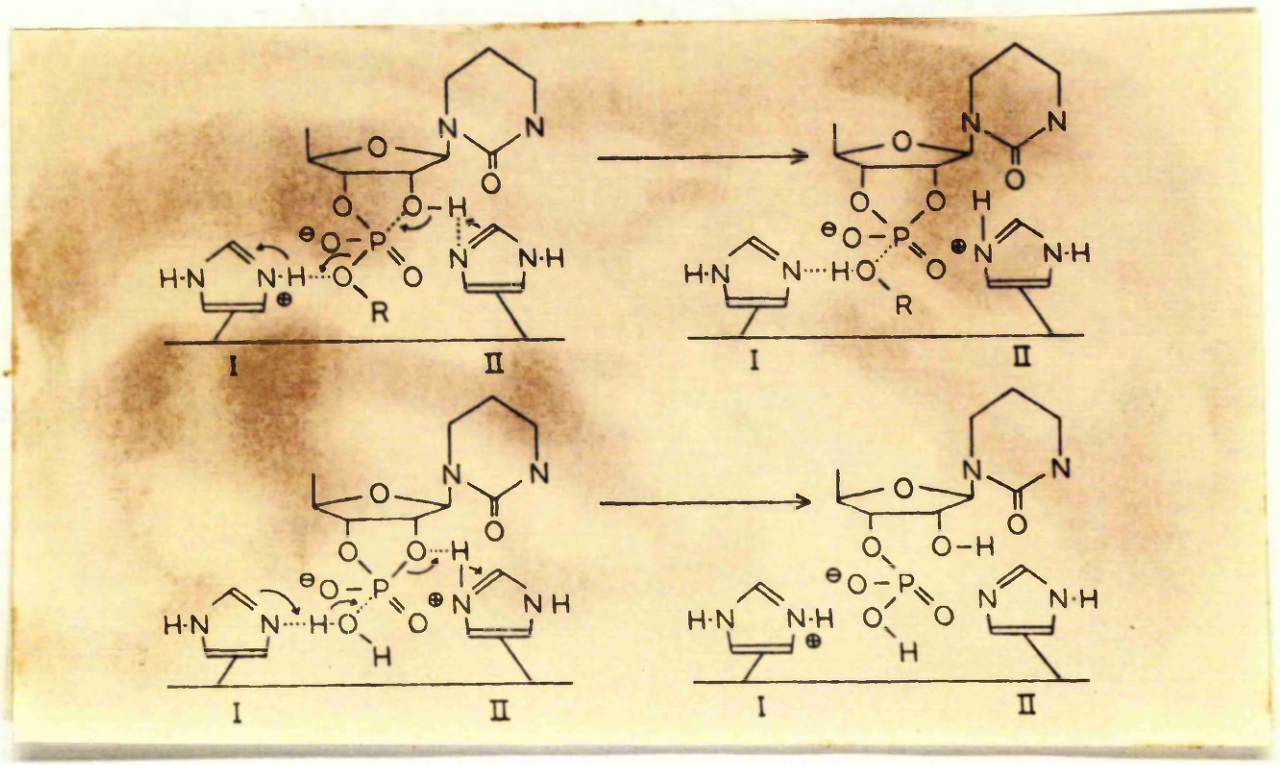


FIGURE 2

The Mechanism of Action of Ribonuclease According to Rabin and his Colleagues: For Explanation See Page 6

(Diagram from Hummel & Kalnitsky, 1964).

1955; Schmidt and Laskowski, 1961; Anfinsen and White, 1961). The reaction is biphasic involving the intermediary formation of oligonucleotides terminated by 2', 3'-cyclic pyrimidine nucleotide residues, and free cyclic pyrimidine nucleotides. The second and slower step involves hydrolysis of the cyclic diester at the 2'-phosphoryl ester linkage with formation of a 3'-nucleotide group* or free nucleotide. The action of the enzyme is not limited to degradation of its substrate. Under appropriate conditions, exchange reactions leading to synthesis of simple esters of mononucleotides (Heppel and Whitfeld, 1955) and even of polynucleotides (Heppel, Whitfeld and Markham) can be demonstrated. Cyclic pyrimidine nucleotides are better participants than nucleoside 3'-phosphates in these reactions, and the synthesis of linkages is restricted to those which the enzyme is capable of hydrolysing.

1955

Various suggestions have been put forward to explain this remarkable ability of a single enzyme to catalyze a transphosphorylation reaction followed by a hydrolysis. Those which have received the most attention have come from Rabin and his colleagues (Findlay, Herries, Mathias, Rabin and Ross, 1962; Mathias, Deavin and Rabin, 1964) and from Witzel (Witzel and Barnard, 1962a, 1962b; Witzel, 1963). According to the former workers, whose studies are largely based upon the hydrolysis of cytidine 2', 3'-cyclic phosphate, one of the imidazole groups of histidine at the active site of the enzyme binds the attacking hydroxylic compound (water or an alcohol); the imidazole group of another histidine residue forms a hydrogen-bond with the oxygen attached to C_2 of ribose, thus weakening its attachment to the phosphorus atom and favouring heterolytic fission. Two other binding sites in the enzyme protein are postulated by these

* end-group

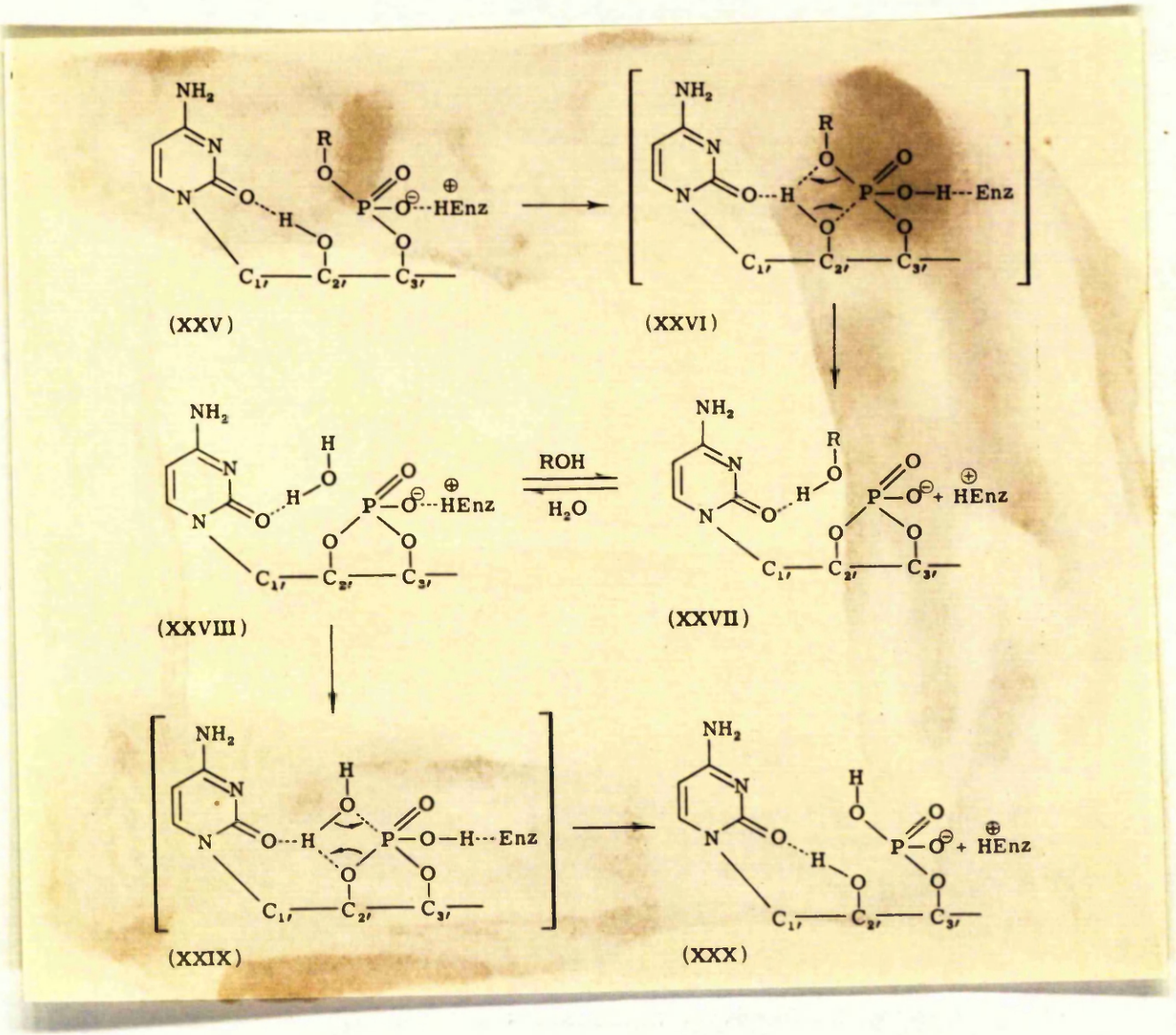


FIGURE 3

The Mechanism of Action of Ribonuclease According to Witzel.

For Explanation See Page 7

(Diagram from Witzel, 1963.)

workers: the first, adjacent to the first histidine serves to bind complex alcohols; the second, adjacent to the other histidine binds the pyridine group. These relationships are shown in Figure 2.

The mechanism proposed by Witzel is quite different. It is based upon the requirement for a pyrimidine group at the C3'-attachment of the internucleotide phosphoryl link and is supported by much experimental work involving rate of enzyme action upon substituted pyrimidine derivatives. In this scheme, which accounts for both transphosphorylation and hydrolysis, seven stages are described, but the essence of the proposal comprises protonation of the phosphate by the enzyme so that it is attacked by the 2'-oxygen of ribose whose proton is transferred to the pyrimidine ring causing a split of the R-O-P linkage and formation of the cyclic intermediate.* In the next stage, the split alcohol which at the end of the previous stage was hydrogen-bonded to the pyrimidine base, exchenges with a water molecule. A renewed protonation initiated by the enzyme enables the activated water molecule to attack the phosphorus atom, while the pyrimidine base transfers the proton to the 2'-oxygen of ribose, causing change of the bonds in the reverse direction, leading to formation of the 3'-monoester as the final product. While neither mechanism is able to meet all the known facts, that of Witzel is favoured by most reviewers (Scheraga and Rupley, 1962; Hummel and Kalnitsky, 1964). One of the main difficulties to be taken account of is the ability of RNAase in very large amounts to carry out the hydrolysis of artificial substrates devoid of pyrimidine groups such as riboapyramidinic acid (Takemura, Takagi, Mujazaki and Egami, 1959), polyadenylic and polyinosinic acids (Beers, 1960), and polyribosephosphate (Rosenberg and Zamenhof, 1961).

* See Figure 3.

As might be expected, for reasons which are well summarised by Hummel and Kalnitsky (1964), the kinetics of the first stage of the reaction are highly complex and the variety of methods employed for measuring enzyme activity based upon depolymerisation of RNA bear witness to the difficulty of obtaining precise measurements of this process. It had been shown by Kunitz (1940) that the ultimate amount of digestion is independent of the amount of enzyme used, and that the rate of formation of titratable acid groups due to liberation of free phosphoryl groups is much slower than the rate of formation of acid-soluble split products. As outlined by McDonald (1955), the reaction is optimal at pH values between 7.2-8.2, proceeds best in the presence of 0.1M-NaCl or $MgCl_2$, and is maximal at 60°. The variation in optimal pH stems from the fact that it is greatly influenced by the ionic composition of the medium - pH optimum and ionic requirements are in fact mutually dependent (Kalnitsky, Hummel, Resnick, Carter, Barnet and Dierks, 1959) Hydrolysis of cyclic cytidylic acid has been employed as a method for measuring enzyme activity based upon the second stage. The initial velocity of the reaction is proportional to enzyme concentration over a fairly wide range (Crook, Mathias and Rabin, 1960), but as an assay proceedure it is much less sensitive than those measuring the depolymerisation of RNA (Stockx, Vandendriessche and Van Parijs, 1960) since cleavage of the cyclic phosphate links requires several hundred-fold as much active enzyme as is required for cleavage of the internucleotide links in the same time (Heppel, Ortiz and Ochoa, 1957).

Heterogeneity of Pancreatic RNAase/.

Heterogeneity of Pancreatic RNAase

The complex nature of RNAase poses an intriguing problem, and the relationship between various fractions identified by column chromatography has received much attention. It is possible to separate no fewer than 8 peaks of activity from extracts of sheep pancreas (Aqvist and Anfinsen, 1959), and the same authors detected 4 peaks in commercial preparations of crystalline bovine RNAase. Three peaks were obtained in neutral homogenates of mouse pancreas, one of which was confined to the supernatant fraction and disappeared on treatment of the homogenate with dilute sulphuric acid (Dickman and Morrill, 1959); bovine pancreas yielded the same 3 peaks, the pH optima of which were identical (Dickman, Morrill and Trupin, 1960); but again only two peaks are present in acid homogenates. Human pancreas, which contains 0.5% of the RNAase activity of bovine pancreas per unit weight, also yields two peaks from acid homogenates; one has been purified 500-fold and shown to have the same substrate specificity as RNAase A (Ukita, Takahashi, Waku and Hoshino, 1964). Three components are present in guinea-pig pancreas (Bartos and Uziel, 1961), though one of the components appears to be unrelated to those of calf pancreas. The microsomes of mouse and guinea-pig contain RNAase bound to ribonucleoprotein granules which becomes soluble in the presence of agents capable of complexing with Mg^{++}, and may represent newly-synthesised enzyme (Dickman and Trupin, 1958; Siekevitz and Palade, 1960). Studies on the biosynthesis of the enzyme do in fact lend strong support to the view that it is synthesised by the ribosomes, transferred to microsomal cisternae, and stored in zymogen granules

prior to secretion (Morris and Dickman, 1960; De Duve, Wattiaux and Baudhuin, 1962), and it is probable that the heterogeneity displayed by the enzyme reflects small molecular changes of the protein or binding of additional compounds at each site.

Activation and Inhibition

To a large extent this question is complicated by the fact that agents may affect different substrates, different assay methods, and different stages of the reaction in diverse manners. Mg^{++} ions activate beef pancreas RNAase when RNA is the substrate, but beyond a certain concentration they render oligonucleotides more readily precipitated by acid, so that when assay is based upon measurement of acid-soluble products pseudo-inhibition is found (Dickman, Aroskar and Kropf, 1956); they are inhibitory when ribonucleoprotein is used as substrate (Shigeura and Chargaff, 1960). Yeast RNAase is activated by increasing ionic strength (Brownhill, Jones and Stacey, 1959), and various pyridine derivatives strongly activate crystalline pancreatic RNAase (Jokay and Toth, 1964). Zn^{++} ions are inhibitory under every experimental situation studied (Houck, 1957; Brownhill et al., 1959; Shigeura and Chargaff, 1960). Heparin is inhibitory when RNAase activity is measured turbidimetrically (Houck, 1957), but it does not appear to affect the initial depolymerisation stage of RNA degradation as measured dilatometrically, its effect being confined to the later stage associated with splitting of cyclic diphosphate links (Vandendriessche, 1956); indeed this property has been utilised for the assay of heparin based upon its

inhibition of hydrolysis of cyclic pyrimidine nucleoside phosphates by RNAase (Hobom and Zollner, 1964). In vivo injection of heparin causes an initial inhibition followed by an increase in activity of acid and alk. RNAase* of mouse liver (de Lamirande, Weber and Cantero, 1956). Pancreatic RNAase is inhibited by various sulphated anionic polymers (Fellig and Wiley, 1959).

A factor present in the supernatant fraction of various rat tissues and inhibitory towards crystalline pancreatic and rat liver alk. RNAase but not towards rat liver acid RNAase was first described by Roth (1956). It is very labile to heat, and can be inactivated reversibly by Pb^{++} ions and irreversibly by p-chloromercuribenzoate (Roth, Bachmurski and Inglis, 1958a), and acts by binding with active enzyme (Roth, Bachmurski and Inglis, 1958b). It has since been purified 6000-fold from rat liver (Shortman, 1961) and shown to be a protein, possibly containing carbohydrate (Shortman, 1962a). Metal ions present in most commercial samples of RNA inactivate the inhibitor thus increasing the activity of bound RNAase (Shortman, 1961; Wojnar and Roth, 1964). Similar factors have been identified in the livers of 5 mammals; in general, they are not species specific, but are inhibitory towards crystalline pancreatic RNAase and alk. RNAase of all mammalian mitochondria (Roth, 1962). During regeneration of rat liver, the concentration of inhibitor increases to reach a maximum of 139% of the initial level at 48 hrs (Shortman, 1962b); this increase precedes that of RNA and of acid RNAase and may be one of the earliest responses to hepatectomy.

* See "Abbreviations" for explanation of these terms.

OTHER RIBONUCLEASES

Liver Ribonucleases

It was simultaneously discovered by Roth (1954) and by de Lamirande, Allard, da Costa and Cantero (1954) that rat liver homogenates show two peaks of activity: an acid peak at pH 5.8-6.0 and an alkaline peak at pH 7.8-8.2. These peaks were shown to be due to at least two distinct enzymes differing in respect of heat stability and response to inhibitory factors, though they were distributed in a similar manner between the various components of the cell, mitochondria containing the highest activity of both, although significant alk. RNAase activity was associated with uncontaminated nuclear preparations. These observations were confirmed with partially purified extracts (Roth, Inglis and Bachmurski, 1957; Zytko, de Lamirande, Allard and Cantero, 1958), although it had earlier been reported that the acid RNAase was primarily associated with lysosomes in this tissue (De Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955). Work in several laboratories has shown that the alkaline enzyme has the same specificity as pancreatic RNAase in so far as all free nucleotides and end-groups of oligonucleotides are cyclic 2',3'-pyrimidine nucleoside phosphates, whereas the acid enzyme is indiscriminate in the links it hydrolyses (de Lamirande and Allard, 1959; Reid and Nodes, 1959; Roth, 1959). There is some uncertainty however regarding the further action of these enzymes. De Lamirande and Allard (1959) and Reid and Nodes (1959) found that alk. RNAase would not hydrolyse cyclic nucleoside phosphates, while Roth (1959) is in agreement so far as the supernatant enzyme is concerned but claimed that the

mitochondrial alk. RNAase hydrolyses cyclic uridine 2'3'-phosphate. It has been claimed that the cyclic nucleoside phosphates of adenosine and uridine are split by the acid RNAase to 2'-phosphates (Zytko et al., 1958; Reid and Nodes, 1959) while cyclic cytidine phosphate is hydrolysed to the 3'-derivative (Reid and Nodes, 1959). Further purification of these enzymes has shown that the production of 2'-nucleoside phosphates is the work of a contaminating enzyme, and has confirmed the specificity of the two enzymes previously reported (Mayer and Greco, 1962).

An interesting RNAase has been identified by ammonium sulphate fractionation of guinea-pig liver (Heppel, Ortiz and Ochoa, 1956) and purified to a point where it is free of all phosphodiesterase and monoesterase activity and is only slightly contaminated by DNAase (Razzell, 1963). The enzyme is active towards polyadenylic and polyuridylic acids as well as native RNA, and it degrades all three substrates to fragments containing 2-6 nucleotide residues. Unlike the products of any other specific RNAase yet discovered, the products are all terminated by free 5'-phosphate groups.

The suggestion of Roth (1960) that rat liver microsomes might possess intrinsic RNAase activity has been confirmed by the demonstration of a RNAase in these particles which is inhibited by Mg^{++} ions and stimulated by EDTA (Morais and de Lamirande, 1965), while evidence has been presented favouring the presence in rat liver cell sap of an acid RNAase which is not released from damaged lysosomes (Reid and Nodes, 1963).

Liver RNAase activity is sensitive to hormonal and dietary stimuli. After adrenalectomy, and after hypophysectomy whether or not growth-hormone was subsequently administered, acid RNAase activity of rat liver

supernatant and whole cytoplasm showed an elevation which occurred much later than changes in other cellular constituents, whereas alk. RNAase activity was unchanged (Stevens and Reid, 1956; Reid and Stevens, 1958). Intense treatment with thyroxine causes a small increase in acid RNAase of rat liver particulate fractions (Reid, 1960). Cortisone administration was without effect, but fasting reduced the activity of rat liver alk. and acid RNAase by 20% (de Lamirande and Allard, 1959a). On the other hand activity was increased by protein-depletion alone (Zigman and Allison, 1959).

Spleen Ribonucleases

An enzyme from calf spleen was purified 700-fold and shown to have the same specificity as pancreatic RNAase, including the ability to catalyse exchange reactions (Kaplan and Heppel, 1956). Chromatography of calf spleen preparations yielded 8 peaks of activity, four associated with activity optimal at alkaline pH values and producing a mixture of 2'- and 3'-nucleoside phosphates as end products, the other 4 associated with activity optimal at pH 5.6-5.8 and producing 2'-nucleoside phosphates only (Maver, Peterson, Sober and Greco, 1959). Further purification of these enzymes has been achieved (Maver and Greco, 1962), and it has been shown that the production of 2'-nucleoside phosphates is due to a contaminant; the ratio of adenine to cytosine nucleotides in digests of the acid RNAase was 4:1 whereas for the alk. RNAase this ratio was 1:7. In rat spleen on the other hand, RNAase activity shows an optimum at pH 5.8, the evidence for an enzyme with more alkaline pH requirements being inconclusive (Maver and Greco, 1956; Eichel and Roth, 1962).

It is concentrated in the mitochondria, but significant quantities also occur in the supernatant. Feeding alters the distribution of the enzyme, a higher percentage of the total residing in the mitochondria, but the specific activity of the whole homogenate is lowered. Delaney (1963) has purified a RNAase from human spleen and reported its molecular weight to be approximately 18,500.

Kidney Ribonucleases

Homogenates of rat kidney contain peaks of RNAase activity at pH 5.9&7.8, the latter being the major component (Roth, 1954). The acid enzyme is associated with intracellular droplets akin to lysosomes (Strauss, 1956; 1957). The activity of alk. RNAase is increased after injection of certain proteins* and azo dyes (Maack, Brentani and Rabinovitch, 1960; Rabinovitch, Brentani, Ferreira, Fausto and Maack, 1961) and also by protein depletion (Zigman and Allison, 1959). Hypophysectomy produced moderate increases in acid RNAase activity in rat kidney supernatant, and adrenalectomy brought about a lesser increase (Reid and Stevens, 1958). An interesting relationship between serum RNAase and the kidney has been described in several species, and it has been suggested that the enzyme, the activity of which increases after bilateral nephrectomy and does not appear to depend upon renal excretion, is taken up by the renal cortex and inactivated (Dohi and Rabinovitch, 1957; Rabinovitch, 1959; Dohi, Terzian, Widman, Brentani, Fausto, Liberman and Rabinovitch, 1959).

* those associated with increased 'droplet' formation such as egg-white and lysozyme.

Blood Ribonucleases

Only one RNAase is present in rat and guinea-pig serum, where its pH optimum is 7.4 (Rabinovitch and Dohi, 1957) and human blood serum likewise contains a single RNAase with a broad pH optimum 7.5-9.0 (Metais and Mandel, 1955; Migliarese, 1958a). Much disagreement concerning the response of the enzyme to various diseases is evident from the literature. Migliarese (1958b) claimed that the enzyme was significantly elevated in cancer patients, but this could not be confirmed by Levy and Rottino (1960) who claimed instead that the activity is elevated in liver disease and in leukaemia. Earlier studies had failed to show elevated activity in leukaemia and other myelo-proliferative disorders (Aleksandrowicz, Urbanczyk, Ostrowska and Sierko, 1958), while Houck and Borman (1958) had been unable to show a rise in liver and other gastro-intestinal disorders, but claimed to find increased activity in myocardial infarction, heart failure, and primary renal disease with uraemia. This association of increased serum RNAase activity with renal failure has been confirmed (Rabinovitch, Liberman and Fausto, 1959; Connolly, Herriott and Gupta, 1962). During azo dye carcinogenesis in the rat, serum RNAase fell to 60% of normal (Zytko and Cantero, 1963) while it has been reported that rats bearing transplantable tumours develop increased serum RNAase activity (Zigman and Allison, 1959).

Attempts to ascertain the origin of the enzyme have been made. Activity rises dramatically after bilateral nephrectomy in the guinea-pig, and this increase is not prevented by pancreatectomy, evisceration, and depletion of lymphoid tissue by administration of cortisone (Rabinovitch and Dohi, 1956) or by total hepatectomy and induction of leucopaenia by

nitrogen mustards (Dohi and Rabinovitch, 1957). Despite this last observation, Houck and Berman (1958) advanced some evidence suggesting that the enzyme may be released from white blood cells. Subsequent investigations have confirmed that the enzyme is present in these cells in the human (Maney, Moloney and Taylor, 1960; Gupta and Herriott, 1963), and in the rabbit, where it is associated with secretory granules (Cohn and Hirsch, 1960). In summary, the origin of the RNAase of serum has not yet been established, and a clear diagnostic use for its assay has not yet emerged.

Urine Ribonucleases

RNAase was purified from human urine by ethanol precipitation and ammonium sulphate fractionation (Dickman, White and Mason, 1958) and shown by column chromatography to contain two peaks of activity. Delaney (1963) has confirmed and extended these observations, and shown that one component is chemically and antigenically identical with bovine pancreatic RNAase, while the other closely resembles human splenic RNAase. This possibility had indeed been raised earlier on the basis of specificity studies and analysis of degradation products formed by an acid-optimal and an alkaline-optimal RNAase preparation from human urine (Hakim, 1959a; 1959b). The activity of these enzymes appears to vary throughout the menstrual cycle (Hakim and Pappas, 1959) and part of the activity is bound to inhibitors. Significant elevation of urinary RNAase activity has been found in chronic granulocytic leukaemia but not in other myeloproliferative disorders (Aleksandrowicz et al., 1958).

Mammary Gland Ribonucleases

A thorough study of RNAases in rat mammary tissue has shown the presence of an acid enzyme with pH optimum 5.8 and probably associated with the lysosomes (Greenbaum, Slater and Wang, 1960), and an alkaline enzyme optimal at pH 8.0 probably mitochondrial in origin (Slater, 1961). Activity was studied at various stages of funotion, both enzymes showing an increase during pregnancy and lactation, but whereas the acid RNAase decreased only slightly when breast involution commenced, alk. RNAase activity dropped dramatically during the first few days of involution. Bovine milk contains a RNAase which is serologically, chromatographically, and chemically identical to bovine pancreatic RNAase A (Dingham and Zittle, 1964; Coulson and Stevens, 1964).

OTHER HUMAN RNAases

Two RNAases, one displaying optimal activity at pH 7.8-8.3 and the other at pH 6.3-6.6, have been reported in saliva collected from human parotid and submaxillary glands, (Eichel, Conger and Chernick, 1964). RNAase with pH optimum of 7.5 has been identified in human skin (Satoianni and Rothman, 1961) and evidence derived from histochemical and conventional proceedures suggest that it is elevated several fold in psoriatic lesions (Steigleder and Raab, 1962; Liss and Lever, 1962). Cerebrospinal fluid also contains RNAase activity with a single pH optimum in the alkaline range, and raised in a variety of neurological disorders especially those involving cerebral white-matter (Kovacs, 1953;

Houck, 1958b).

Other Animal RNAases

Rat epididymal fat pad contains RNAase activity showing two peaks, one at pH 6.2-6.7 and the other at pH 7.5-8.2; both are present chiefly in the supernatant fraction of homogenates prepared from the tissue (Eichel, Figueroa and Goldenberg, 1961). Acid and alk. RNAases were found in ribosomal preparations of goat cerebral cortex, the former being inhibited by divalent cations and producing all four mononucleotides terminated by 3'-phosphoryl groups in equimolar amounts after prolonged action upon RNA (Datta, Bhattacharya and Ghosh, 1964). The presence of acid RNAase has also been detected in lysosome-like particles from rat brain (Beaufay, Berleur and Doyen, 1957). Muscle tissue from rabbits, chicks and mice also contain RNAase of lysosomal origin, enormous increases of which are encountered in nutritional and genetic dystrophies (Zalkin, Tappel, Desai, Caldwell and Peterson, 1960).

To sum up, it is virtually certain that enzymes capable of degrading RNA are present in every cell. In many cells, perhaps even in most cells, more than one such enzyme is present. These differ in respect of their pH optimum and their distribution within the cell.

Biological Function of Ribonucleases

The presence of RNAase in pancreatic juice is clearly related to the role of this exocrine secretion in the digestion of foodstuffs. The reason for the presence of related intracellular enzymes is more intriguing, and the question is made more confusing by the complex system of RNAases and of RNAase inhibitors found in many diverse tissues. Attempts to answer these problems have not been lacking, but the final verdict is still to come.

The activity of alk. RNAase relative to DNA in human placenta decreases with age*(Brody, 1957). On the other hand, an increase of activity with ageing of the animal was found in various organs of the rat (Stavitskaya, 1957); the specific activity of yeast ribosomal RNAase shows a similar increase with age of the culture (Danner and Morgan, 1963). A significant relationship between RNAase activity and RNA content was reported for normal rat and mice tissues (Ledoux, Pileri, Vanderhaeghe and Brandli, 1957) and for regenerating rat liver (Pileri, Ledoux, Liu and Vanderhaeghe, 1959), but this did not hold for the Landschutz ascites tumour which, from time of implantation until death of the animal lost twice as much acid RNAase as RNA per cell, while the alk. RNAase showed no change (Ledoux, Pileri, and Vanderhaeghe, 1958a). The same workers claimed that in various tumours of rat and mouse, and in human cervical carcinoma, for a given ratio of RNA to protein, the ratio of acid RNAase to protein is very much less than that found in the homologous normal cells (Ledoux, Pileri and Vanderhaeghe, 1958b; Ledoux, Brandli and Paepe, 1958).

* this refers to age of the placenta

To a limited extent, the suggestion made by these workers that tumours are characterised by a low content of RNAase is supported by their finding of a cancerostatic effect of crystalline pancreatic RNAase upon ascites tumour cells *in vivo* accompanied by a fall in RNA/DNA ratio (Ledoux, 1956a), and *in vitro*, where the effect of the enzyme is dependent upon the age and rate of growth of the cell strain (Ledoux, 1956b); this latter experimental situation is very complex since incorporation of labelled adenine is stimulated while that of labelled phenylalanine is inhibited (Ledoux, Daltus and Vanderhaeghe, 1956), and interference microscopy has shown that the uptake of the enzyme by the cells probably depends upon pinocytosis (Easty, Ledoux and Ambrose, 1956). This *in vitro* effect commences with an increase in RNA/DNA ratio and is followed by a second phase during which the ratio falls (Ledoux and Vanderhaeghe, 1956). Evidence has been obtained implicating modifications in the base ratios of RNA leading to formation of abnormal RNA in the process, since ascites cells treated with RNAase incorporate labelled adenine much less rapidly than labelled orotic acid (Pileri, Ledoux and Vanderhaeghe, 1957) and while the rate of incorporation of adenine and guanine is affected only to a moderate extent, that of uracil and cytosine is increased by 100%. This preferential modification of RNA turnover by pancreatic RNAase leading to a preponderance of pyrimidine nucleotides has been confirmed by further experiments *in vivo* and *in vitro* (Ledoux, Pileri, Poznanska and Vanderhaeghe, 1958) and is in line with the known bond specificities of the enzyme as regards both hydrolysis and transfer reactions.

Certain of these findings have been confirmed in other biological systems. Thus protein synthesis was inhibited in B.megaterium by addition of RNAase to the culture medium (Groth, 1956), and in the lysogenic strain of the same organism the enzyme inhibited synthesis of phage protein even more than that of bacterial protein (Jeener, 1958; 1959b). Addition of the enzyme to cultures of chick fibroblasts and myoblasts resulted in cessation of cell division and accumulation of DNA (Firket, Chevremont-Comhaire and Chevremont, 1955. When RNAase is included in the culture medium of He La cells, incorporation of labelled thymidine into DNA is increased by 48% at 3 hrs; this phase of stimulation is followed by inhibition of thymidine incorporation which at 72 hrs is only 40% of control values (Shah, 1963). Cell division was also interrupted by the enzyme in non-induced lysogenic cells of B.megaterium, and the base composition of the RNA isolated from the organism showed a 10% increase in uridylic acid (Jeener, 1959a); in induced cells of the same organism subjected to RNAase treatment, the phage protein isolated by column chromatography was quite different from that of normal phage protein and showed only partial fixation of antibody to normal phage protein (Jeener, Dupont-Mairesse and Vansanten, 1960). Uncoupling of oxidative phosphorylation in isolated mitochondria in the presence of RNAase has been demonstrated (Hanson, 1959); but it does not appear to affect the activity of the amino-acid incorporating enzyme of rat liver (Von der Decken and Hultin, 1959).

The above studies with added RNAase point to a possible role for the enzyme in the control of cell growth and cell division, but they are open to the criticism that the effects described could all be predicted

as a consequence of RNA degradation, and the action of endogenous RNAases may be quite different. That this may be so appears probable from work in plants and micro-organisms. Regenerating chick marrow shows a two-fold increase in alk. RNAase activity (Brody and Thorell, 1957). In bean hypocotyls, RNAase activity is increased by growth stimulants such as kinetin and β-indoleacetic acid (Maciejewska-Potapczyk, 1959). A parallelism between RNA content and acid RNAase activity has been found in barley seedlings (Ledoux, Galand and Huart, 1962). Detailed investigation of changes of RNAase activity in microsomes and supernatant of apple leaves has shown that the former increases to reach a maximum at the third week of development and thereafter declines sharply due to accumulation of an inhibitor, while the supernatant activity is low in the early stages due to the presence of a large amount of inhibitor in this fraction but increases with the age of the leaf; the authors postulated that the microsomal enzyme has a synthetic function while the supernatant enzyme has a catabolic role (Kessler and Engelberg, 1962).

Studies of RNAase activity in Micrococcus lysodeikticus led Barker and Cannon (1960) to suggest that in this organism the enzyme is concerned with breakdown and resynthesis associated with metabolic turnover of RNA. The RNAase activity of leaves infected by tobacco mosaic virus was higher than that of non-infected leaves (Reddi, 1959), and infection of chorio-allantoic membrane with influenza virus increased its RNAase activity (Klamerth, 1959). The difference in behaviour between endogenous and exogenous RNAase is strikingly illustrated by their relationship to virus growth, since it has been shown that the infectivity of several different

viruses is prevented by addition of RNAase to the medium (Hamers-Casterman and Jeener, 1957; Tamm and Bablanian, 1960; Norman and Veomett, 1961).

Taking into account the close relationship between RNAase and ribonucleoprotein particles (Roth, 1960), the RNAase activity of histones isolated from nuclei and microsomes and subsequently more fully reported by Martin, England, Turkington and Leslie (1963), and the double-helical structure of DNA (Watson and Crick, 1953) from which it follows that each strand of DNA could synthesise RNA so that two converse molecules would be produced, only one of which would give expression to the required genetic function, Leslie (1961) has proposed that the correct RNA is protected by a histone with RNAase activity against the converse molecule.

In summary, it appears probable that RNAase activity in different parts of the cell is due to different molecular species, and that a unique function cannot be ascribed to this activity since this may vary with the locus in the cell and the stage of development reached by it. Whatever else might be said, it remains a fact that depolymerisation of RNA, whether for the purpose of degradation, turnover, or as a prelude to nett synthesis, must count as the principle function of these enzymes.

Ribonuclease Content of Malignant Tissues

Some aspects of this question have been considered in the previous section. The topic is confused by the variations in the method of expression of activity employed by different workers quite apart from the wide range of tissues examined in these investigations. When rats were fed azo dyes, an increased activity of RNAase per unit weight of liver

occurred with the carcinogenic 3-Methyl-DAB but not with the non-carcinogenic 2-Methyl-DAB (Schneider, Hogeboom, Shelton and Striebich, 1953). The activity of acid and alk. RNAase of two rat hepatomas exceeded that of normal liver (Mayer and Greco, 1956).

An extensive study on DAB carcinogenesis in rat liver was carried out by Allard, de Lamirande and Cantero (1957). These authors reported an increase in specific activity of acid RNAase throughout tumour development and maximal in the induced tumour. This increase was not found in the Novikoff hepatoma. It occurred in all cell fractions of the liver and primary tumour, but the percentage of the total activity recovered in the mitochondria was low in the tumour though not in the surrounding liver. With alk. RNAase a rather different situation prevailed. The activity of the whole homogenate per mg. nitrogen did not differ in the DAB-fed animals from control levels, but it was greatly increased in the primary tumour. The mitochondrial fraction of the tumour was the only one to show an increase in specific activity over that of the corresponding fraction from control animals, but when the percentage activity of the various fractions was calculated, it was found to have diminished in the mitochondria and increased in the supernatant of the tumour. The results in Novikoff hepatoma were variable, some tumours containing enormous increases in alk. RNAase while others had low levels of activity; an increase in the percentage of the total activity recovered in the supernatant was the only consistent finding.

When the carcinogen 2-acetylamino fluorene was fed to rats, alk. RNAase of liver mitochondria was depressed by 50% while no change in acid RNAase of the mitochondria was found (Roth, 1957). The depression of

mitochondrial alk. RNAase occurred immediately upon feeding the dye so that it may be related to dye metabolism rather than to the carcinogenic process. Reid and Lotz (1958) studied the effect of 3-Methyl-DAB upon liver RNAases, and examined the activities of both enzymes in pre-cancerous liver, tumours, and the livers of tumour-bearing animals. They were able to make only two generalisations: a) that the level of neither enzyme is lowered in the whole cytoplasm in any of the above conditions compared with normal livers; b) that the acid RNAase in the supernatant fraction is increased in precancerous liver.

Brody and Balis (1958) reported the results of a small series in which the RNAase activity per mg. DNA of animal and human tumours was compared with the value found in homologous normal tissue. Only in human pancreatic carcinoma did the activity exceed that of normal tissue. The authors were unable to confirm the suggestion of Ledoux and his co-workers (Ledoux, Pileri and Vanderheeghe, 1958b; Ledoux, Brandli and Paepe, 1958) that the ratio of RNAase to RNA was lowered in tumours compared to normal tissue. Various ascites tumours of the mouse have low RNAase activity at physiological pH values due to formation of a complex between the native enzyme, optimally active at pH 7.3 in the free state, and a potent inhibitor (Ellem, Colter and Kuhn, 1959; Colter, Kuhn and Ellem, 1961). Differences in the rate of mercaptide formation by purified rat liver acid RNAase from that found with purified acid RNAase preparations from two hepatomas led to the suggestion that the enzymes from normal and malignant tissue differ in respect to the number and location of active SH-groups in the molecule (Mayer and Greco, 1962).

RNAase activity per g. of tumour and per mg. DNA increased in parallel

with the growth and development of Walker 256 tumour in the rat; however treatment with the anti-tumour drug N,N'-diethylene-N"-phenethyl phosphoramide produced a further increase in RNAase activity (Wannamacher, Allison, Chu and Crossley, 1962). Using the histochemical substrate film method, Daoust and Amano (1963) reported that no RNAase activity could be detected in the malignant cells of a large series of human and animal tumours; these findings have been strongly criticised by Roth, Hilton and Morris (1964) who carried out an extensive investigation of RNAase activity in transplantable rat hepatomas and whose findings merit close attention. With two exceptions, the level of RNAase inhibitor in the tumours was lower than that of the surrounding normal liver; the percentage of acid and alk. RNAase in the mitochondrial fraction was greatly diminished; the percentage of acid and alk. RNAase in the nuclear fraction was unchanged or elevated; the percentage of alk. RNAase in the microsomes was nearly doubled and was accompanied by a large increase in specific activity; while the acid RNAase activity of the supernatant was greatly increased. Summarising the position in a recent review, Roth (1963) stated that "scarcely a beginning has been made in our understanding of the relation of RNAase activity to cancer or even to RNA metabolism in the normal cell".

DEOXYRIBONUCLEASES

Deoxyribonuclease I
(Deoxyribonucleate Oligonucleotido-hydrolase) EC 3.1.4.5

Although the existence of enzymes capable of degrading DNA had been recognised in many tissues through the work of several investigators summarised by Schmidt (1955), the crystallization of the pancreatic enzyme by ammonium sulphate fractionation of an acid extract of the bovine organ (Kunitz, 1950a) paved the way for fundamental research into the behaviour and mode of action of these enzymes. The molecular weight reported by Kunitz (1950a) was 60,000. Using a preparation purified by electrodecantation, Polson (1955) found the enzyme to have a molecular weight of 40,000 with an isoelectric point of 4.7, but Gehrmann and Okada (1957) reinvestigated the problem and concluded that the molecular weight was 61,566; the latter authors also reported on the amino-acid composition of the enzyme, but further work on its covalent structure does not appear in the literature, although evidence has been presented for the involvement of tryptophan at the active centre (Okada and Fletcher, 1962).

The pH optimum of the enzyme is close to 7.0, a concentration of NaCl in excess of 0.05M is inhibitory, and Mg^{++} is required in a concentration which is dependant upon that of the substrate and not upon that of the enzyme (Kunitz 1950b). Removal of Mg^{++} by chelating agents causes profound inhibition of the enzyme (Gilbert, Overend and Webb, 1951; Feinstein and Green, 1956) while Ca^{++} will activate a system which is already optimal with regard to Mg^{++} (Wiberg 1958). Since the stimulating effect of Mg^{++} was found even when magnesium deoxyribonucleate was employed

as substrate, it may be concluded that the divalent cation complexes with the enzyme protein as well as with the substrate (Erkama and Suutarinen, 1959).

Mode of Action

Agreement is general that the products of enzyme action upon DNA are terminated in 5'-phosphoryl groups (Sinsheimer and Koerner 1951, 1952; Privat de Garilhe and Laskowski, 1955). The same authors also identified all four deoxynucleotides of DNA in the digest, although they only constituted 1% of the products of enzyme action.

In a study of the dinucleotides present in the digest, Sinsheimer (1955) concluded that the sequence d-$_p$Pu.$_p$Py was scarce compared with the sequence d-$_p$Py.$_p$Pu, while the Milwaukee group were unable to find it at all and inferred that impurities in the analytical enzyme preparations employed by the former worker had resulted in a degree of molecular rearrangement (Privat de Garilhe, Cunningham, Laurila and Laskowski, 1957; Potter, Laurila and Laskowski, 1958). This led to the proposal, summarised by Laskowski (1961), that the missing linkage was the one preferentially attacked by the enzyme. Re-examining the problem of specificity, Vanecko and Laskowski (1962) were forced to abandon this view, since of all dinucleotides tested d-C$_p$.G$_p$ was the most resistant. Furthermore, they made the interesting observation that during the course of the digestion of DNA, the bond which is preferentially hydrolysed changes from being the d-G$_p$.G$_p$ linkage in the earlier stage to that between deoxyadenosine in the 3'-terminus and deoxycytidine in the 5'- terminus in the later stage, though at neither stage is the preference absolute. In the presence of

Mn^{++} ions, however, a different specificity prevails, with the $^{d}_{p}Py.{}_{p}Pu$ linkage being the most susceptible to enzymic hydrolysis (Becking and Hurst, 1963).

It is clear that the reaction mechanism is a complex one, so much so that Hurst and Findlay (1960) distinguished between a nucleodepolymerase activity and an oligonucleotidase activity in various preparations of pancreatic DNAase, the latter activity being much more unstable than the former. This claim has since been challenged by Vanecko and Laskowski (1961), but there is support for a mechanism whereby DNAase I activity commences with cleavage of double-stranded DNA into double-stranded fragments followed by a slower phase in which the enzyme attacks the single stranded oligonucleotides separating from these fragments (Desreux, Hache and Frederiq 1962; Matsuda and Makino, 1963). Kinetic studies in general bear witness to this complexity, though variations in conditions of digestion and techniques of measurement render comparison of results a hazardous procedure. Employing light-scattering techniques to follow the digestion of DNA, Reichmann (1956) reported an initial retardation in molecular weight decay followed by a period when, although the molecular weight of the fragments falls, their configurational properties are akin to those of the original substrate; the reaction slows long before the stage of mono- and di-nucleotide formation commences. These results, as well as those reported by Schumaker, Richards, and Schachman (1956) and by Thomas (1956) in which viscosity and sedimentation data were compared with the number of splits as measured in a pH-Stat supported the view that splits occurred at random, many single bonds being broken on each

chain before scission of the double helix occurred. Comparison of the titration curve of the products of digestion of DNAase with native DNA and a mixture of deoxynucleotides led Fredericq (1958) to suggest that the enzyme is capable of degrading only the double-helical form of DNA. Using a turbidimetric method, Houck (1959) found the activity of DNAase measured at pH 5.9 to tail off after 30 min. Williams, Sung and Laskowski (1961) correlated bond rupture measured in a pH-Stat with increase in the extinction of the substrate at 260 mμ; the relationship between the two could best be expressed by a sigmoid curve indicating a three-phase kinetic reaction. In contrast with the above results, Le Talaer, Le Pecq, Festy and Paoletti (1964) studied degradation of DNA by the enzyme at variable substrate concentrations and measured the reaction viscosimetrically as well as spectrophotometrically. They found that the Michaelis law was obeyed, and that the Km was 9.5×10^{-4} M, independent of the assay technique used; this they interpreted as evidence for the constancy of enzyme affinity for substrate throughout the course of hydrolysis. It is clear that despite intensive investigation, a definitive picture of the mode of action of the enzyme has not yet been attained.

Deoxyribonuclease II
(Deoxyribonucleate 3'-nucleotidohydrolase) EC 3.1.4.6

An enzyme active upon DNA and differing in several important respects from the above was identified in calf spleen by Catcheside and Holmes (1947) and in calf thymus by Mayer and Greco (1949). Both activities were purified 100,000 fold by Oth and Fredericq (1958) who concluded that the nature of

the protein involved was the same in each tissue.

Although precise measurement of the pH optimum for the enzyme is rendered difficult because it is influenced by the nature of the cation present (Koerner and Sinsheimer, 1957a) and by the ionic strength of the medium (Oth, Fredericq and Hacha, 1958), this lies well to the acid side of neutrality, values from 4.2 to 5.6 having been reported (Laskowski 1961). The requirement for a monovalent cation is 0.2 - 0.3M, and Mg^{++} is inhibitory (Shimomura and Laskowski, 1957).

The reaction of enzyme with substrate proceeds to a point where 25-30% of the phosphoric ester linkages are broken; the products include all four deoxynucleotides of DNA - these, as well as the dinucleotides and oligonucleotides formed are terminated in a 3'-phosphoryl group (Koerner and Sinsheimer, 1957b). The kinetics, under the conditions employed by these latter authors, displayed a biphasic course, and no preference for specific linkages could be deduced. On the other hand, Laurila and Laskowski (1957) examined the reaction products and were unable to find the sequence $^{d-}Py_p\text{-}Pu_p$, from which evidence they concluded that this linkage was particularly susceptible to the action of the enzyme. Oth et al (1958) compared the characteristics of enzyme action as measured viscosimetrically and spectrophotometrically and noticed a time lag of about 15 min. when assay was by the latter method; their studies led them to suggest that both strands of DNA are broken simultaneously, and further work appears to have confirmed this view (Desreux et al, 1962).

Occurrence of Deoxyribonucleases

Investigation of many animal tissues has revealed the widespread

occurrence of deoxyribonucleases. Although few have been characterised, it is conventional to attribute the activities to DNAase I and DNAase II where the method of assay was designed to permit conditions allowing selective measurement of pancreatic DNAase I and splenic DNAase II. It must be borne in mind that for most of these studies, the results indicated the ability of the extracts in question to degrade DNA under two different sets of conditions, and it does not follow that the estimates obtained arose from the unique function of two individual proteins.

Allfrey and Mirsky (1952) in an extensive study of various animal tissues found DNAaseI present in pancreas only, whereas DNAase II was detected in every tissue examined. It has since become clear through work carried out on ox pancreas that the enzyme is largely stored in zymogen granules in company with other enzymes of pancreatic juice (Keller and Cohen, 1961). On purifying DNAase of rat liver, spleen, two hepatomas and lymphosarcoma, Maver and Greco (1956) concluded that only one enzyme was present and that it had the characteristics of DNAase II. On the other hand, Cunningham and Laskowski (1953) identified both DNAase I and DNAase II activities in veal kidney, the presence of the former being revealed by treating the homogenate with dilute sulphuric acid. More recent work has indicated the presence of enzymes resembling DNAase I in homogenates of kidney, thymus and lymphoma of the mouse (Shack 1957); the mitochondria of guinea-pig liver (Goutier-Pirotte and Oth, 1956) and of rat liver (Dounce, O'Connell and Monty, 1957; Beaufay, Bendall, Baudhuin and De Duve 1959; Baudhuin, 1959); and the nuclei and cytoplasmic particles of ascites tumour cells (Keir and Aird, 1962). The presence of DNAase I activity in human serum has been known for more than a decade

(Wroblewski and Bodansky, 1950). This finding was confirmed by Kurnick (1953) who also reported that the activity of cat serum was one-hundred times greater than that of human serum. It was also shown in the rat that passage through the liver reduced the DNAase activity of plasma, thus raising the possibility of biliary excretion (Kurnick and Gazzera, 1953). Acute haemorrhagic pancreatitis increases the activity of serum DNAase I (Kowlessar and McEvoy, 1956) while malignancy lowers it (Wroblewski and Bodansky, 1950; Gavosto, Buffa and Maraini, 1959). The enzyme of cat serum has been highly purified, and shown to belong to the α^2 globulins (Riou and Paoletti, 1963), while that of human plasma was purified 600-fold and shown to belong to the α^1 acid glycoprotein fraction (Doctor, 1963). Human platelets have a fairly high concentration of DNAase I (Connolly, Herriott and Gupta, 1962a) which can also be detected in human leucocytes by special procedures designed to remove inhibitors (Maney, Moloney and Taylor, 1960). DNAase I has been found in a bound and in a free form in human urine (Hakim, 1959d); the binding is affected by various hormones, and the activity appears to vary throughout the menstrual cycle in the female.

As already indicated, enzymes akin to DNAase II enjoy a wide distribution. In addition to those tissues mentioned previously, the enzyme has been found in sources as diverse as cow's milk (Haessler and Cunningham, 1957), rat brain (Beaufay, Berleur and Doyen, 1957), human prostate (Böman, 1958) and rabbit leucocytes (Cohn and Hirsch, 1960). Reviewing their own work and that of others, de Duve, Wattiaux and Baudhuin (1962) concluded that the enzyme is situated primarily in the lysosomes. On the other hand, it appears that considerable DNAase II activity is present in rat liver nuclei,

the presence of Ca^{++} ions being obligatory for preservation of activity (Swingle and Cole, 1964); and the activity of DNAase II in nuclei of ascites tumour cells was twice that found in cytoplasmic particles from the same cells (Keir and Aird, 1962). The enzyme has been separated from human urine (Koszalka, Schreier and Altman, 1954), though minute amounts only are present in the normal subject (Hakim, 1959c). Claims for the presence of the enzyme in human serum have also been made (Houck, 1959; Gavosto et al, 1959).

Some enzymes have been discovered which cannot be placed in the same category as DNAase I or DNAase II. Two of the most interesting are an enzyme from chicken erythrocytes optimal at pH 5.5 and inhibited by Mg^{++} ions which degrades DNA without the production of dialysable material (Bernardi, Champagne and Sadron, 1961), and one from rat liver which, unlike previously identified DNAase II from this source whose properties are otherwise similar hydrolyses thermally denatured DNA more rapidly than native DNA (Burdon, Smellie and Davidson, 1964).

Inhibitors of Deoxyribonucleases

Much of the early work on deoxyribonucleases produced conflicting results, the reasons for which became clear with the recognition of the widespread occurrence of tissue inhibitors of these enzymes, although various non-protein inhibitors were shown many years ago to be effective by one of two mechanisms - removal of Mg^{++} ions and direct action upon the enzyme protein (Gilbert et al., 1951).

The identification of a protein inhibitor of DNAase I in pigeon crop

gland (Dabrowska, Cooper, and Laskowski, 1949) was followed by the recognition of a similar inhibitor in several other tissues and tumours, although no relationship with cellular growth could be established (Cooper, Trautmann and Laskowski, 1950). Further work demonstrated the inhibitory capacity towards pancreatic DNAase I possessed by extracts of many organs, inhibition being maximal at pH 6.75 and lost upon standing for time intervals over 4-7 hours (Henstell and Freedman, 1952). Studies with human white blood cells have shown that mature cells contain much more of this inhibitor than immature cells (Henstell, Freedman and Ginsburg, 1952). A different inhibitor, active against serum DNAase I but not against pancreatic DNAase I, and predominating in immature cells, was detected in the cytoplasm of human leucocytes (Kurnick, Schwarz, Pariser and Lee, 1953). Human blood represents a particularly complex system as regards inhibition of DNAase I; the purified enzyme of serum is inhibited by all other fractions of serum (Dodor, 1963) and by haemolysates of erythrocytes (Chepinoga and Rukina, 1955), while serum itself inhibits added pancreatic DNAase I (Kurnick, 1950). Recent work in this field has shown the presence of an inhibitor of serum DNAase I in rat serum (Berger and May, 1964) and an inhibitor of pancreatic DNAase I in many organs of the rat, notably liver, brain, and lung, (Festy and Paoletti, 1963). The liver factor is cytoplasmic in situation, probably a β-globulin, and is inhibitory towards the DNAase I of liver mitochondria (Loiselle and Carrier, 1963); it is very labile to heat, but is unaffected by SH-reagents (Zalite and Roth, 1964). An inhibitor of pancreatic DNAase I which has been partially purified from calf spleen resembles the above in being thermolabile, but appears to be stabilised by mercaptoethanol and glutathione (Lindberg, 1964).

The evaluation of tissue inhibitors of DNAase I is difficult, not only because of their lability and the number of different types discovered, but because of the existence in many tissues of a heat-stable activator which can be removed by exhaustive dialysis but which has not been further identified (Feinstein, 1960). Certain proteins such as albumin may also increase the activity of DNAase I in many tissues through a mechanism believed to involve protection against proteolytic enzymes (Feinstein and Hagen, 1962).

Much work, to be reviewed later on the effects of radiation upon tissue levels of DNAase II has inferred the existence of inhibitors of this enzyme. To date, none has been identified, and the only inhibitor of DNAase II for which convincing evidence exists is a heat stable dialyzable factor present in normal human urine unrelated to any of the known urinary constituents (Kowlessar, Okada, Potter and Altman, 1957).

Relationship of Deoxyribonucleases to Cellular Functions

With the exception of pancreatic DNAase I, whose function is clearly related to intestinal digestion of dietary DNA, the role of deoxyribonucleases in cellular processes is far from certain. A number of studies have shown a relationship between the DNAase II content of tissues and their mitotic rate (Allfrey and Mirsky, 1952; Goutier and Goutier-Pirotte, 1961; Goutier and Leonard, 1962). Regenerating chick bone marrow cells showed no increase in DNAase I, but a six-fold increase in DNAase II activity was detected (Brody and Thorell, 1957). Chick erythro-leukaemia cells had higher DNAase II activity than both normal and regenerating

cells, the activity showing a relationship to the mitotic rate of the tumour (Brody, Johannison and Thorell, 1959). An increase in DNAase II activity occurs as soon as 12 hours after partial hepatectomy in the rat (Brody and Balis, 1959; Goutier-Pirotte and Goutier, 1962), though a decline in particulate enzyme precedes cell division and has been attributed to cessation of lysosomal synthesis (Adams, 1963). Earlier work had shown a steady decrease in DNAase II/DNA-P ratio in the human placenta as it developed from early pregnancy towards term (Brody, 1953), and study of a number of aniral and human tissues and tumours led Brody and Balis (1958) to suggest that the enzyme has either a synthetic function or a growth regulating effect.

Chevremont, Chevremont-Comhaire and Baeckeland (1959) applied DNAase II to cultures of fibroblasts and observed increase in Feulgen staining of the nucleoli coupled with the appearance of Feulgen-positive material in the mitochondria, the latter not being removed by additon of RNAase; they interpreted their findings as evidence for the mitochondrial synthesis of DNA under the influence of DNAase II. The increase in activity of this enzyme in the early stages of liver regeneration in the rat is accompanied by the appearance in the cytoplasm of material having some of the properties of DNA, though this may be nuclear in origin and released in order to serve as a primer for DNA synthesis (Goutier-Pirotte and Goutier, 1962). Studies on rat hepatomas induced by feeding azo dyes have shown an increase in DNAase II activity in pre-cancerous liver and in hepatomas compared to liver from control animals; the intracellular distribution of the enzyme was also altered in the hepatoma, a much higher percentage being located in the nuclei and in the supernatant than was the case for normal

rat liver (Schneider, Hogeboom, Shelton and Striebich, 1953; de Lamirande, Allard and Cantero, 1954). These findings have been challenged by Brody (1958) on the basis of a single hepatoma, and by Daoust and Ameno (1963) whose histochemical substrate - film technique has already been criticised (Roth, 1963). On the other hand, indirect support stems from the work of Tunis and Regelson (1963) who demonstrated that anionic polyelectrolytes, strongly inhibitory in vivo to the growth of transplantable tumours, are powerful in vitro inhibitors of DNAase II but not of DNAase I.

Work in lower organisms has revealed the possibility of a less exalted role for DNAase II. From a study of the distribution of the enzyme in embryos of chick, frog, and sea-urchin, Blumenthal (1957) concluded that the presence of the enzyme in high concentration at certain stages was concerned with the clearing of debris following metamorphosis. A similar conclusion in respect of tail resorption was reached in studies of the development and metamorphosis of two different species of frog (Coleman, 1962; Coleman, 1963), while work on the development of the mouse embryo lends support to this view (Solomon, 1964).

The role of DNAase I other than that of pancreas has until recently been obscure, but a number of experiments have shown that it is capable of stimulating the activity of DNA polymerase in a mouse leukaemia system low in DNAase I activity (Mantsavinos and Canellakis, 1959b) and in two other polymerase systems (Sarkar, 1961), as well as in an extract of Landschutz ascites cells (Keir, Binnie and Smellie, 1962; Keir, 1962). On the other hand, addition of pancreatic DNAase I to the DNA polymerase of regenerating rat liver, a tissue rich in DNAase content, inhibits

DNA synthesis (Mantsavinos and Canellakis, 1959a). The action of DNAase I is optimal when the molecular weight of the fragments produced is about 10^6; sonic disruption of native DNAase to fragments of similar size results in similar stimulation of polymerase activity; degradation of native DNA by DNAase I to smaller fragments inhibits polymerisation (Sarkar, Mukundan and Devi, 1963) although the methods used were not capable of detecting synthesis of acid soluble oligonucleotides. The views of all the above workers are in accord in assigning to DNAase I an important role in reducing native DNA to a size suitable for functioning as a primer for DNA polymerase, with the unmasking of free hydroxyl end groups to which addition of deoxynucleotides can take place. Such speculation assists in explaining the relationship of DNAase activity to plant growth stimulants (Maciejewska-Potapczyk, 1959) as well as the stimulation of the growth rate of Euglena induced by addition of pancreatic DNAase to the medium (Zahn, 1959).

Related to these observations, are those on lysogenic induction of E. Coli by bacteriophage in which the DNAase content of the cells increases five-fold (Wormser and Pardee, 1957), and the appearance of a new exonuclease degrading native DNA much faster than heat-denatured DNA (Korn and Weissbach, 1962). Such activity appears as a prelude to synthesis of viral DNA. Strong support for this relationship comes from work on the growth of Herpes simplex virus in mammalian cells in tissue culture, where it has been observed that an early increase in activity of DNAase I and of DNA polymerase precedes the synthesis of viral DNA (Keir and Gold, 1963; Russell, Gold, Keir, Omura, Watson and Wildy, 1964). Furthermore, the nuclear fraction showed the highest percentage increase in DNAase I

activity (161%) compared with an increase of 105% for the mitochondria-microsome fraction and one of 107% for the cell sap. The ability of ribosomes of E. Coli to bind and release DNAase under various conditions has also been reported (Tal and Elson, 1961), the authors speculating on the possibility that this mechanism may have a part to play in the control of DNA metabolism. Finally, it has been suggested that the pathogenicity of staphylococci may be related to their DNAase content (Jacobs, Willis and Goodburn, 1963).

PHOSPHODIESTERASES

Orthophosphoric Diester Phosphohydrolase EC 3.1.4.1

The principal feature of these enzymes is their ability to degrade both RNA and DNA (Heppel and Rabbinowitz, 1958); this they do with apparently little specificity in the majority of cases towards the purine or pyrimidine base on either side of the diester linkage.

Spleen Phosphodiesterase

This enzyme has been purified several hundred-fold from beef spleen by Hilmoe (1960) and by Razzell and Khorana (1961). The preparation shows optimal activity near pH 7.0 and releases products terminated in 3'-phosphoryl radicals by an action that is predominantly exonucleolytic. It is active upon a wide variety of RNA preparations but is inactive towards native and highly polymerised DNA (Hilmoe, 1960). Nevertheless it rapidly hydrolyses oligonucleotides derived from DNA to deoxynucleoside 3'-phosphates provided that they do not have a free 5'-phosphoryl end group (Gilham and Khorana, 1958; Turner and Khorana, 1959). Like pancreatic RNAase, enzymic action under appropriate conditions leads to exchange reactions resulting in synthesis of internucleotide links (Heppel and Whitfield, 1955; Heppel, Whitfield and Markham, 1955). A similar enzyme has been demonstrated in homogenates of rat, hog, and human tissues (Razzell, 1961a). It is optimally active at pH 6.0, is stimulated by EDTA, and appears to be present chiefly in the supernatant of rat liver and kidney, while in the hog kidney activity is maximal in the mitochondria.

Intestinal Phosphodiesterase

The discovery of this enzyme which leads to the formation of products terminated by 5'-phosphoryl groups was first made by Carter (1951) in extracts of intestinal mucosa.

A method for its purification and separation from another diesterase present in the extract and which forms products terminated by 3'-phosphoryl groups has been described (Heppel and Hilmoe, 1955), but the specific activity of the material isolated by this proceedure does not greatly exceed that of the original homogenate.

A similar type of enzyme which forms 5'-terminated products by step-wise exonucleolytic action upon RNA and DNA has been found in leukaemia cells (Anderson and Heppel, 1960), while Razzell (1961a) has detected phosphodiesterases of this type in homogenates of rat, hog, and human tissues. It is optimally active at pH 9.0 and inhibited by EDTA, and appears to be distributed throughout all tissue fractions, including the nuclei, but chiefly in the lysosome-microsome fraction. A partial purification of the enzyme from hog kidney has been achieved (Razzell, 1961b) and the step-wise action whereby it sequentially liberates nucleotides terminated by 5'-phosphoryl radicles has been demonstrated. A report on the localisation of this enzyme in various rat tissues by a histochemical technique has been presented (Sierakowska and Shugar, 1963); kidney and pancreas contained the highest activity, spleen and liver and thyroid contained moderately high activity, while brain contained little activity; it was absent from the nuclei of all tissues examined, in contrast to the findings of Razzell (1961a). Studies on ribosomal preparations of goat brain (Datta and Ghosh, 1963) and lamb brain homogenates

(Healy, Stollar, Simon and Levine, 1963) have revealed the presence of an enzyme described as a phosphodiesterase which resembles the above in having a pH optimum well to the alkaline side of neutrality, in requiring Mg^{++} ions for activity, and in being inhibited by EDTA. However it has not yet been established that either enzyme is active towards both RNA and DNA, though Healy et al., (1963) have claimed that their preparation produces fragments terminated by 5'-phosphoryl residues from DNA by an endonucleolytic action; a similar enzyme has been detected in human cerebrospinal fluid (Healy, 1964).

Other Phosphodiesterases

The presence of a phosphodiesterase in the venom of certain snakes was first discovered by Uzawa (1932); it has been extensively purified by chromatographic proceedures (Bowman and Kaletta, 1957; Razzell and Khorana, 1959a) and has proved to be a powerful analytical tool in the determination of base sequences and end group residues of nucleic acids. Studies of its action on a variety of synthetic deoxyribo-oligonucleotides (Razzell and Khorana, 1959b) and ribo-oligonucleotides (Singer, Hilmoe and Heppel, 1958) bearing 3'-hydroxyl end-groups have shown that hydrolysis begins from that end, is stepwise, and results in the successive liberation of nucleoside 5'-phosphate units.

The phosphodiesterase of mung bean sprouts, as mentioned previously, operates in a manner that is predominantly endonucleolytic, and shows no preference towards the sugar moiety (Sung and Laskowski, 1962). It appears that linkages involving the 3'-phosphoryl group of adenine

nucleotides are most susceptible to hydrolysis by the enzyme, and the products are terminated by phosphoryl groups in the 5'-position and hydroxyl groups in the 3'-position. A similar enzyme has been purified from Azotobacter agilis (Stevens and Hilmoe, 1960a and b) which cleaves RNA and DNA to small oligonucleotides with 5'-phosphomonoester end groups. Once again the action is predominantly endonucleolytic, and polyadenylic acid is cleaved at a very much faster rate than polynucleotides consisting entirely of one of the other bases.

An enzyme isolated from the culture medium of Staphylococcus aureus has been purified and shown to attack both RNA and DNA with the production of mononucleotides and oligonucleotides terminated by 3'-phosphomonoester end groups (Cunningham, 1959). The mechanism of action of this enzyme has received close study, since it provides an example of changing affinity during the course of digestion of the substrate, switching from an endonucleolytic attack chiefly in regions of high concentration of adenine and thymine or uracil residues in the early stages to an exonucleolytic attack ceasing with the formation of dinucleotides in the late stages (Dirksen and Dekker, 1960; Sulkowski and Laskowski, 1962).

FURTHER DEGRADATION OF NUCLEOTIDES

The ultimate products formed by the action of nucleases, including phosphodiesterases, upon their substrates are nucleoside phosphates, as we have just seen. These may be dephosphorylated to yield the corresponding nucleoside by one of a number of phosphomonoesterases which are widely distributed in cells and tissues, some of which are specific for 5'-nucleotides while others are non-specific regarding the type of nucleotide towards which they are active. The action of these enzymes is essentially irreversible.

The next stage in nucleotide catabolism is the rupture of the bond uniting the sugar component and the nitrogenous base. This step, which is freely reversible, is catalysed by the enzyme nucleoside phosphoxylase. The enzyme is active towards both ribose and deoxyribose derivatives, and the nucleosides of all the bases, with the apparent exception of cytosine, may participate in this reaction. The reaction involves inorganic phosphorus according to the following equation:

Sugar - Base + Inorganic Phosphate $\rightleftharpoons$ Sugar-1-Phosphate + Base

Purine Catabolism

The main excretory product of purine catabolism in man, birds, and reptiles is uric acid; in mammals other than primates, this product is further degraded to allantoin (Figure 4). The formation of uric acid can be considered to commence at

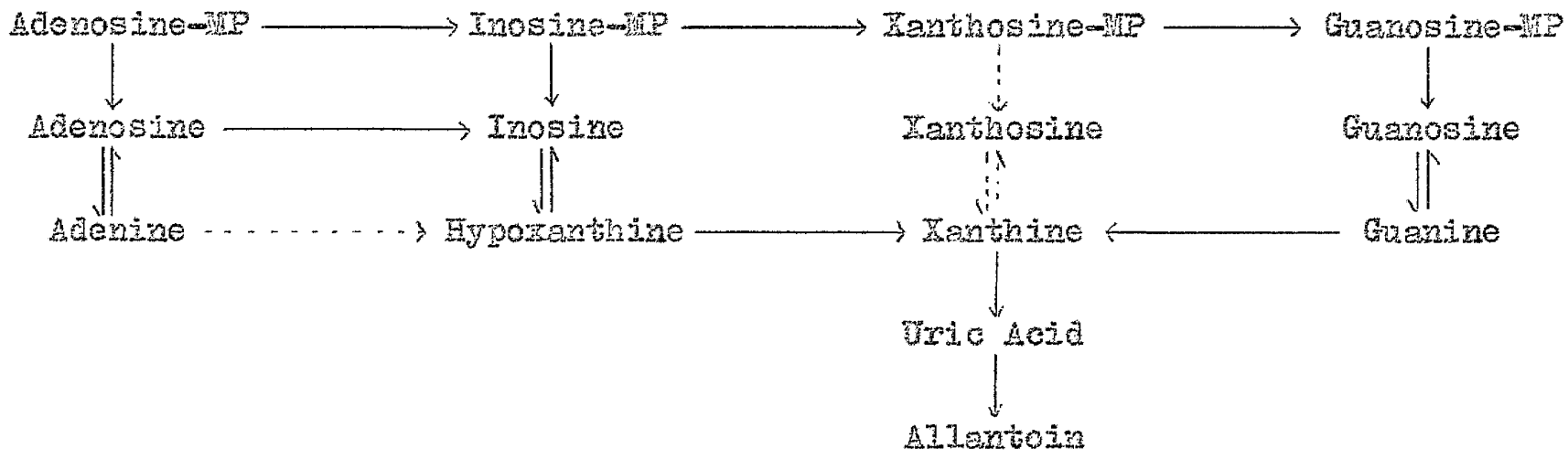


FIGURE 4

CATABOLISM OF THE PURINES

The above scheme is concerned only with the essential catabolic steps and is derived from Potter (1960). Reversible reactions are indicated by a double arrow. Those absent from animals but occurring in micro-organisms and/or plants are shown by a broken arrow. Allantoin is not formed in primates.

The suffix 'MP' stands for 'Monophosphate'.

the level of inosine and proceeds from there in three stages. The first of these involves removal of the sugar component to yield the base, hypoxanthine, and this step is catalysed by nucleoside phosphorylase in the manner described above. The next two stages are both catalysed by the same enzyme, xanthine oxidase, which brings about successive oxidation, firstly of hypoxanthine to xanthine; secondly of xanthine to uric acid. The action of this enzyme is irreversible and the products of its action are lost to the organism for energy-yielding and synthetic purposes. Moreover, it appears to be the rate-controlling enzyme in purine catabolism and probably determines, directly or indirectly, the concentration of purine derivatives available for synthetic reactions leading to the build-up of nucleic acids.

Derivatives of those purines present in nucleic acids, adenine and guanine, are able to reach various points on this pathway by means of a number of interconversions which may take place at the level of the nucleotide, the nucleoside, or the free base. These interconversions are shown in Figure 4 which is based upon the scheme of Potter (1960). Adenine derivatives may gain access to the outlet pathway in two ways: by deamination of adenosine monophosphate to inosine monophosphate under the influence of the enzyme adenylic deaminase; or by deamination of adenosine to inosine through the mediation of adenosine deaminase. Further consideration will be given

to this enzyme later in this Introduction. In some plants and micro-organisms, the conversion of adenine to hypoxanthine by means of an enzyme, adenase, is also possible, but this enzyme appears to be lacking from the tissues of animals. Guanine derivatives also have two routes towards the outlet pathway; one is at the nucleotide level where guanosine monophosphate may be converted to inosine monophosphate; the other is at the level of the free base where guanine can be deaminated to yield xanthine by means of a specific enzyme, guanase, which unlike adenase is certainly present in animal tissues.

Pyrimidine Catabolism

The end-product of catabolism of uracil derivatives is β-alanine, and of thymine derivatives β-amino isobutyric acid. Neither of these are excretory products in the sense with which this term can be applied to uric acid, since they are both capable of further metabolic reactions bringing them within the orbit of the tricarboxylic acid cycle. The free bases are formed by the consecutive action of phosphatases and nucleoside phosphorylase in exactly the same manner as that described for purine derivatives, and a summary of the reactions is set out in Figure 5 which is based on the scheme of Potter (1960). Cytosine derivatives do not have a separate pathway of their own and follow the routes taken by uracil or by thymine. The ribonucleotide, cytidine, is converted to uridine by the enzyme cytidine deaminase which

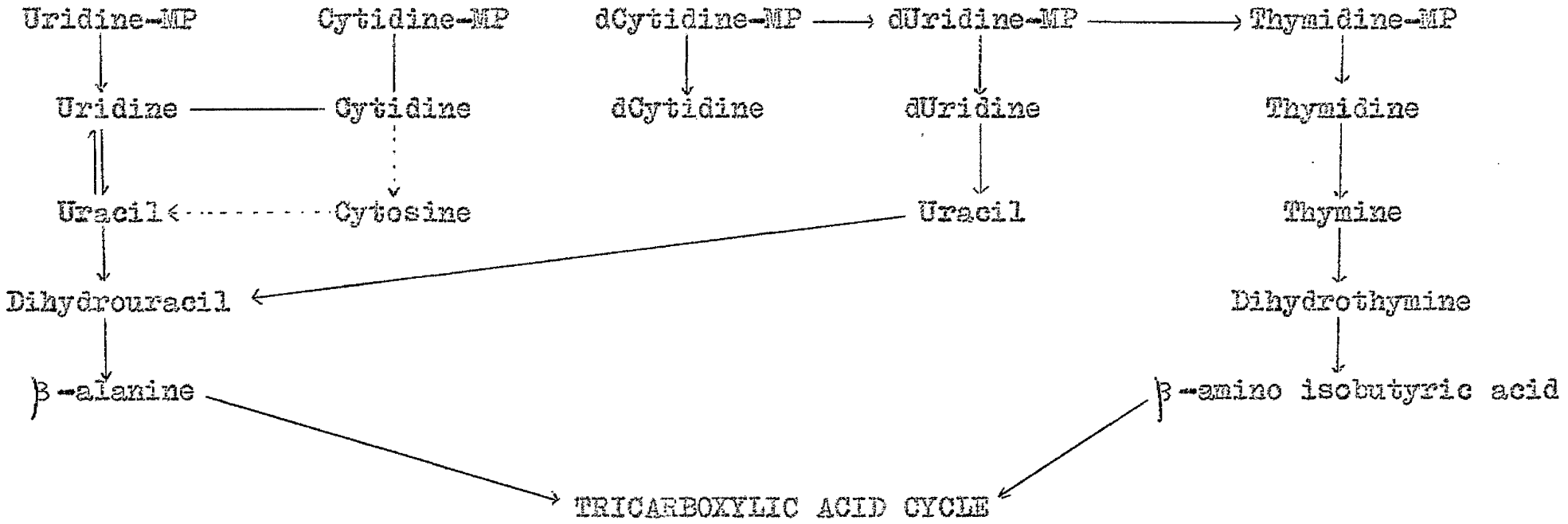


FIGURE 5

CATABOLISM OF THE PYRIMIDINES

The above scheme is concerned only with the essential catabolic steps and is derived from Potter (1960). Reversible reactions are indicated by a double-arrow. Those absent from animals but occurring in micro-organisms and/or plants are shown by a broken arrow.

The prefix 'd' stands for 'deoxy'; the suffix 'MP' for 'monophosphate'.

is present in animal tissues. Micro-organisms have a cytosine deaminase which achieves this conversion at the level of the free base. The deoxyriboside of cytosine is deaminated at the level of the nucleotide to yield deoxyuridine monophosphate. This, in turn, may be degraded to uracil by the consecutive actions of a nucleotidase and nucleoside phosphorylase. An alternative pathway for deoxyuridine monophosphate exists in the form of its conversion to thymidine monophosphate by the enzyme thymidylate synthetase in a very complex reaction involving tetrahydrofolate and a 1-carbon-donor. This reaction is probably an important step in the formation of thymidine derivatives and should perhaps be regarded as synthetic rather than catabolic; but it does nevertheless constitute a device whereby the cytosine ring might eventually be degraded to -amino isobutyric acid in company with thymine.

ADENOSINE DEAMINASE

The discovery of this enzyme is attributed to Gyory and Rothler (1927). Its distribution in animal tissues was studied by Conway and Cooke (1939) who reported that the highest concentrations were in appendix, intestine, spleen and testicles. Considerable purification of the enzyme from aqueous extracts of calf intestinal mucosa was achieved (Brady, 1942; Kalckar, 1947b; Kaplan, 1955). More recently, column chromatography has been employed to purify it still further (Coddington, 1960; Brady and O'Connell, 1962). These preparations are absolutely specific for adenosine and deoxyadenosine; substrates bearing a phosphate radical or incorporating minor changes in the purine or sugar moiety are quite immune (Schaedel, Waldvogel and Schlenk, 1947; Coddington, 1960). Brady (1942) reported that although deoxyadenosine combined more rapidly with the enzyme than adenosine did, it was deaminated at only half the rate of the latter. Coddington (1960) on the other hand, found the rate of deamination of deoxyadenosine to be a little more rapid than that of adenosine.

The pH optimum for the enzyme is close to 7.0 though considerable activity is found on either side of neutrality (Kalckar, 1947; Brady and O'Connell, 1962). The iso-electric point of a preparation homogeneous in the ultracentrifuge was at pH 4.85, but electrophoresis on starch gel showed the presence

of at least 4 individual components, (Brady and O'Connell, 1962). The effect of various possible inhibitors was studied by Brady (1942) who reported pronounced depression of activity by heavy metals and strong oxidants.

The distribution of adenosine deaminase activity in plants and micro-organism as well as in animal tissues has been widely reported (for bibliography consult Brady and O'Connell, 1962), Among those enzymes which have been partially characterised, mention should be made of the following: an enzyme from rat epithelioma which has been purified by ammonium sulphate fractionation and shown to have the same specificity as adenosine deaminase from calf intestine (Yagi and Khouvine, 1956); an enzyme present in rat kidney homogenates and separating with the albumin fraction of the supernatant (Zydowo, 1959); an enzyme from E. Coli which resembles that of calf intestine in its pH optimum, specificity, and behaviour with various inhibitors (Koch and Vallee, 1959); an enzyme of chick duodenum which is distinguishable from the calf intestinal enzyme in respect of the ratio of its activity towards various substrates, its energy of activation, and the effect of pH upon its Michaelis Constant (Chilson and Fisher, 1963); and an enzyme purified from the supernatant of human leucocytes which behaves electrophoretically as an α_2-globulin (Karker, 1965). The enzyme is present predominantly in the supernatant fraction of mouse and rat liver (Schneider and Hogeboom, 1952) and in the supernatant fraction

of rat and rabbit brain (Jordan, March, Houchin and Popp, 1959); it has been classified as a supernatant enzyme by De Duve et al., (1962).

Factors Affecting Intracellular Activity

A number of factors have been shown to influence the adenosine deaminase activity of cells and tissues. In E. coli, for instance, it is increased if purines are added to the culture medium (Koch and Vallee, 1959). It has been claimed that a similar adaptive increase can be obtained in chick embryos by injecting adenosine (Gordon and Roder, 1953) and in mouse liver by injecting xanthine (Feigelson and Wood, 1954). Subsequent investigation has however failed to confirm the chick experiments (Solomon, 1960). There is some evidence to relate adenosine deaminase activity to growth. Gordon and Roder (1953) claimed that during the growth of the chick embryo, activity per mg. nitrogen increased. Solomon (1960) was unable to confirm this finding; but subsequently it was reported that whereas the activity of the whole embryo was unaltered during embryonic development, a 40-fold increase in enzyme content per mg. of tissue took place in the duodenum of the chick embryo over a two-week period (Fisher, Chilson and Chan, 1962).

An increase in adenosine deaminase activity takes place in regenerating rat liver (Thomson and Moss, 1955); this

increase is not prevented by adrenalectomy. In normal rat liver, neither adrenalectomy, or hypophysectomy with or without subsequent administration of growth hormone affect adenosine deaminase activity (Reid and Stevens, 1958); nor does massive administration of thyroxine (Reid, 1960). Livers of rats maintained on a low protein diet display low adenosine deaminase activity unless a glucocorticoid is simultaneously administered (Nakata, Itoh, Nakata and Sakamoto, 1964), but this effect could be indirect, and due to the action of the hormone on protein or carbohydrate metabolism.

Adenosine deaminase activity of rat liver was found to be unaltered by 600r whole body irradiation (Eichel, 1955). A similar dose increased the specific activity in the spleen by 32% over the first three days, but since this was accompanied by shrinkage of the organ, the total activity was diminished by 21% (Eichel and Roth, 1960). These results with spleen were confirmed in experiments employing deoxyadenosine as substrate and using a dosage of 1000r (Roth, Wagner and Koths, 1964); the same paper describes a fall in the activity of the whole thymus under these conditions.

Activity in Relation to Cancer

In contrast to most other enzymes of purine catabolism, adenosine deaminase is higher in Novikoff hepatoma transplants than in normal liver whether activity is related to wet weight,

protein content or cell number (deLamirande, Allard and Cantero, 1958). Activity also increases during the development of Flexner-Jobling carcinoma, (Fodor, Tomashefsky and Funk, 1958). Reid and Lewin (1957) found no change in adenosine deaminase per g. wet weight of liver during azo dye carcinogenesis despite a fall in activity of other purine catabolising enzymes. But subsequent workers have found that activity per mg. nitrogen doubles in the pre-cancerous liver, no further increase taking place when the actual cancers develop (Chan, McCoy and Kizer, 1959). This increase in adenosine deaminase activity during azo dye carcinogenesis has been confirmed by de Lamirande and Allard (1959) and by Fiala and Kasinsky (1961), though the latter workers have produced evidence suggesting that the increased activity might be related to the metabolism of the dye rather than to the initiation of the malignant process. Recently, Roth, Sheid and Morris, (1963) examined the ability of several hepatomas to deaminate deoxyadenosine; compared with normal liver, activity was increased in Novikoff and McCoy hepatomas, decreased in the Dunning hepatoma, while different strains of Morris hepatoma showed marked variation in activity, some being increased and some decreased. If increased adenosine deaminase is a characteristic of tumours, it might be expected to diminish if the tumour regressed. But activity in Flexner-Jobling carcinoma remained high during regression (Fodor et al., 1958); and in Sarcoma 37, it was not affected by administration of the anti-tumour agent acetylpodophylotoxin-ω-pyridinium chloride

(Waravdekar, Paradis and Leiter, 1955).

Adenosine deaminase activity was first detected in the blood of man and other mammalism species by Drury, Lutwak-Mann and Solandt (1938). The level of enzyme in the serum is high in infants and falls gradually during growth and development (Sassowa, 1962). A report that over 90% of human cancer subjects have activity outside the range shown by normal subjects (Straub, Stephaneck and Acs, 1957) received partial confirmation from a study in which the majority of the subjects had bronchial carcinoma (Letnansky and Seelich, 1958). It was subsequently shown that rats bearing fibrosarcomata develop higher levels of serum adenosine deaminase activity although this is neither an early nor an invariable concomitant of tumour growth (Villela, Abreu and Abreu, 1962). Other investigators, while confirming that cancer populations have statistically high levels of serum adenosine deaminase activity than normal populations, have been unable to obtain such precise separation of the two populations as was claimed by Straub et al., (1957) and have concluded that the estimation is valueless as a means of cancer diagnosis (Schwartz and Bodansky, 1959; Koehler and Benz, 1962; Smyth, 1963).

BIOLOGICAL EFFECTS OF RADIATION

The use of radiation in cancer therapy, based upon the sensitivity shown by rapidly dividing cells to ionising radiations and the relative insensitivity of non-dividing cells, has long been established as a procedure of the utmost value. Unfortunately, the biochemical steps which initiate the sequence of events leading to genetic damage, inhibition of mitosis, and cell death are but little understood at present. This is particularly so for the intact mammalian organism, where the variability in response shown by mixed cell populations and the effects of such ill-defined factors as the liberation of tissue break-down products, the reduction of food intake, and the activity of the endocrine glands will conspire to mask the primary mechanism (Kelly, 1957; Errera, 1959).

Attempts to define the locus of the initial radiation effect in terms of a primary metabolic lesion have focused considerable attention upon the role that might be played in this process by damage to DNA and to interference with specific enzyme systems. These two topics will now receive brief consideration, after which a fuller account will be presented of the effects manifested by ionising radiations upon nucleases.

Effect of Radiation upon DNA and its Metabolism

It is obviously easy to irradiate solutions of DNA in the test-tube and observe the nature and extent of the damage.

Early work on these lines pointed to the formation of labile phosphate esters; attack on the sugar component, most probably at -C(4'); and liberation of free bases (Scholes and Weiss, 1953; Daniels, Scholes and Weiss, 1953). Further studies have shown that 80% of the free radicals formed by ionising radiations in the presence of oxygen attack the bases and 20% the sugar component of DNA (Scholes, Ward and Weiss, 1960; Scholes, 1963). Unfortunately they tell us little about what happens to the DNA inside an irradiated cell.

In vitro studies on cellular components and on DNA isolated from irradiated cells have shown that spontaneous depolymerisation may take place with separation of the nucleic acid from its accompanying histone and release of acid-soluble components, loss of the ability of DNA and isolated nuclei to form gels and to swell in solution (Holmes, 1957).

From animal experiments it appears that in general, irradiation causes a fall in DNA concentration which is accompanied by an accumulation of nucleotides and nucleosides in radiation-sensitive tissues, but the time sequence of these changes is complex, suggesting an initial inhibition of DNA synthesis followed by stimulation of DNA catabolism (Bishop and Davidson, 1957). This may lead to the appearance of breakdown products of these compounds in the urine. Thus, rats after irradiation with 300-600r showed an increase in the excretion of deoxyribose-containing compounds in the urine, mainly accountable as deoxycytidine; if nephrectomy was carried

out prior to radiation, deoxyribosides accumulated in the tissues (Parizek, Arient, Dienstbier, and Skoda, 1958). Moreover, the amount of deoxycytidine excreted was proportional to the dosage of radiation (Parizek, 1960). More recently, studies have been reported on two humans subjected to whole-body irradiation; one of the patients excreted deoxycytidine for 24 hours after treatment while the other excreted deoxycytidine and thymidine for 42 hours after treatment, the urines of these patients being free of both deoxynucleosides during a 3-day control period (Berry, Saenger, Perry, Friedman, Kereiakes and Scheel, 1963). β-amino isobutyric acid, a substance produced in the catabolism of thymine (Fink, Henderson and Fink, 1951 ; 1952 ; Fink, Cline, Henderson and Fink, 1956) was elevated in the urine of 8 humans exposed to excessive doses of radiation as a consequence of explosion in a nuclear reactor (Rubini, Cronkite, Bond and Fliedner, 1959), but no such increase could be found during therapeutic radiation of human subjects (Berry, 1960).

In so far as the other aspect of DNA metabolism - namely synthesis - is concerned, the situation is not by any means clear-cut. This is partly due to the variety of cellular systems in which the process has been examined and the multiplicity of methods used to measure DNA synthesis. In a review published several years ago, Kelly, (1957) concluded that there was very little evidence for a specific lesion of DNA synthesis, and that mitotic inhibition was the result of an independent

process which might ultimately inhibit DNA synthesis by blocking cell division. As summarised by Lajtha (1960) the available evidence strongly suggests that inhibition of mitosis and chromosome damage occur with much smaller doses of radiation than would be necessary to produce inhibition of DNA synthesis. Nevertheless, while X-rays have no effect upon these synthetic processes in cells already synthesising DNA, if the radiation is administered before synthesis commences, marked delay or even complete inhibition may result, depending on the dosage. Goutier (1961) who has discussed this topic from an enzymological standpoint considers that in these situations where interference with DNA synthesis occurs, inhibition may be ascribed to two factors: a) decrease in phosphorylation of nucleotides leading to nucleotide triphosphates; b) inhibition of DNA polymerase.

General Effect of Radiation upon Enzyme Systems

There has been no lack of experiments in which purified enzymes have been irradiated *in vitro*, but their relevance to the present discussion is limited because the dosages involved were much higher than those employed for therapeutic purposes and in animal experiments. Moreover, even in such a simple experimental system, the observed effects depend largely on whether the enzyme is irradiated as a dry powder or in solution, whether the substrate is present, what ions are present, and whether oxygen is excluded from the environment (Augenstine, 1962). It is obviously hazardous to try to extrapolate the

results of such experiments to the intact organism. There is however an extensive literature on the effects of irradiation in vivo on the enzyme activities of animal tissues. Indeed there have been few enzyme systems which have escaped such examination; the most important findings have been summarised critically in the reviews by Holmes (1957) and by Stocken (1959). The general picture which emerges is as follows:

1) Profound changes in the enzymes controlling fat metabolism lead to great increases in the rate of incorporation of labels into neutral fats, phospholipids, and cholesterol; these changes are not affected by adrenalectomy.

2) An increase takes place in liver glycogen which is attributable to reduction in the rate of phosphorolysis, of glycolysis, and of aerobic oxidation as well as to increased gluconeogenesis from amino acids. None of these changes occur in the adrenalectomised or hypophysectomised animal.

3) Break-down of tissue proteins with increase in the excretion of urea occurs. This is partially dependent upon an intact pituitary-adrenal axis.

4) Oxidative phosphorylation in isolated mitochondria decreases. This may be due to loss of cytochrome C, since it can be partially offset by addition of purified cytochrome C to the system.

5) Alkaline phosphatase is lost from bone; liver transaminases are increased, but equally marked elevations occur during starvation or cortisone administration.

6) Increases occur in adenosine triphosphatase and 5'-nucleo-

tidase of spleen when the whole animal is irradiated but not when radiation is confined to the spleen alone.

7) It is a difficult matter to separate the effects of radiation upon an organ from those occurring in non-specific stress reactions or as a response to damage to neighbouring or distant parts.

EFFECT OF RADIATION UPON NUCLEASES

1. Ribonucleases

a) In Vitro Studies

A comparison of various alterations occurring in solutions of pancreatic RNAase exposed to irradiation under aerobic and anaerobic conditions pointed to a non-specific protein denaturation in the latter while specific oxidative effects were found in the former (Romani and Tappel, 1959). When solutions of RNAase were irradiated by UV-, X-, or Y- radiation, the inactivation which resulted could be reproduced by chemically-induced free radicals; protection against radiation and free radicals was afforded by the addition of a disulphide compound (Brighenti and Falaschi, 1962). When dry RNAase was irradiated, the presence of oxygen greatly reduced the subsequent enzyme activity while nitric oxide, if present during irradiation, had a protective effect. Changes in electron spin resonance demonstrated a correlation between loss of activity and stable free radical production (Hunt, Till and Williams, 1962).

Controversy exists as to the nature of the chemical changes resulting. Evidence was produced suggesting that -SH bonds are ruptured and that as a consequence a number of the non-ionised tyrosine residues are exterior-ised and subsequently ionised (Williams and Hunt, 1963). This view was refuted by Hayden and Friedberg (1964) who claimed that, with doses of Y-radiation up to 45 megarads, solid RNAase was resistant to splitting of disulphide links, and that when the enzyme was irradiated in solution, the rate of destruction of amino acids ran parallel to the decrease in

"hard to exchange amide hydrogens".* However, support has come from fluorimetric assay, and examination of the difference spectrum of irradiated RNAase, these procedures demonstrating a linear decrease of tyrosine content with increased radiation dosage which extrapolates to a loss of 3 tyrosine residues at 100% inactivation (Smith and Adelstein, 1965).

Two other studies have produced interesting results, though at megarad doses. RNAase in aqueous solution shows aggregation in the absence of oxygen; amino-acid analysis of the aggregate demonstrated that 1 lysine residue per molecule had been chemically altered (Shapira, 1963). When substrate analogues competitively inhibiting RNAase were added to solutions undergoing Y-irradiation, pyrimidine, but not purine, analogues showed a correlation between radioprotective effect and inhibitory capacity; a new protein with distinct ultracentrifugal, chromatographic and electrophoretic properties but with full enzymic capacity was isolated from solutions irradiated in the presence of 2' (+3')-cytidylic acid (Ukita and Waku, 1964).

b) Animal Experiments

The activity of RNAase per mg. nitrogen after 600 r whole body radiation was measured at pH 6.0 in rat liver homogenates; a slight elevation during the first three post-radiation days was followed by a fall to 25% of the normal activity by the 8th day, with a return to values considerably above those of controls by the 12th day (Roth, Eichel, Wase, Alper, and Boyd, 1953). In a later report, Roth (1956b) stated that a slight fall in liver mitochondrial RNAase measured at pH

* amide hydrogens of glutamine and asparagine

5.8 occurred during the first three days after radiation but the activity at pH 7.8 fell to 48% of the control value at day 5, recovering once again by day 12; the content of RNAase inhibitor in the supernatant decreased after 24 hours but was normal by the second day.

When rats were given 700 r whole body radiation there was a marked shift in enzyme activity of spleen homogenates from nuclei and particles to supernatant which, at 64 hours, had doubled its percentage share of the whole homogenate activity (Roth and Eichel, 1958). The specific activity of the whole spleen homogenate basedupon nitrogen content had doubled by 16 hours, but at 64 hours had fallen below the control value. The activity of the whole spleen at 16 hours had risen by 54%, representing a clear case of enzyme activation (Roth and Eichel, 1959).

Weymouth (1958) has published the results of a detailed investigation into the effects of radiation upon mouse thymus nucleases. After 160r, increased activity of both RNAases per mg. DNA was found, beginning at 20 minutes after radiation for alk. RNAase and 8 hours after for acid RNAase. Similar, but less pronounced, changes were provoked by injecting hydrocortisone. The author concluded that increase in alk. RNAase was likely to be due to destruction of an inhibitor, while increase in acid RNAase was probably related to shifts in the cell population of the organ.

The results cited above have received confirmation in an extensive report by Maor and Alexander (1963) who found no change in rat liver and kidney RNAase and only small changes in brain RNAase after 500-1000 r whole body radiation. A rise in activity of the whole thymus RNAase was found, the concentration per cell increasing 400-fold. Whole spleen activity was unchanged but the concentration per cell increased f

The elevations in activity of thymus and spleen RNAases were less when radiation was confined to the head, and were further diminished when whole body radiation was administered with head-shielding, but these modifications did not alter the loss of weight, of DNA, and of cells in the two organs.

Two reports of work outwith the animal kingdom merit attention. When seedlings of Zea mays were irradiated, RNAase activity was increased in the meristematic tissues and decreased in the scutellum, but the distribution of the enzyme between subcellular fractions was unaltered (Cherry, Hageman and Hanson, 1962). Survival of irradiated E. Coli was increased ten-fold when RNAase was added to the medium, this effect being accompanied by break-down of ribosomal RNA into sub-units; it was suggested that this may permit the salvage of RNA damaged by the radiation (Gardner, 1963).

2. Deoxyribonucleases

a) In vitro Studies

Pancreatic DNAase I is inactivated by ionising radiation, an increase in the rate even at low doses being observed in the presence of oxygen (Butler and Robins, 1962). Adsorption of the enzyme on to various surfaces exercised a protective effect which may have relevance for bound intracellular DNAase I (Fletcher and Okada, 1962). While irradiation destroys the catalytic function of DNAase I, its ability to bind with substrate is actually increased (Okada and Fletcher, 1962). Such increase in formation of enzyme-substrate complex also follows irradiation of the substrate (Okada, Kraunz and Gassner, 1960).

Exposure of isolated rat liver mitochondria to large doses of

Y-rays induced an increase in the level of DNAase II; subsequent treatment with sonic vibrations raised the level still further. The activity could not be attributed to solubilisation of the enzyme, which still remained attached to mitochondrial membranes. Surprisingly, when the whole homogenate was irradiated, no increase in DNAase II activity was found, the authors suggesting that the mitochondria might be protected by unidentified factors in the homogenate.

b) Animal Experiments

When rats were exposed to 500r, a slight and unsignificant elevation of liver DNAase I was found, DNAase II showing no change; in the spleen, on the other hand, the DNAase I activity was depressed and the specific activity of DNAase II per mg. nitrogen was significantly increased; but when the latter was related to the total weight of the organ, no change was apparent (Douglass, Fellas, Meschan and Day, 1954 ; Fellas, Meschan, Day and Douglass, 1954 ; Douglass and Day, 1955).

Assay of rat spleen homogenate prepared 30 minutes after radiation demonstrated a shift in the pH optimum of DNAase II towards the acid side, this shift being proportional to the dose administered and possibly due to solubilisation of a mitochondrial enzyme, since the percentage of the whole homogenate activity recovered in the supernatant was greatly increased (Goutier-Pirotte and Thonnard, 1956). A comparable study in rat liver showed the appearance of a second peak of DNAase II activity attributed to unmasking of inactivated enzyme by destruction of an inhibitor (Goutier and Goutier-Pirotte, 1957).

When rats were exposed to 750 r whole body radiation, a two-fold

increase in DNAase II activity of spleen and thymus was detected and was accompanied by an increase in deoxyribose derivatives in the tissues, but no changes were found in liver and kidney (Okada, Gordon, King and Hempelmann, 1957). These workers found that cysteine had no effect upon DNAase II activity, but in a re-examination of this problem, Goutier (1959) found that activity in the spleen of rats given a protective injection of cystine dihydrochloride did not rise beyond 2 days after 850 r, whereas this elevation continued in unprotected animals. Although DNAase II activity of sucrose homogenates of rat spleen is less than that of aqueous homogenates of the same organs, the relative increase in DNAase II after radiation is more pronounced when the spleens of test and control animals are homogenised in sucrose (Okada, Schlegel and Hempelmann, 1958). Further work by these investigators on the effects of radiation upon rat spleen and thymus demonstrated that, while the specific activity was elevated, the total organ activity fell to one-third of control values by the 7th day. Injection of cortisone caused increases in the specific activity of DNAase II in spleen and thymus comparable to those occurring after radiation; and even when the thymus was shielded, specific activity of DNAase II of this organ was still considerably elevated (Gordon, Gassner, Okada and Hempelmann, 1959). Evidence from histochemical investigations points to localisation of radioresistant cells rich in DNAase II with loss of radio-sensitive cells poor in this enzyme as an important mechanism in the increased specific activity of DNAase II in rat spleen and thymus (Aldridge, Hempelmann and Emmel, 1960), while a shift of activity from nuclei and particles to supernatant is a subsidiary mechanism in spleen (Roth and

Hilton, 1963). The view that increased DNAase II specific activity in thymus after radiation is due to changes in the cell population derives support from the work of Weymouth (1958) in mice.

A different view has been put forward by Kurnick and his associates, who examined the effects of radiation on a large series of mouse tissues. Enormous increases in acid DNAase of spleen, thymus, and bone marrow relative to DNA content occurred after radiation, and could be curtailed by injecting bone-marrow cells from healthy control animals, or, in the case of the thymus, by shielding the organ; few changes occurred in the liver or kidney. Because the level of DNAase I inhibitor of spleen fell with irradiation and rose to normal subsequent upon injection of marrow cells, destruction of a similar and hitherto unidentified inhibitor of DNAase II was postulated (Kurnick, Massey and Sandeen, 1958 ; 1959). This suggestion has however been modified as a result of further work on mouse spleen after whole body radiation with and without spleen shielding, and radiation to spleen alone. When the spleen is directly irradiated, there is a loss of lymphocytes poor in DNAase II together with inactivation of the enzyme in the DNAase II - rich cells remaining, so that the overall activity per cell is unchanged. When whole body irradiation with spleen shielding is carried out, an even greater loss of DNAase II - poor lymphocytes takes place while the activity of the remaining cells is increased, leading to an enormous increase in the activity of the organ when measured per cell, this increase being proportional to the dose of X-rays up to 800r (Kurnick, Massey and Montano, 1960 ; Kurnick and Nokay, 1962).

It is clear from the work cited above that the normal liver shows little sensitivity to radiation, for which reason it is of special interest

that Goutier-Firotte and Goutier (1962) have demonstrated what they believe to be a lesion of the lysosomes in irradiated regenerating rat liver. This at any rate is their interpretation for a doubling of the percentage of the total DNAase II content of the homogenate recoverable in the supernatant fraction when regenerating rat liver was irradiated within 18 hours of hepatectomy. Although normal liver does not demonstrate this effect, a latent lesion must exist, since identical findings were obtained if hepatectomy was carried out two hours after irradiation.

Administration of 700 r to rats brought about a gradual elevation in the urinary DNAase I excretion to 5 times the pre-irradiation level by the 5th day, the level remaining high for a further 8 days. On the other hand the level of urinary DNAase II rose to 5 times the basal level at 18 hours, and was normal once more by the 9th day. When 300r were administered, no change was detected in urinary DNAase I, and the level of DNAase II was only 50% above that of the control period at 18 hours (Kowlessar, Altman and Hempelmann, 1953 ; 1954). After an unspecified dose of radiation, rat plasma DNAase I was doubled at 18 hours and 5 times the basal level at 6 days, whereas DNAase II was ten times elevated at 18 hours and only 4 times the basal level at 6 days (Kowlessar, Altmann and Hempelmann, 1955). The increase in serum and urine DNAases occurs when splenectomised rats are subjected to irradiation; moreover, splenectomy by itself raises DNAase activities in serum and urine to the same extent as irradiation, from which it was concluded that irradiation performs a functional splenectomy resulting in loss of metabolic regulation of enzyme activity by the spleen (Jovanović and Voncina, 1959).

Scope of Present Work

The present investigation was undertaken in an attempt to obtain data which might assist in providing answers to certain questions regarding the behaviour of nucleases, the related enzyme adenosine deaminase, and possible break-down products derived from DNA catabolism, in several human tissues subjected to a number of diseased states with particular reference to hyperplastic and neoplastic involvement. The questions to which answers were sought were as follows:

1) What is the level and distribution of these enzymes in the normal cytoplasm of these tissues, and what changes in level and distribution occur in hyperplastic and neoplastic states?
2) What are the effects of therapeutic radiation upon the level and distribution of these enzymes in malignant tissues, in the blood serum, and in their excretion along with DNA metabolites in the urine?

It was hoped that data pertinent to the first question might also throw some light upon the intracellular function of these enzymes and upon possible lesions in nucleic-acid catabolism arising as primary or secondary features of malignant growth. From these considerations it might be expected that suggestions leading to the development of diagnostic tests for the detection of malignancy would present themselves; and clear proof of an enzymological lesion would be an obvious target at which rational therapy could be directed. Moreover, comparison of a spectrum of enzymes in several quite different tissues and cancers thereof might give an indication of a pattern of behaviour common to

human carcinomata, or alternately might point to the lack of a unifying feature in these conditions.

So far as the second question was concerned, it was not unreasonable to assume that such an investigation, while unlikely to reveal the nature of the initial lesion in radiation, might at least provide some insight into possible modifications of the enzyme content of tumours occasioned by its use. Such changes, either in the tumour itself, or in the levels of enzyme activity in tissue fluids and urine, might suggest the importance of certain enzymes in tumour metabolism, and might form the basis for simple laboratory procedures that could be used to evaluate the response of a patient to therapeutic radiation.

To these ends, samples of three quite different human tissues were examined, these being an endocrine gland - the thyroid; an exocrine gland - the breast; and an epithelial tissue - the cervix uteri. In addition, the effects of radiation were studied in tumours of the latter organ and in the blood serum and urine of patients bearing such tumours.

MATERIALS AND METHODS

MATERIALS AND METHODS

Specimens of human breast, cervix uteri, and thyroid gland were obtained in the fresh state from patients undergoing operative proceedures or radium implantation, with the exception of the normal thyroid glands employed for study of DNA content; these were removed post-mortem within 12 hours of death.

Thyroid Gland

A total of 20 specimens was obtained from patients in whom the gland was removed because of failure to control the symptoms of thyrotoxicosis by medical means. Histological examination revealed the features of thyrotoxicosis in these specimens - namely epithelial hyperplasia with papillary growth into the vesicles, and areas of focal thyroiditis. The severity of these two changes varied enormously from gland to gland, but within the same gland these changes were uniformly present to the same extent.

Ten samples of thyroid adenomata were obtained from 9 patients; two of the adenomata were removed from the same patient at the same time, were found to have quite different morphological and histological features, and were situated in different lobes of the thyroid gland, hence each was treated as a separate sample. Of these ten samples, eight were discrete solitary adenomata and two consisted of glands replaced by multiple adenomata. One sample consisted of a well-defined Hurthle-cell adenoma; for the remainder, the histological changes displayed marked heterogeneity,

varying degrees of cyst formation, calcification, stromal degeneration, haemorrhagic necrosis, and focal thyroiditis.

Four glands taken from patients with Hashimoto's thyroiditis were studied. These tissues were macroscopically homogeneous, and on histological examination displayed the characteristic intense round-cell infiltration found in this condition, with varying degrees of Askanazy-cell change.

Samples of thyroid carcinomata were obtained from 5 patients. In four, the lesion was a primary one situated in the thyroid gland itself; in the fifth, the lesion was a metastasis to a neighbouring lymph-node, the substance of which was entirely replaced by adenocarcinoma of thyroid. Three of the primaries were papillary adenocarcinomata; the fourth was a highly anaplastic tumour with many mitoses and aberrant cells.

Samples of normal thyroid tissue were obtained from four of the patients with carcinoma and from 6 of the patients with a solitary adenoma. Histological examination confirmed that the tissue was essentially normal and was not involved in the pathological process which occasioned the removal of the gland.

The above material comprises those tissues used in the study of the enzyme activity of the thyroid gland and its distribution within the sub-cellular fractions of the cytoplasm. Henceforth, this material will be referred to as the First Series. A representative example of each histological type is shown in high-power magnification in Figure 6.

Upon collection, the samples were placed in ice-cold distilled water and washed repeatedly until no further blood could be removed from the tissue, whereupon they were thoroughly dried on absorbent paper, weighed,

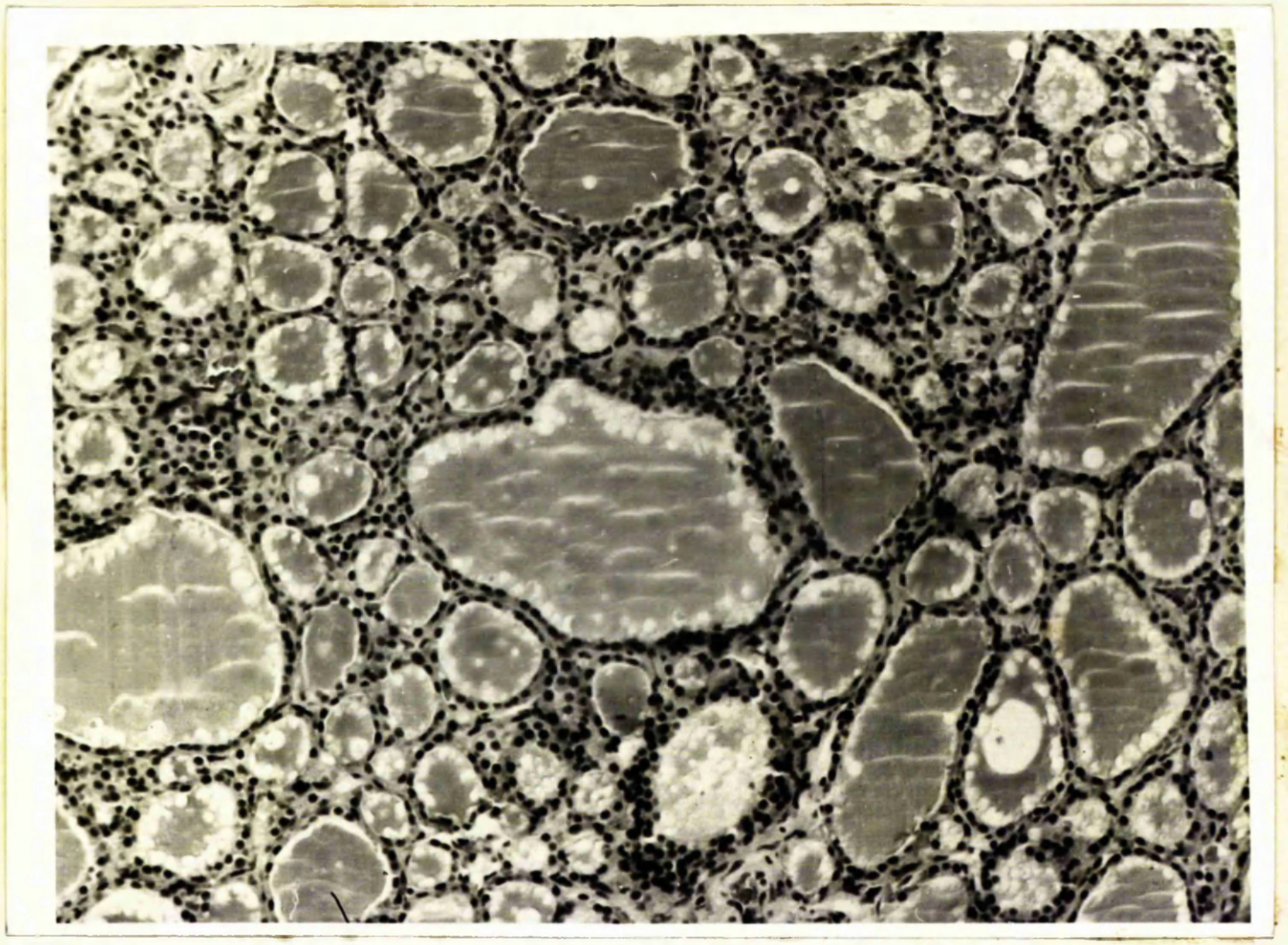


FIGURE 6a

NORMAL THYROID

The cells are uniform in size, flat in shape, and the vesicles are well filled with 'Colloid'.

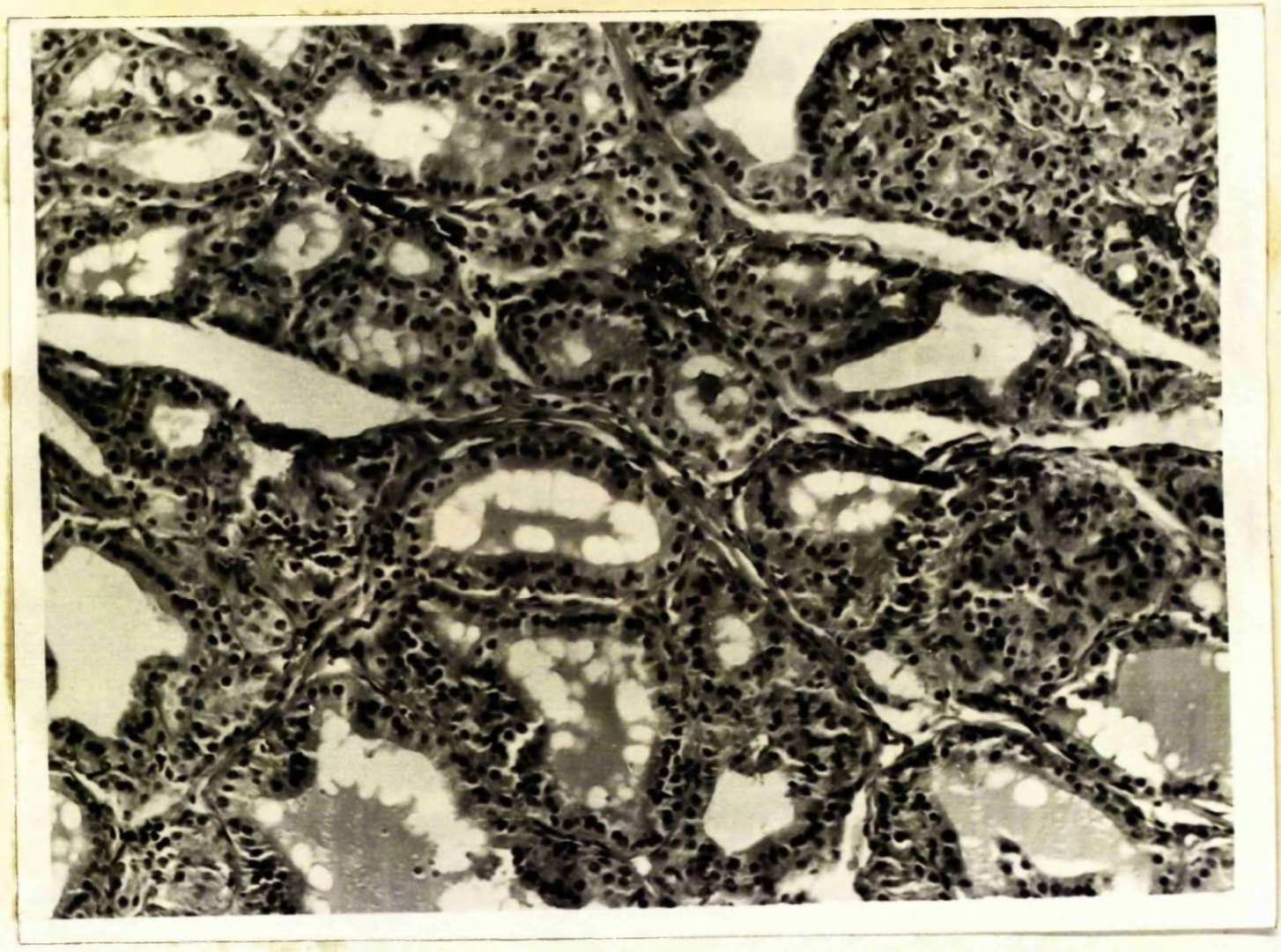


FIGURE 6b

TOXIC THYROID

This gland displays marked hyperplasia of the thyroid epithelium. The cells are columnar, and the ratio of cytoplasm to nucleus is increased. The vesicles are deficient in 'Colloid' and there is slightly increased stroma between the vesicles.

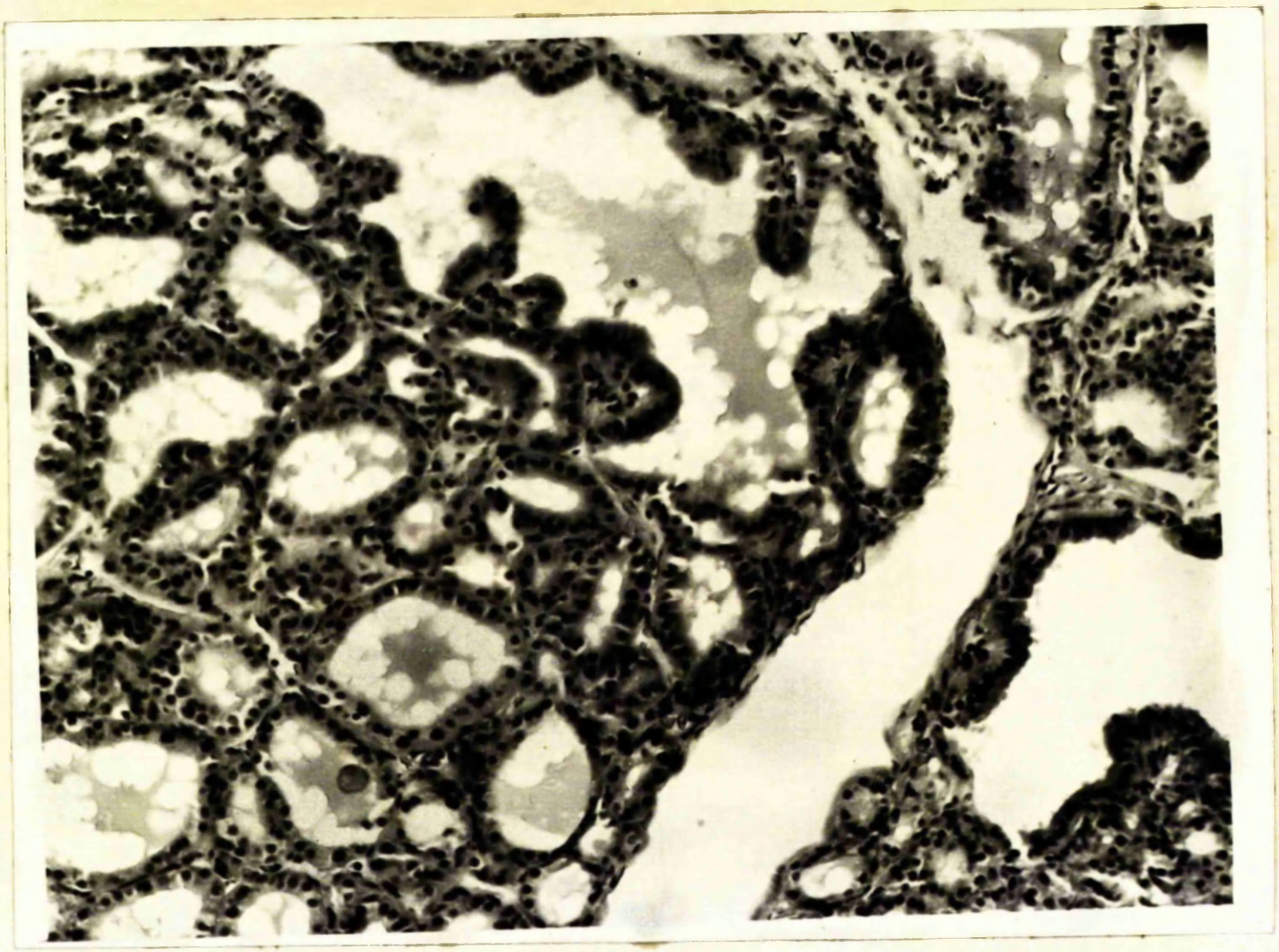


FIGURE 6a

TOXIC THYROID

Another sample of a hyperplastic gland taken from a patient with thyrotoxicosis. In addition to the features described in 6b, there are many cells, especially to the right of the field, whose nuclei are very much larger than those of normal cells.

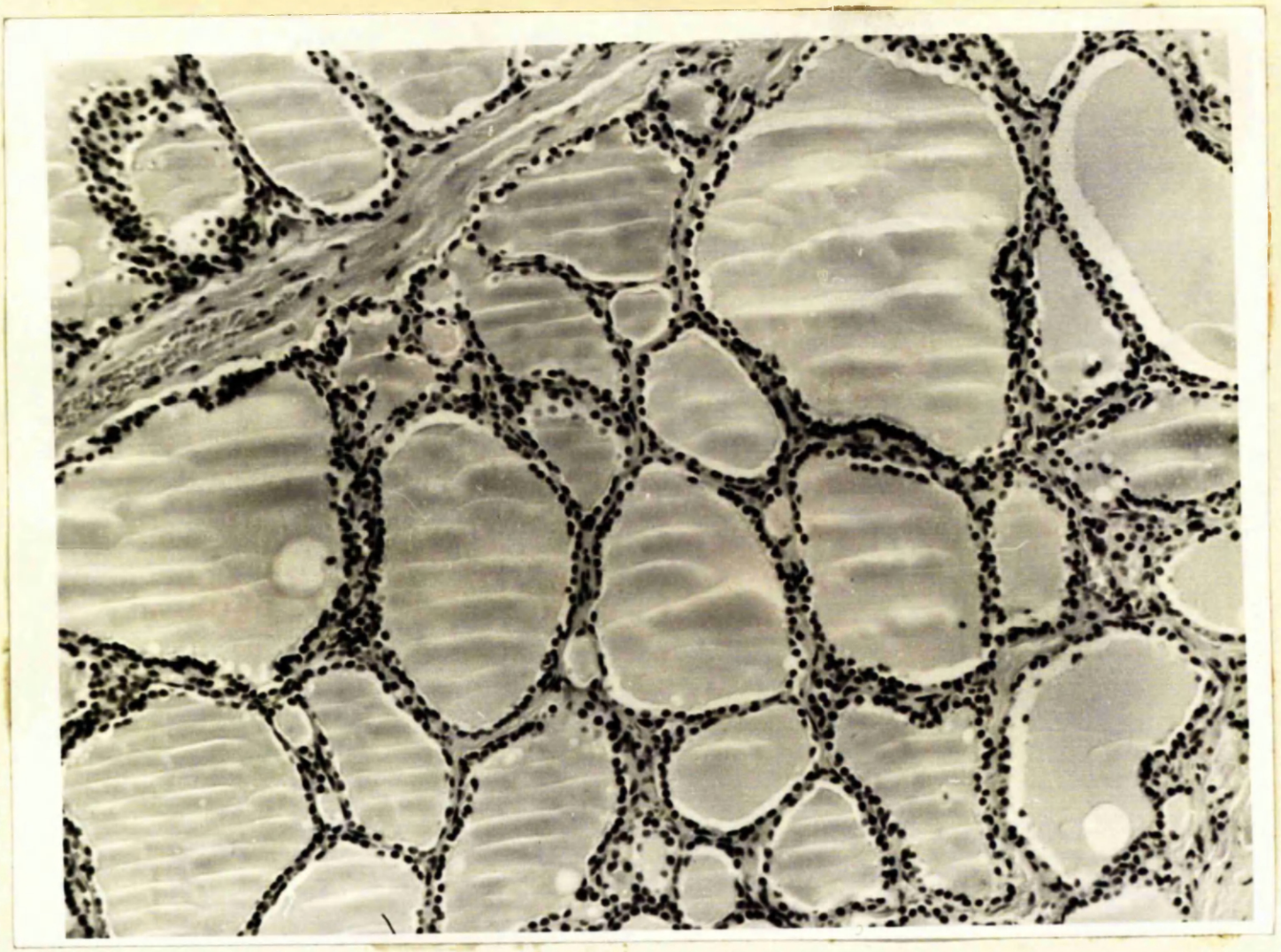


FIGURE 6d

TOXIC THYROID

This specimen bears no resemblance to those shown in 6b and 6c. Histologically speaking, it is more 'normal' than the 'normal' gland shown in 6a. Yet the subject was clinically thyrotoxic, and the diagnosis was confirmed by laboratory procedures.

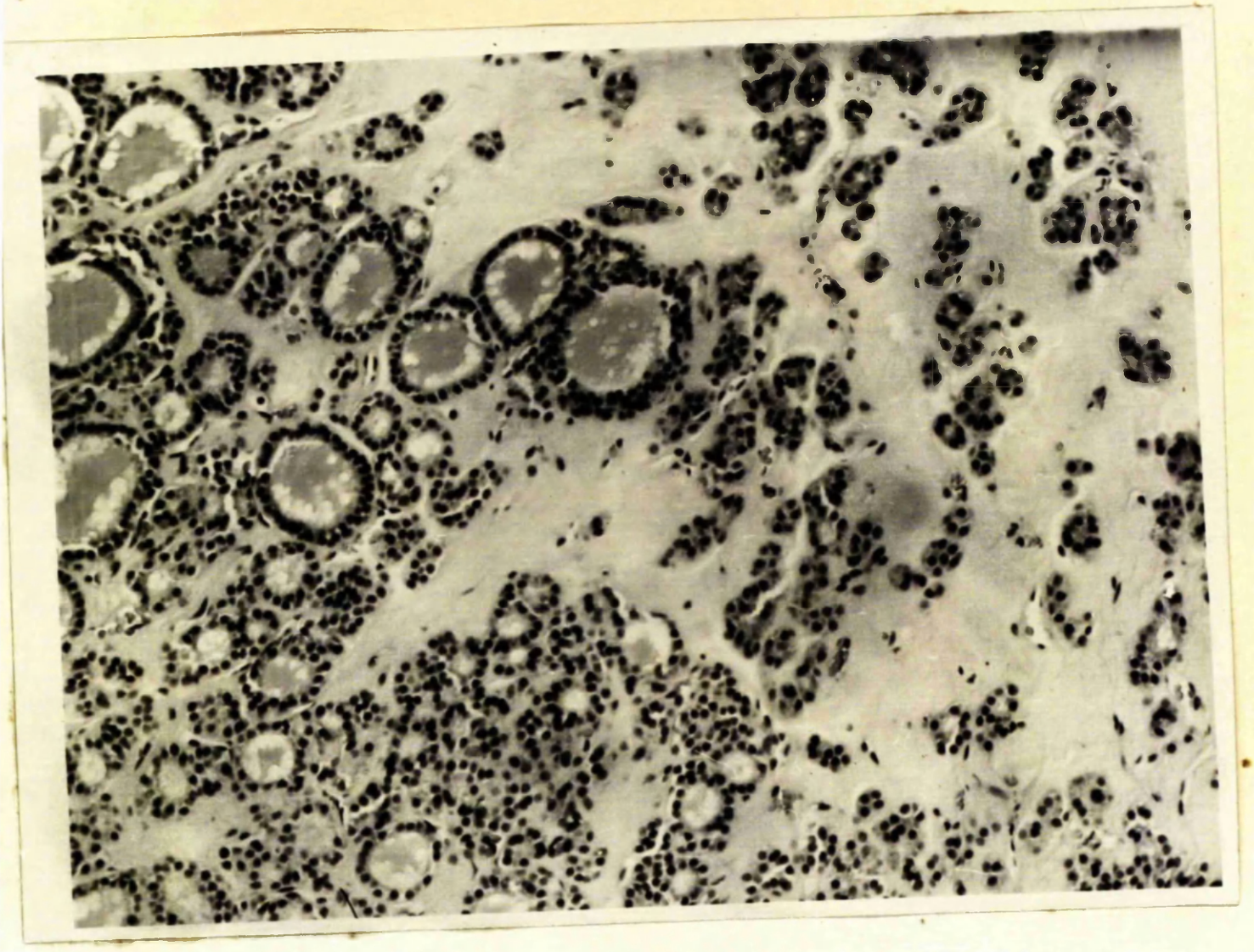


FIGURE 6e

THYROID ADENOMA

This field shows an area of micro- and macro-acini bounded by cells which are normal in appearance. The ratio of cytoplasm to nucleus is essentially normal. 'Colloid' storage is diminished and the architecture of the gland has largely disappeared. About 50% of the field consists of oedematous stromal tissue.

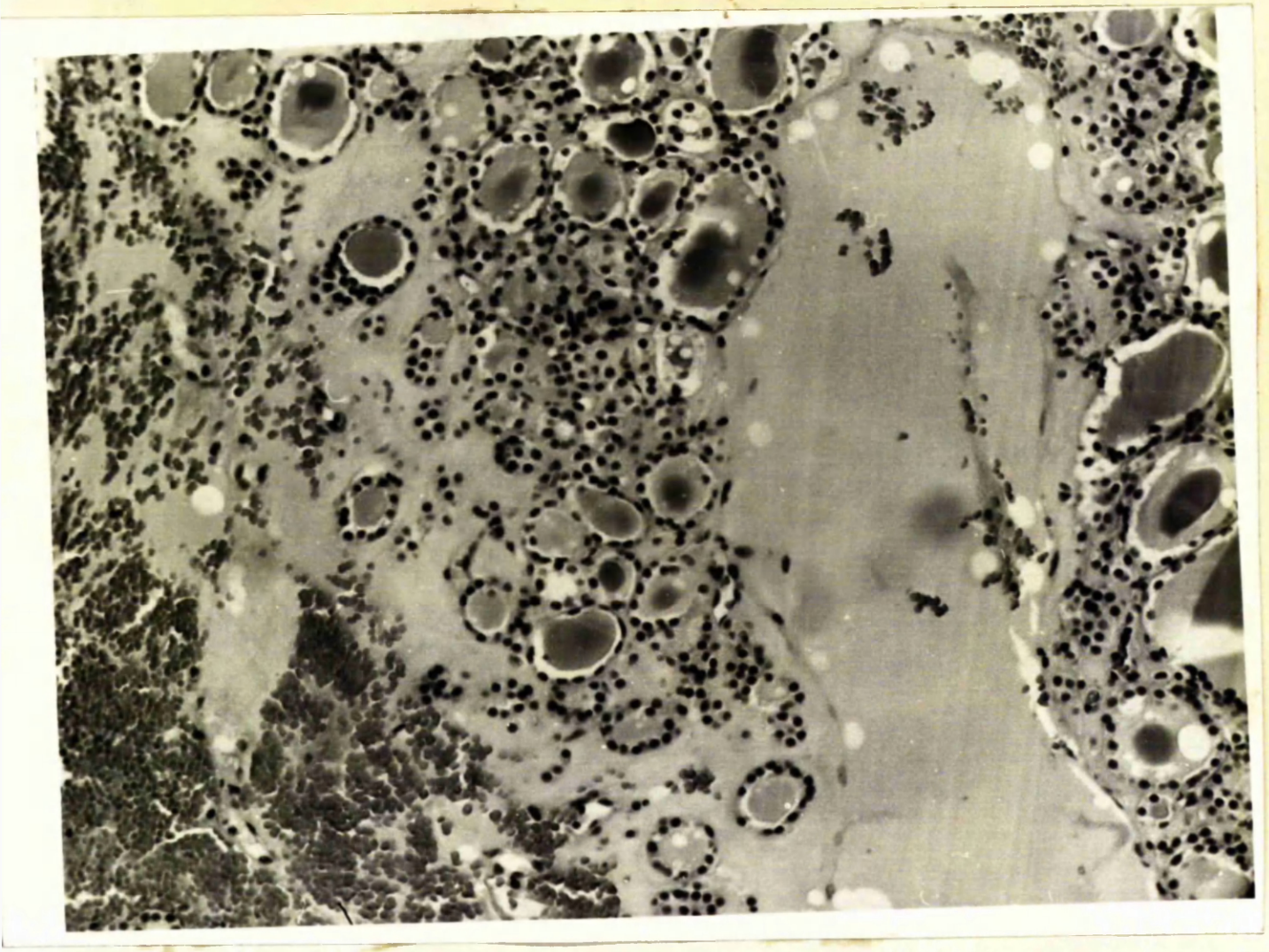


FIGURE 6f

THYROID ADENOMA

The cell type in this adenoma is much flatter than that of the previous adenoma shown in 6e. A large area of haemorrhagic degeneration may be seen in the bottom left-hand corner.

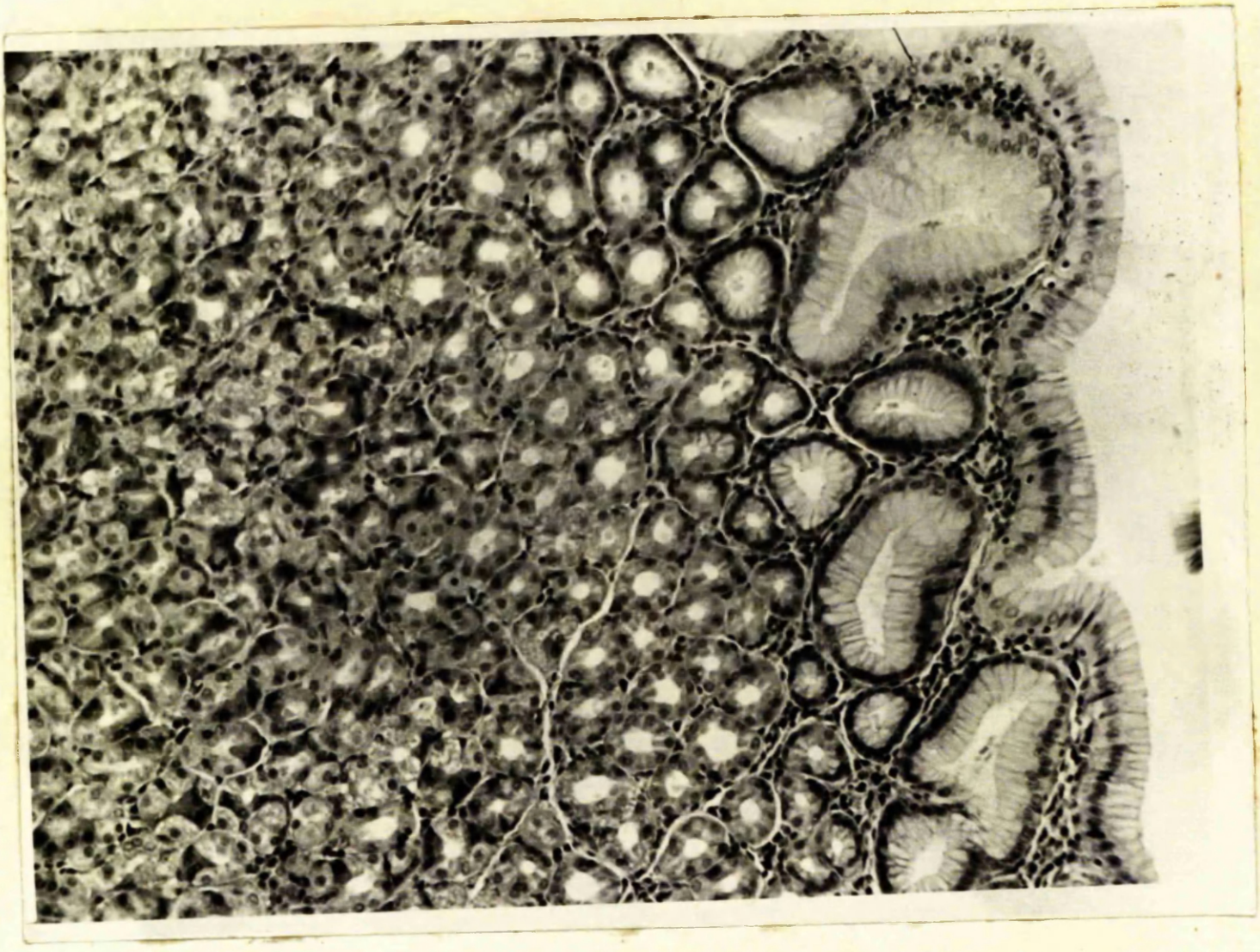


FIGURE 6g

HURTHLE CELL ADENOMA

The architecture of the thyroid is completely lost in this tumour which is relatively homogeneous, and is composed of large cells with normal nuclei and an increased ratio of cytoplasm to nucleus.

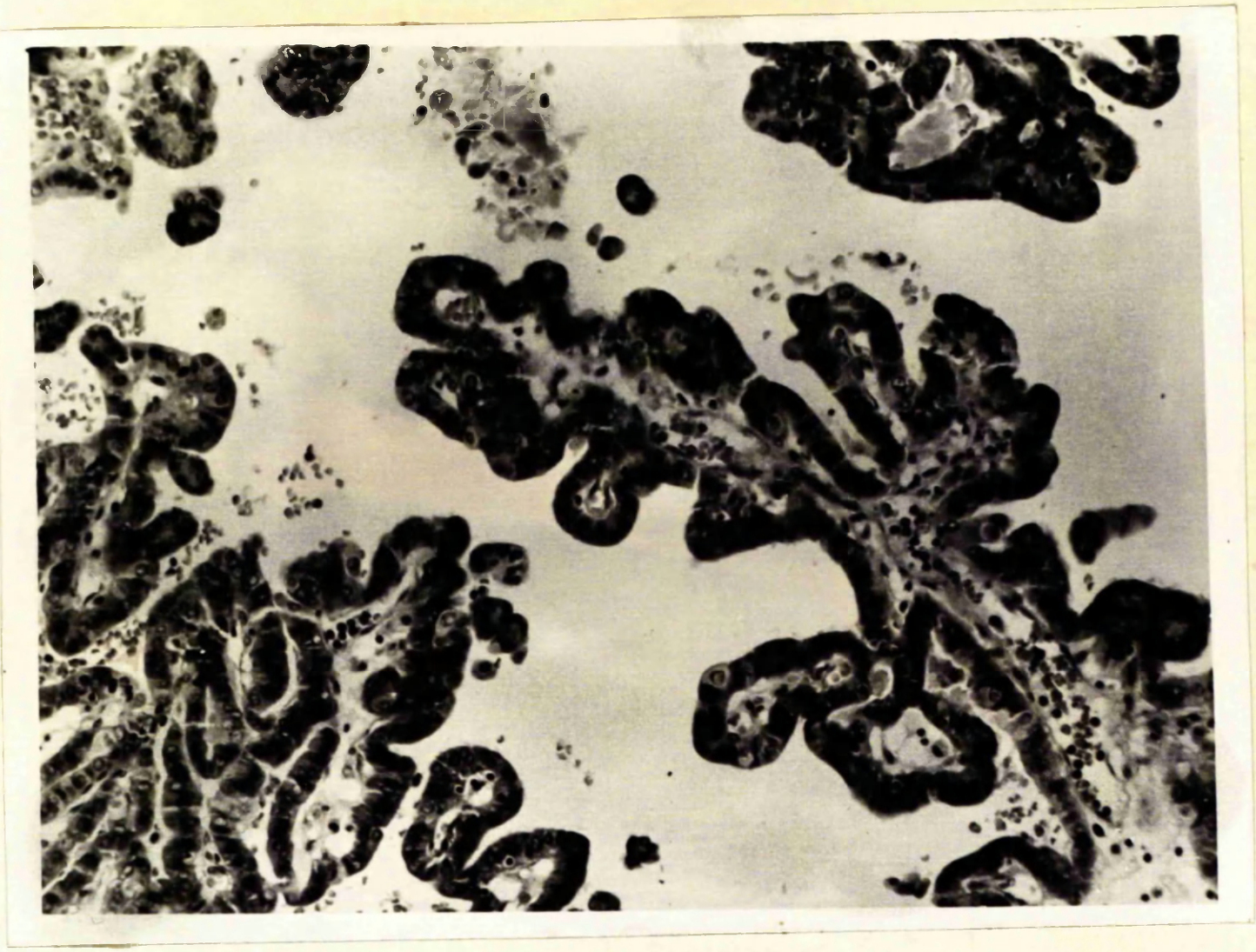


FIGURE 6h

THYROID CARCINOMA

This field shows part of a papillary carcinoma of thyroid. The cells are large, with enormous nuclei, and are supported on a backbone of fibrous stroma.

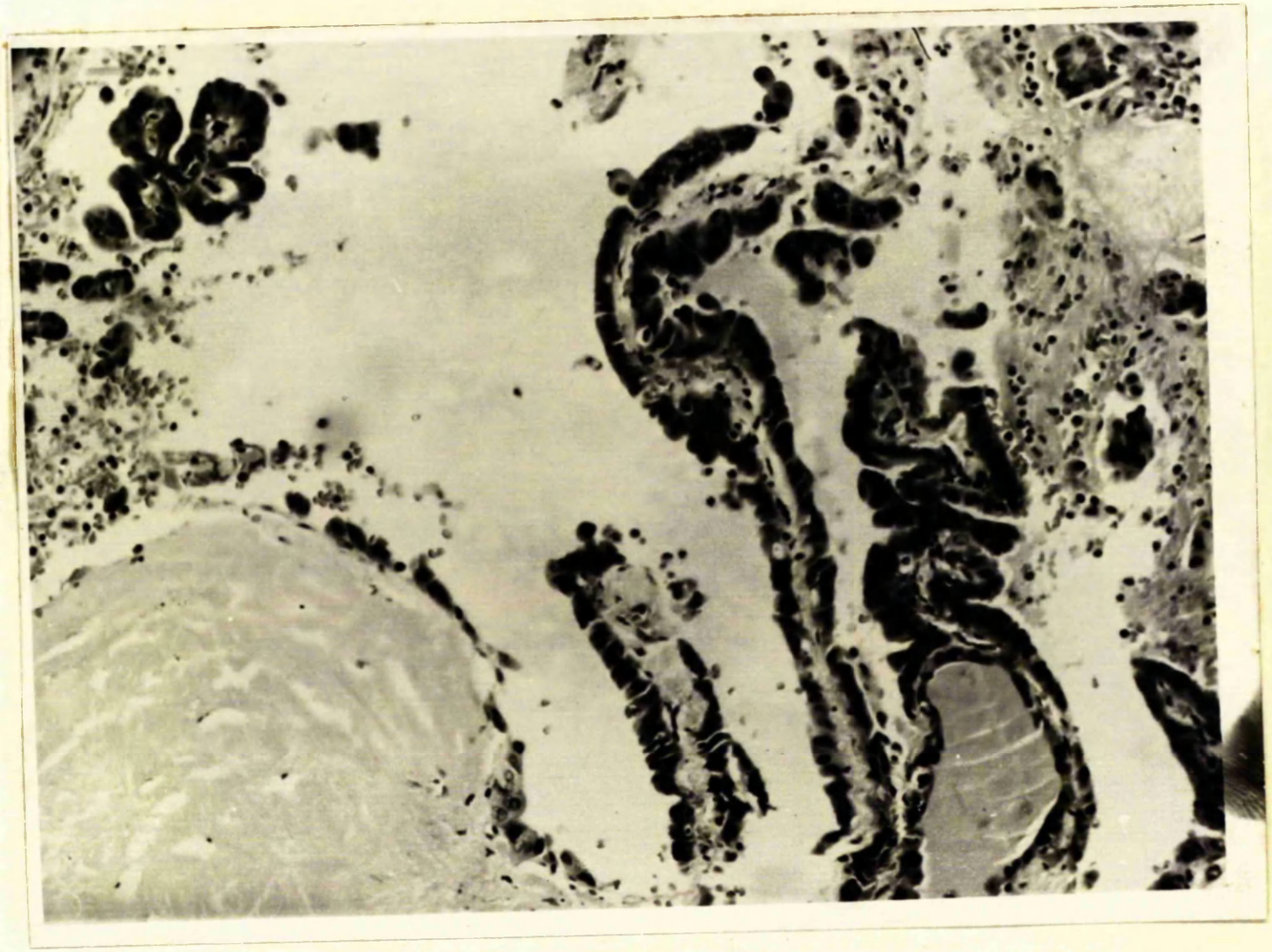


FIGURE 6i

THYROID CARCINOMA

This field is taken from the same papillary carcinoma as was shown in 6h. An area of hyaline degeneration is apparent in the lower left quadrant.

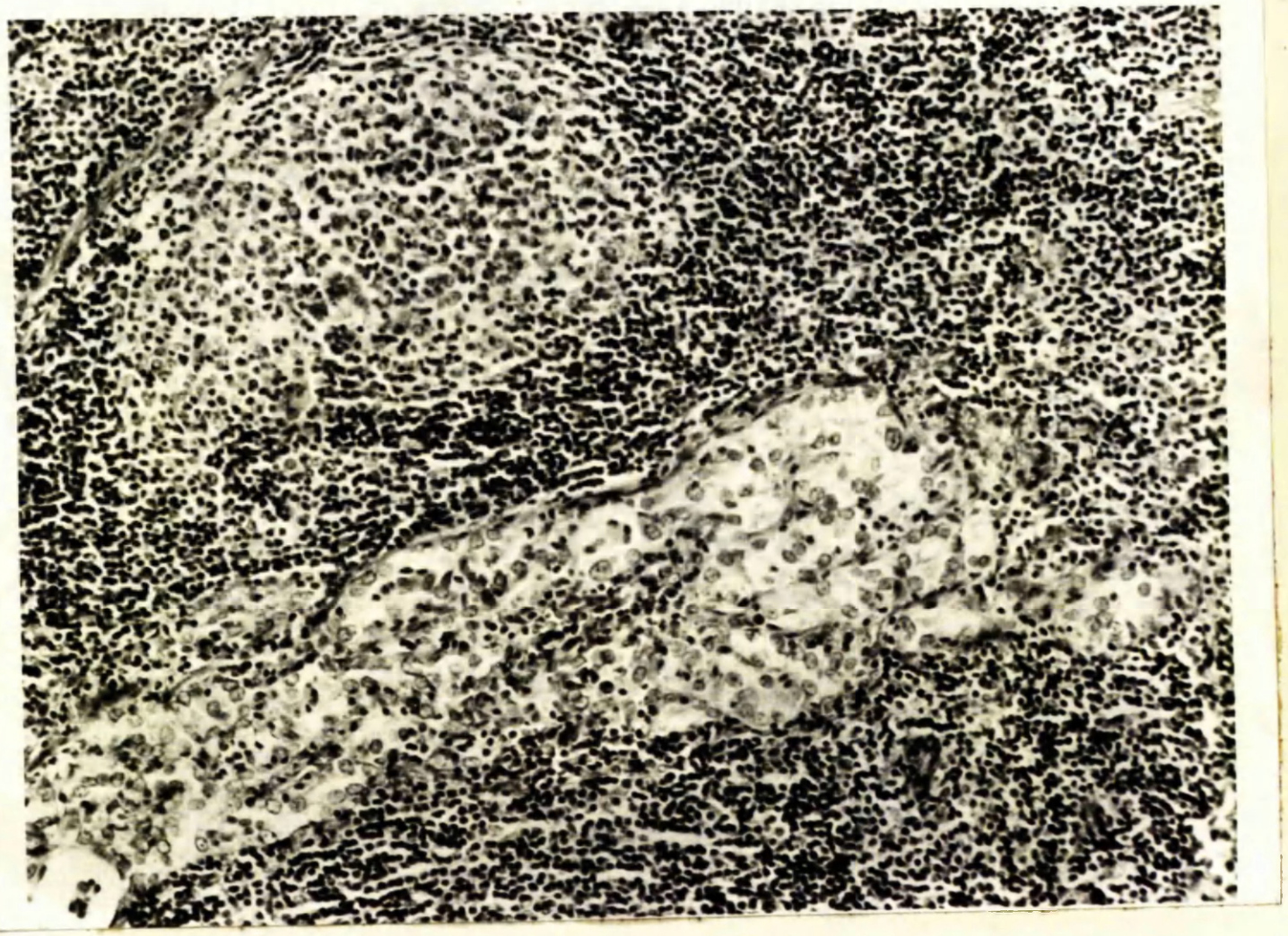


FIGURE 6i

HASHIMOTO'S THYROIDITIS

The cells found in the thyroid in this condition comprise large numbers of lymphocytes. These contain a mere rim of cytoplasm outside a nucleus which tends to be smaller than that of the normal thyroid epithelium. A well-organised lymphoid follicle may be seen in the upper left quadrant.

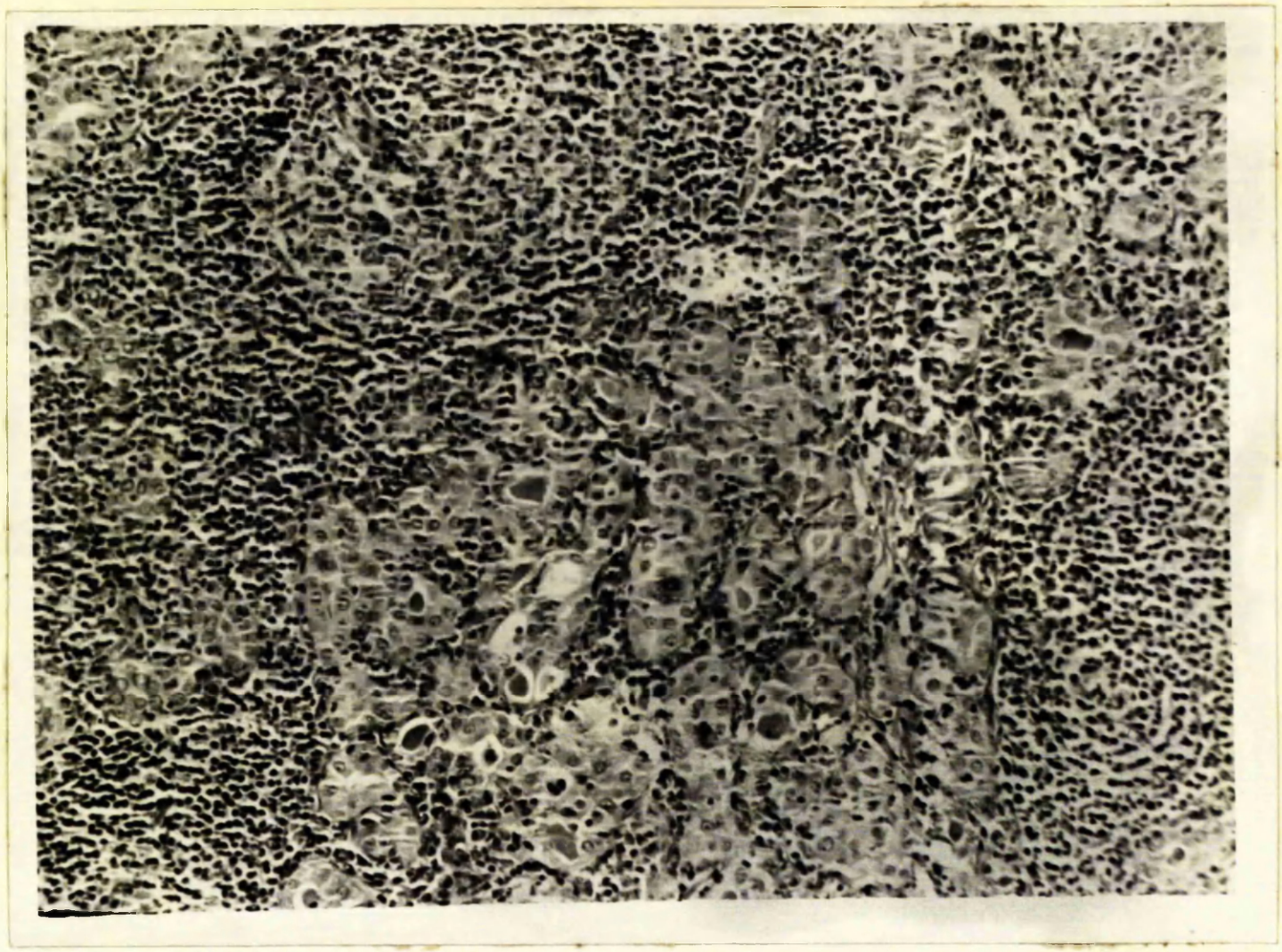


FIGURE 6k

HASHIMOTO'S THYROIDITIS

In addition to the features described in 6j for the previous example of this condition, the gland in the present subject displays the presence of groups of epithelial cells with increased cytoplasm, and morphologically similar to those comprising the Hürthle Cell Adenoma (See 6g).

and stored at -20°. Because these tissues were removed primarily for routine histological examination, it was necessary to await completion of certain formalities by the histologist during which interval the material was kept at 4° in a domestic refrigerator. The time elapsing between removal by the surgeon and deposition in the deep-freeze was of the order of 45 min. Rarely was an interval of one hour exceeded; on one occasion, the interval was more than two hours.

Specimens were kept at -20° for periods of one to four weeks. They were removed as required, allowed to thaw at room temperature, minced by hand, transferred quantitatively to a vessel containing five volumes of ice-cold 0.25M-sucrose, and homogenised for three minutes in an M.S.E. Blendor Model No. 7700 employing crushed ice as the refrigerating agent.

The homogenate was centrifuged for 10 min. at 500g in the M.S.E. 'Minor' Centrifuge Model No. 84220 to bring down the nuclei, this procedure being carried out in the Lightfoot cold-room to ensure refrigeration. The unsedimented cytoplasm was transferred quantitatively, avoiding nuclear contamination, to clean nylon centrifuge tubes. Three fractions were prepared from the cytoplasm by further centrifugation in an M.S.E. 'Superspeed 17' Refrigerated Centrifuge at 4°. A "Mitochondrial" fraction was obtained by centrifugation at 5,000g for 20 min. and from the unsedimented liquid a "Microsomal" fraction was obtained by centrifugation for 60 min. at 35,000g. The unsedimented liquid surviving this latter centrifugation is designated the Supernatant. Care was taken to ensure that the separation of unsedimented from sedimented layers was quantitatively carried out within the limitations imposed by the necessity of avoiding contamination of one fraction by another. After collection,

the precipitated particles were washed separately by resuspension in a large volume of ice-cold 0.25M-sucrose and thoroughly dispersed by sucking them up and down in a Pasteur pipette, whereupon they were reprecipitated by centrifugation at 35,000g for one hour at 4°. Following this, the sucrose was decanted and each particle fraction was dispersed in an appropriate volume of ice-cold distilled water, the volume of the suspension then being accurately measured prior to rupture by ultrasonic disintegration. This was achieved by exposing the suspensions to 20Kc/sec. under refrigeration for 90 sec. in the M.S.E. Ultrasonic Disintegration Model 60W with Titenium Vibrator Probe of End Diameter $\frac{3}{8}$" and End Ratio 10:1. Rupture of the "Mitochondrial" fraction was accompanied by clarification of the suspension and formation of a coagulum of mitochondrial membranes which could be removed without difficulty. No such coagulum appeared when the "Microsomal" fraction was ruptured, although clarification occurred almost to the point where the fluid became transparent.

Enzyme assays were carried out on all three fractions on the following day with the exception of adenosine deaminase; this was estimated on an aliquot of Supernatant stored at -20° for one to four weeks. During the 12 hours elapsing between completion of the preparation and commencement of the enzyme assays, the material was stored in crushed ice and placed in a domestic refrigerator at 4°.

In addition to the material of the First Series, 15 other specimens of thyroid tissue were examined in an attempt to study the influence of cellularity, as measured by DNA content, upon the enzyme activity of the gland. These tissues consisted of 5 samples removed from patients undergoing surgical treatment of thyrotoxicosis; five samples of solitary

thyroid adenomata, two of which were from the same patient but presented quite different morphological and microscopical features; and five samples of thyroid tissue removed post-mortem from patients succumbing to diseases not known to affect the endocrine system in a specific fashion - these samples being regarded as "Normal" in what follows. The remarks made concerning the histological features of the various tissues used in the First Series apply with equal force to those of the Second Series.

After collection, the samples were washed, dried, weighed, and stored exactly as described previously. On the day of preparation, they were removed from the deep-freeze, allowed to thaw at room temperature, minced by hand, transferred quantitatively to a vessel containing 10 volumes of ice-cold 0.15M-potassium chloride and homogenised for five minutes in an M.S.E. Blendor employing ice as the refrigerating agent.

An aliquot of the homogenate was centrifuged for one hour at 35,000g in the M.S.E. 'Superspeed 17' Refrigerated Centrifuge at 4°, and the resulting Supernatant was removed by Pasteur pipette into small plastic containers for storage in the deep-freeze at -20°. Enzyme assays were carried out on the Supernatant one to four weeks after preparation. From the remainder of the homogenate, quadruplicate aliquots were taken for estimation of DNA by the methods of Ceriotti (1952) and of Munro and his associates (Hutchison and Munro 1961; Fleck and Munro 1962; Mallinan, Fleck and Munro 1963). Despite intensive research, the method of Ceriotti (1952) could not be made to yield consistent and reproducible results and was reluctantly abandoned. The method of Munro, though more tedious, proved more reliable. All the DNA results reported in this thesis have been derived from this method which will be described later in this section.

Cervix Uteri

A total of 25 specimens were obtained by curettage from patients with established carcinoma of the cervix under anaesthesia for insertion of the primary radium implant. These specimens comprise the Pre-Radiation Series. In 13 of these patients, a further sample was obtained by curettage one week later at the time of insertion of the second radium implant. In 3 of the 25, a second sample was obtained at the time of the second radium insertion, but in these three the interval between the two insertions was four weeks, and included a three-week course of deep X-Ray therapy. All 15 samples, obtained from patients already subjected to radiation, comprises the group which will be referred to as the Post-Radiation Series.

A report on the histology of the lesion was available in every case. This was based upon biopsy material taken one to three weeks before the patient presented for radium treatment. Two of the 25 lesions were adenocarcinomata and the remainder squamous carcinomata of the cervix. Special attention was devoted to the degree of differentiation of the lesion, its mitotic activity and its invasiveness, and whether some other feature such as stromal reaction or inflammatory reaction was prominent. In addition to the histological features of the tumour, information was carefully gathered concerning the clinical staging of the disease. This is carried out according to a universally agreed classification (Novak and Woodruff, (1962).

The stages recognised in the progress of this disease are as follows: Stage 0: Carcinoma in Situ. Stage 1: Confined to cervix. Stage 2: The tumour extends beyond the cervix but does not reach the pelvic wall. Stage 3: The tumour has spread to the pelvic wall. Stage 4: The tumour involves rectum or bladder, or both.

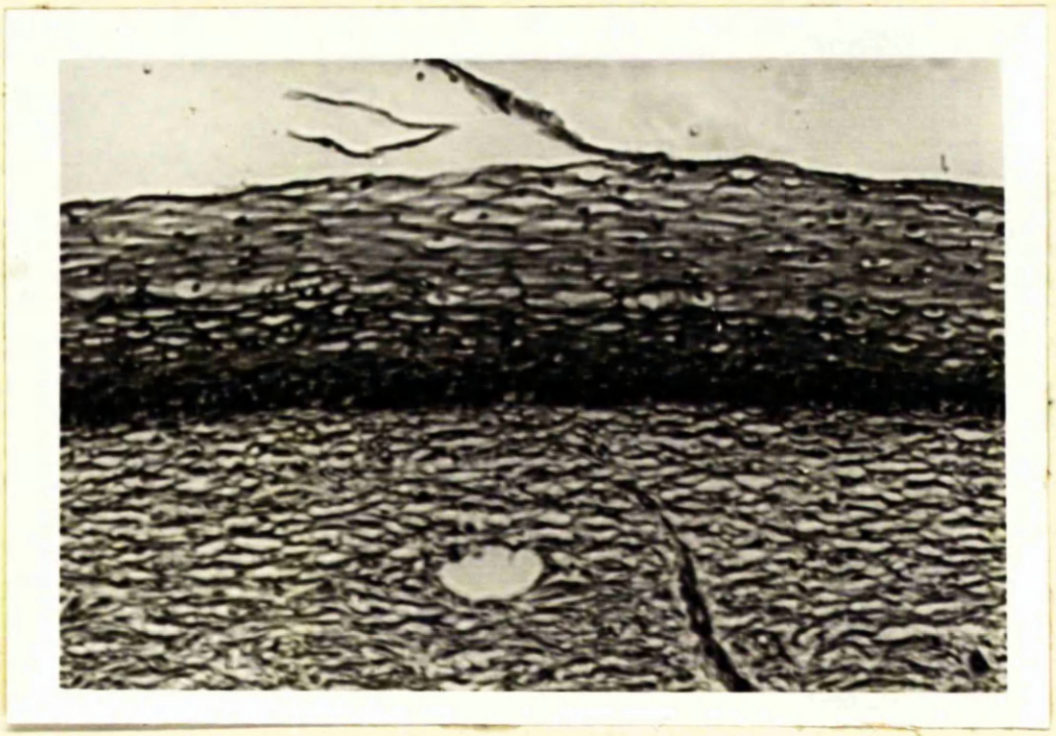


FIGURE 7a

NORMAL CERVIX UTERI

The superficial cells are flat or polygonal and are arranged in a regular pattern of stratification. Their nuclei are small or absent, in contrast with the cells of the basal layer which have prominent well-staining nuclei. The junction with the underlying stroma is linear and well-defined.

FIGURE 7b

CARCINOMA OF CERVIX UTERI

This is an early lesion. The large bizarre cells with peculiarly shaped nuclei are visible in the centre of the field. The pattern of epithelial stratification is lost, and the junction between epithelium and stroma is irregular.

A series of "Normal" samples, sixteen in all, was obtained from patients undergoing amputation of the cervix uteri as part of the operative repair of uterine prolapse. A wedge of tissue was cut from the cervix, angling the incision as widely as possible so as to include a large area of surface epithelium with the minimum of underlying fibro-muscular layer. Although on naked eye examination the tissue appeared normal in all cases, microscopic examination of neighbouring areas revealed varying degrees of epithelialisation, inflammatory change, and Nabothian follicle formation in the majority of specimens.

Representative illustrations of the material used are shown in high power magnification in Figure 7.

The tissue was placed in ice-cold distilled water as soon as it was removed from the patient and washed repeatedly to remove all blood-clot and inflammatory exudate. It was dried thoroughly on absorbent paper, the biopsy shavings in particular being treated with great care and moulded into a solid lump of material which rendered the subsequent steps more manageable. After drying, the tissues were weighed, and stored in the deep-freeze at -20°.

They were removed after an interval of 7 to 10 days, transferred to a freezing microtome M.S.E. Model No. 4400, and cut into sections 10-40 microns thick. Thereafter the sections were quantitatively added to a known volume of ice-cold 0.25M-sucrose and homogenised for 3 min. in an M.S.E. Blendor. Separation of the three cytoplasmic fractions was achieved employing the system of differential centrifugation described previously for Thyroid: First Series. In certain samples of tissue from cervical carcinomata, the size did not warrant an attempt to prepare

particulate fractions and the Supernatant alone was collected; in others, the size of the particulate fractions obtained did not warrant separate analysis, and they were combined for this purpose, being referred to subsequently as "Combined Particles".

Collection of particles from specimens of Normal cervix gave rise to serious technical problems which could only partially be overcome. The principal problem was that of obtaining a clear line of demarcation following the first centrifugation at 500g occasioned by the presence of collagen fibres in large numbers. Straining the whole homogenate through muslin offered a partial solution, but it was impossible to select a thickness of muslin which held back only the collagen fibres and unbroken cells; consequently in those samples where a deliberate attempt was made to collect particle fractions quantitatively, the possibility has to be admitted that perhaps a third of the particles were trapped in the muslin, or dragged down by the network of collagen where the thickness of muslin employed was not sufficient to exclude this completely from the homogenate prior to centrifugation.

Breast

Five samples of each of four histological types of human breast tissue were examined. One group consisted of fibroadenomata removed as a solitary lesion in an otherwise healthy breast, the sample examined containing proliferation of cells predominantly of mesenchymal origin, with moderate hyperplasia of acinar tissue. These changes were distributed throughout the lesion in a uniform manner. Three of the specimens were pericanalicular fibroadenomata and two were intracanalicular.

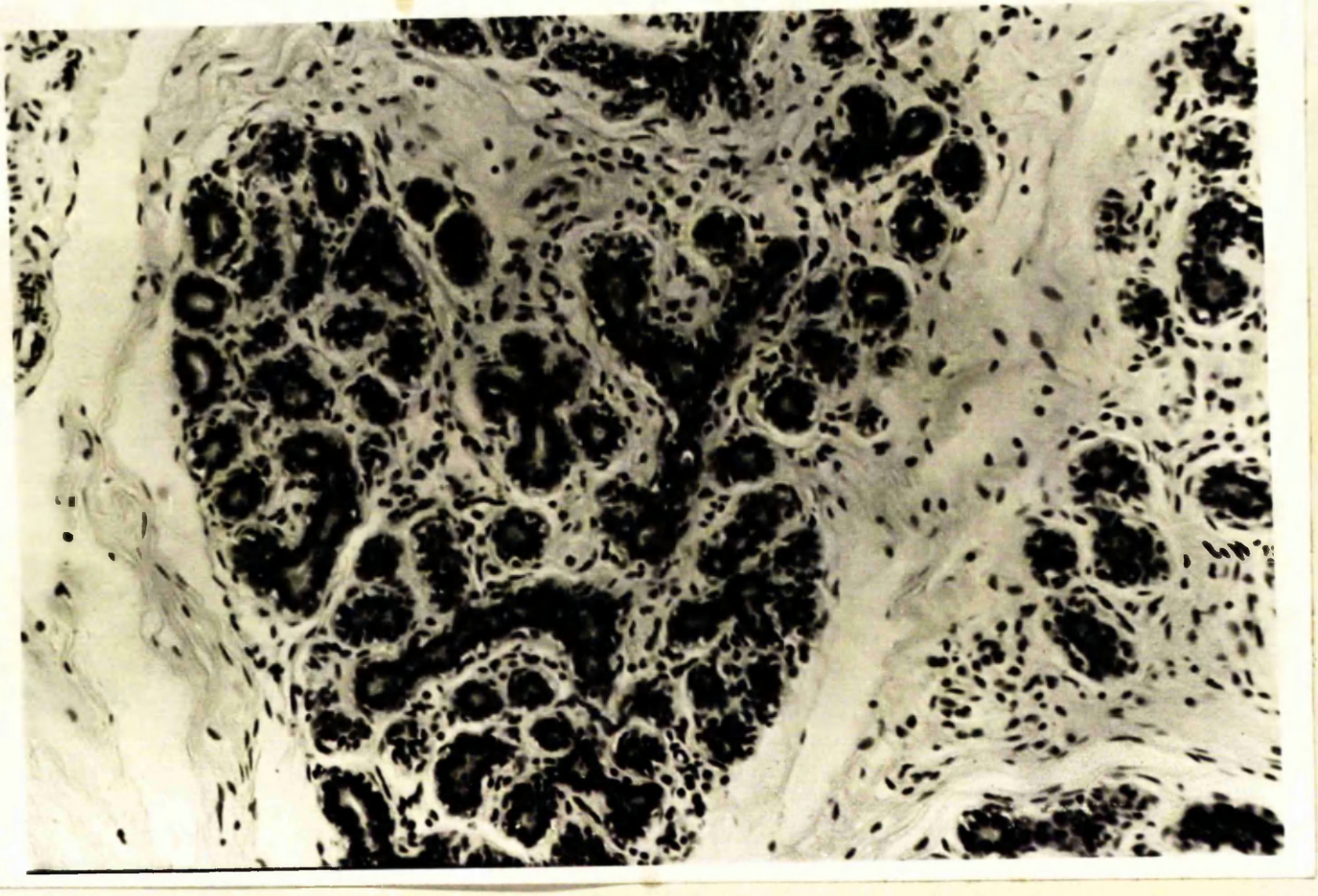


FIGURE 8a

NORMAL BREAST

The sample consists of groups of flattened epithelial cells arranged in acini, and embedded in loose connective tissue.

The ratio of cytoplasm to nucleus is low in these cells.

FIGURE 8b

CYSTIC MASTOPATHY

The specimen contains large cystic spaces bounded by hyperplastic epithelium with increased ratio of cytoplasm to nucleus. The connective tissue in which these cysts are embedded is very much denser than that of the normal gland.

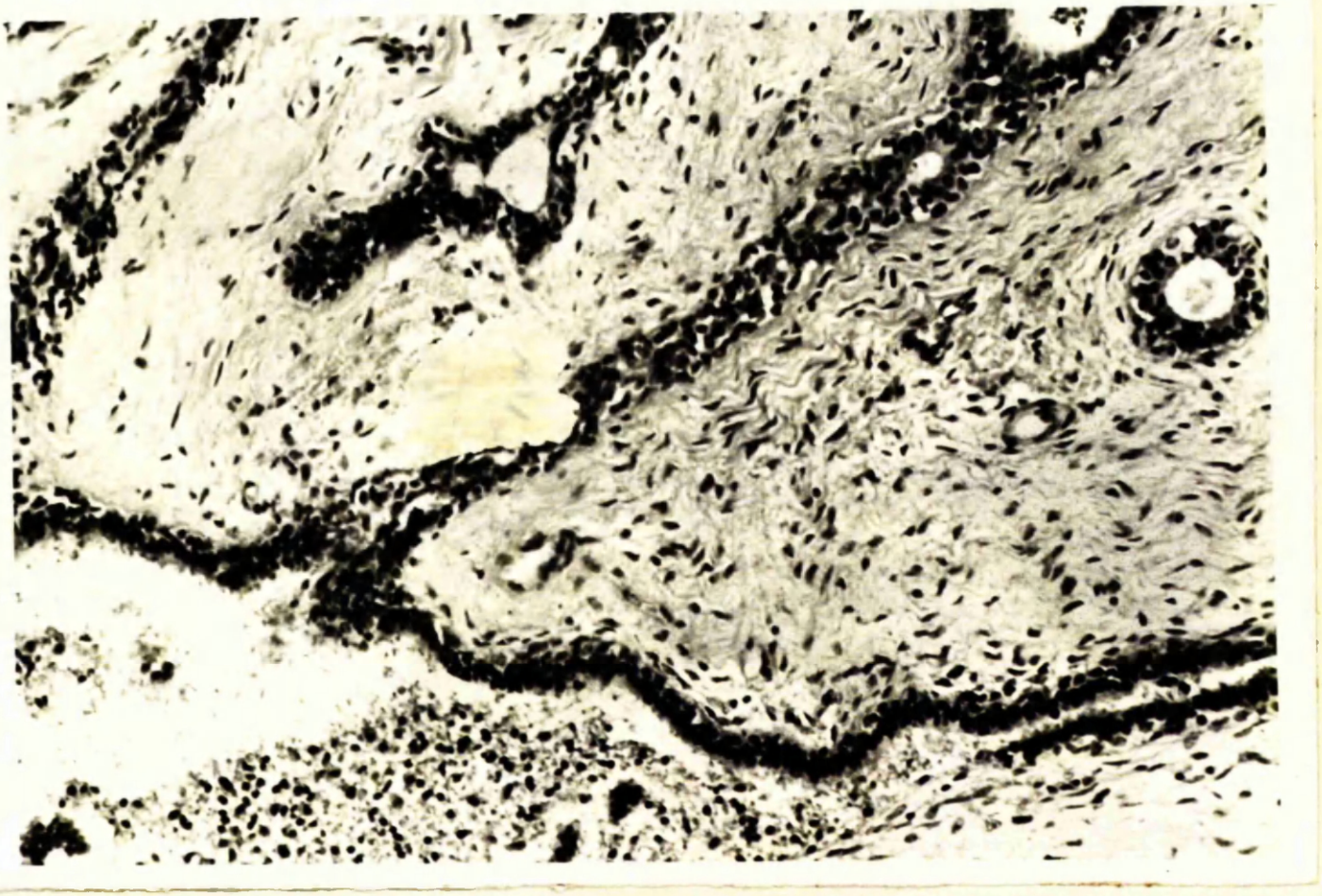


FIGURE 8e

FIBROADENOMA OF HUMAN BREAST

Morphologically, the epithelium present is not dissimilar to that of the normal breast. The acinar architecture, however, has almost completely disappeared, and there is a great proliferation of connective tissue.

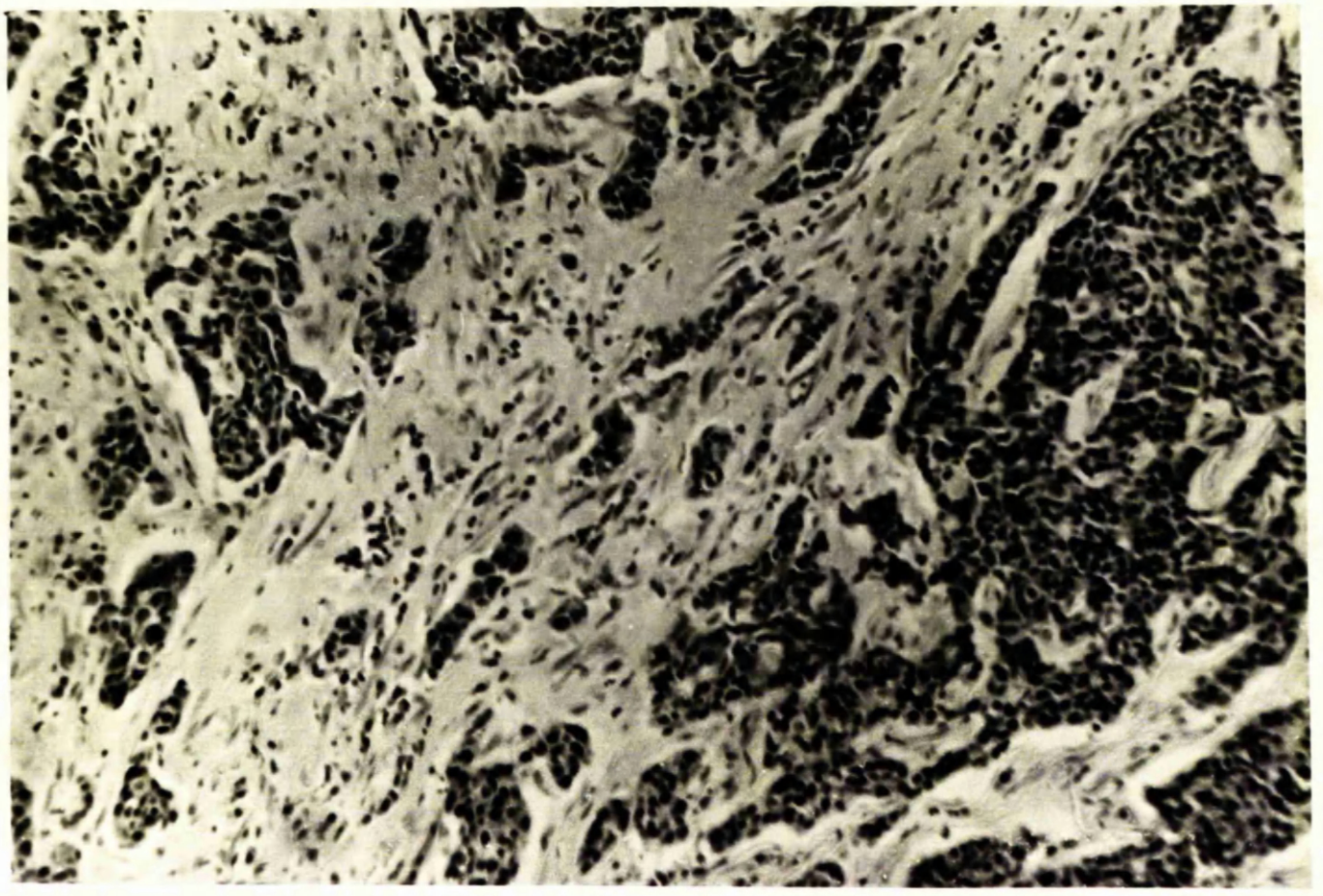


FIGURE 8d

SCIRRHOUS CARCINOMA OF BREAST

The fibrous stroma of the breast, which is normal in appearance, is invaded by nests of cancer cells devoid of acinar structure. The nuclear size of the cancer cells, and their ratio of cytoplasm to nucleus, are not very different in this specimen from those of normal breast epithelium.

A second group consisted of five carcinomata: one being a spheroidal cell carcinoma with pronounced malignant features, another an intra-duct carcinoma showing early invasive properties, and the remaining three being scirrhous carcinomata displaying a moderate degree of mitotic aberration. The line of demarcation between normal and abnormal in these samples was imprecise since none of the lesions was encapsulated, and the sample which was cored out for examination was as free from fat and reactive fibrous tissue as was possible under the circumstances.

The third group of samples was drawn from patients subjected to the operation of simple mastectomy for diffuse cystic mastopathy. This is a condition in which varying degrees of intraductal hyperplasia, intraductal papillomatosis, and cyst formation occur, accompanied by fibrous reaction and round-cell infiltration. The disease is usually present to a varying extent in different parts of the same breast, and variations from one patient to another can be very considerable. However, in all the patients studied, a very severe degree of involvement was present and no part of the breast could truly be described as normal. The sample cored out for examination was as representative as could be obtained.

As controls, a fourth group consisting of samples taken from pre-menopausal women undergoing removal of solitary cysts or fibroadenomata was examined. The breast was essentially healthy in all these patients, and the sample, which consisted predominantly of acinar tissue, was taken from the sub-areolar region. For obvious ethical reasons it could only be a small sample. In what follows, this group shall be described as Normal, although it is realised that the application of this term to tissue other than that taken from the lactating female requires reservation.

Examples of each histological group are shown in Figure 8.

Immediately upon removal from the patient, the samples were dissected free of fat, washed in ice-cold distilled water, dried on absorbent paper, weighed, and stored at -20°. They were removed after an interval of approximately one week and treated in exactly the same manner as were the specimens of cervix uteri. Two problems require special comment. One concerns the large amount of collagenous tissue in the homogenates which presented difficulties even more acute than those described in the preparation of particle fractions from normal cervix; thus in only a handfull of tissues could "Mitochondrial" and "Microsomal" fractions be separated quantitatively. The second problem was that of fat in the homogenates; however this was easily disposed of since it came to the surface as a firm disc after the 5,000g centrifugation and could be removed with a wooden spatula.

Blood and Urine

Ten patients undergoing radiotherapy for carcinoma of cervix uteri were chosen for study. The patients presented consecutively, the only conditions governing selection being adequate bladder control, the ability to co-operate in urine collections, freedom from renal tract involvement at the time of presentation, and the work-load which prevented more than two patients being studied simultaneously.

Before commencement of therapy, two or three blood samples were withdrawn for base-line estimations, and two 24-hour urine collections were obtained (in four patients, a random urine specimen had to be substituted for one 24-hour collection). The blood was drawn off by the investigator

into sterile syringes, transferred to clean test-tubes, allowed to clot, and centrifuged without delay at 3,000 revs/min. in the M.S.E.'Major' Centrifuge. The supernatant serum was transferred to a fresh test-tube and re-centrifuged at the above speed. The serum was then removed from the button of red cells into yet another clean test-tube, and maintained at 4° in a domestic refrigerator.

The urine was collected by the patient who was given detailed instruction in the proceedure to be adopted. It was passed into a bed-pan and transferred by means of a filter funnel into a Winchester containing 5-10ml. chloroform as preservative. When the collection was complete, the volume was accurately measured after thorough mixing of the specimen, and an aliquot was centrifuged to remove any undissolved chloroform or urinary sediment that was present. The clear liquid was transferred into a clean test-tube and maintained at 4° in a domestic refrigerator.

All enzyme assays on blood and urine were carried out within 24 hours of collection, with the exception of a very few specimens which were taken at week-ends. The urinary creatinine was measured on the day of collection by the laboratory staff, and the urinary deoxynucleotides were measured on samples of the clarified urine which had been stored at -20°, the period of storage not exceeding six weeks in any sample.

The dose of radiation to which the patients were exposed during therapy ranged from 5,000 to 8,500 rads. This was divided between conventional radium implantation for periods ranging from 48 to 100 hours, given as one or two treatments and supervoltage therapy administered by the A.E.I. Linear Accelerator at 4 m.e.v. Only one radium treatment was given to patients with advanced carcinoma, the balance being added

in the form of supervoltage therapy.

During the time of the first radium implantation, a specimen of blood and a 24-hour urine collection were obtained. In the last four patients of the series, the urine was collected by catheterisation since the low urine volumes obtained in one or two of the earlier cases raised the possibility of inadequate drainage due to pressure of the radium pack upon the urethra. Another blood and urine collection were obtained within three days of withdrawal of the radium pack and before the commencement of any other therapy. Six of the ten subjects were exposed to a second radium implant, and in these subjects, blood and urine collections were obtained during the period of implantation and following the withdrawal of the implant as before. Thereafter, at least two, and occasionally three blood and urine collections were obtained per week from each patient by the investigator during the course of their treatment by supervoltage therapy up to the time of their discharge from hospital.

Estimation of Ribonuclease Activity

The principle of the method employed involves incubation of the test material with RNA previously treated to provide a macromolecular substrate free of inhibitory nucleotides and heavy metals, and precipitation of the undigested polynucleotide material with a mixture of uranyl acetate and perchloric acid. The extinction of the digestion products may be read at 260 mμ by diluting an aliquot of the filtrate.

Technique The method which has been followed in this work is based upon that described by Kalnitsky, Hummel, Resnick, Carter, Barnett and Dierks (1959). These workers employed the method for the assay of crystalline bovine pancreatic RNAase. The modifications used in the present work were governed by the necessity of increasing the time of incubation to allow measurement of the weaker activity associated with the crude biological material which formed the enzyme source; and since the work reported was carried out on a wide range of material over a span of several years, it was regarded as desirable to standardise the RNA substrate in accordance with the recommendation of McDonald (1955) after preliminary purification.

The following reagents are used:

a) Substrate Yeast Nucleic Acid (Pabst Laboratories: British Agent, V. A. Howe and Co. Ltd., 46 Pembridge Road, London, W.11) purified according to the method of Zytko, de Lamirande, Allard and Cantero (1958) as described below, the concentration being adjusted to 0.5 mg. phosphorus per ml., in distilled water.

b) Buffers Tris/HCl 0.1M pH 7.4

Sodium Acetate/Acetic Acid 0.1M pH 5.6

c) Precipitant Uranyl Acetate 0.75% (W/V) in 4.17N-Perchloric Acid (ice-cold).

In the standard technique, 1 ml. of substrate was added to 1 ml. of the appropriate buffer and placed in a water bath at 37°. After a period of not less than two minutes, an aliquot of test material ranging from 0.05-0.50 ml. was added with vigorous shaking and the mixture incubated for 30 min. A volume of distilled water to bring the total reaction mixture to 3 ml. was added prior to addition of the enzyme; the water was pre-warmed at 37° in some cases, but was added at the beginning of the pre-incubation period in most assays.

At the end of incubation, the reaction was stopped by the addition of 2 ml. of precipitant, the tubes being stoppered and shaken thoroughly, after which they were allowed to stand in ice for 30 min. before being filtered through Whatman No. 42 filter-paper. A dilution of the filtrate was prepared by adding 0.5 ml. to 10 ml. distilled water, and after stirring well, the extinction was read at 260 mμ in the Uvispek Spectrophotometer (Hilger and Watts Ltd.) in silica cells having a light path of 1 cm., employing a dilution of the uranyl acetate/perchloric acid precipitant 1:50 in distilled water as a blank. With each test, a control was run in which the addition of enzyme source to the substrate took place after each had been incubated separately for 30 min. at 37° and 2 ml. of precipitant had been added to the buffer-substrate-water mixture. Where possible, tests were carried out in duplicate and the results averaged.

Purification of Substrate

The Yeast Nucleic Acid was dissolved in distilled water to which the

minimum volume of 0.4 N-NaOH was added intermittently to maintain the pH at 7.0 and a final adjustment to pH 7.0 was carried out upon complete solubilisation of the starting material. To the solution, two volumes of glacial acetic acid were added. The precipitate was collected at the filter-pump and washed twice with 66% (v/v) acetic acid, redissolved in distilled water with minimum addition of 0.4 N-NaOH as before, and dialysed for 24 hours against distilled water in the cold with frequent changes, the dialysis being followed by measurement of the extinction at 260 mμ of the bath fluid in order to ensure that dialysable nucleotide material had been completely removed from the dialysate. The RNA solution was then adjusted to 10% with respect to NaCl, and two volumes of ice-cold ethanol were added, the mixture being allowed to stand overnight in the refrigerator. The precipitate was collected by centrifugation in the cold, washed twice with 66% (v/v) ethanol, twice with absolute ethanol, and twice with ether, dried in a vacuum desiccator, and dissolved in distilled water. The purified preparation was negative for protein by the biuret reaction, and chloride free as tested by addition of silver nitrate to the filtrate resulting from precipitation of the nucleic acid by glacial acetic acid and passage through Whatman No. 42 filter-paper.

Standardisation of Substrate

This was carried out by estimation of the phosphorus content of the solution according to the method of Gomori (1941) after acid digestion of the organic material.

The following reagents were used:

a) Ammonium Molybdate: 5% (w/v) $(NH_4)_6Mo_7O_{24}.\ H_2O$ in 5.4N-sulphuric acid.

b) Reducing Solution : 3% (w/v) hydrated sodium sulphite $Na_2S_2O_3.\ 7H_2O$ in 1% (w/v) p-methylamino-phenol sulphate.

1 ml. of purified RNA in solution was digested with 0.3 ml. conc. sulphuric acid in a microkjeldahl flask over a hot flame in the presence of a glass bead, the final digest being clarified by repeated addition of hydrogen peroxide (100 vols.), after which the flask was cooled and the contents made up to a suitable volume with distilled water. 1 ml. of this solution was placed in a graduated tube, followed by 2 ml. ammonium molybdate and 2 ml. reducing solution and the contents adjusted to the mark with distilled water. After shaking, the tube was left in the dark for 30 min. and the extinction of the solution measured on the SP300 photometer (Unicam Instruments Ltd.) using the red filter. A reagent blank was processed through the stages of digestion and of colour development and subtracted from the value given by the test solution. The final extinction was compared with that of phosphorus standards carried through the entire procedure. From this, the extent to which stock RNA solutions required to be diluted in order to give a solution containing 0.5 mg. phosphorus per ml. was calculated. The stock solutions were kept at -20° for a period not exceeding one year, and the diluted substrate containing 0.5 mg. phosphorus per ml. was kept in the domestic refrigerator for a period not exceeding one week.

Units

The units reported in this thesis refer to the extinction of the final one-in-twenty dilution of the filtrate following precipitation of the

undigested substrate. This value was multiplied by the necessary factor required on the basis of the volume of test material taken for the assay to give the extinction that would have been obtained from the acid-soluble nucleotides released by 1 ml. of material.

One unit of activity is thus defined as that amount of enzyme which, when present in 1 ml. of test material, liberates acid soluble material whose extinction at 260 mμ in a 1 in 20 dilution of the filtrate in a light path of 1 cm. is 1.00 under the conditions of assay.

The specific activity per mg. protein was obtained by dividing the protein content of 1 ml. of the test material into the units of activity per ml. of test material as defined above. The activity per g. wet weight of tissue was derived by multiplying the volume of the cytoplasmic fraction in question by the enzyme activity in units per ml. and dividing the product by the wet weight of the whole tissue sample. The percentage of the whole cytoplasmic activity present in a given fraction was calculated by summing the activity per g. wet weight of tissue for each separate cytoplasmic fraction and dividing the sum into the individual value for that fraction. The activity per 24-hours in the urine was calculated by multiplying the units per ml. of the urine by the volume of urine produced over the 24-hour period. The activity per mg. creatinine was obtained by dividing the urine activity in units per ml. by the creatinine concentration in mg. per ml.

Estimation of Deoxyribonuclease Activity

The principle involved in this estimation is similar to that of the previous estimation, namely precipitation of undigested substrate by strong acid and measurement of the acid soluble nucleotides in the filtrate. The technique is closely related to that of Allfrey and Mirsky (1952) as modified by Kowlessar, Altman and Hempelmann (1954).

Technique Certain modifications were introduced through necessity. It was essential to scale down the method to permit assay of smaller volumes of material than those employed by the original authors; and facilities did not exist for centrifugation of the precipitated substrate at 10,000 r.p.m. in the cold. Finally, the diphenylamine reaction first described by Dische (1930) which was used by the above authors for the measurement of acid soluble deoxynucleotides, was replaced by measurement of the extinction in the ultraviolet.

The following reagents were used:

a) Substrate Deoxyribosenucleic acid prepared from salmon sperm by the method of Chaikoff (1953) was purchased as the sodium salt from the California Corporation for Biochemical Research. The material was highly polymerised and had a phosphorus content of 8.34%, the ratio of nitrogen to phosphorus being 1.67 and the extinction at pH 12 of a 1% solution was 200. The material was dissolved in 0.005 N-NaOH to a concentration of 0.4% (w/v); it remained stable for prolonged periods but fresh batches were usually required at monthly intervals. The dried salt was stored at -20° in a desiccator.

b) Buffers Tris/HCl 0.16M pH 7.5 for DNAase I.

Sodium Acetate/Acetic Acid 0.2M pH 5.6 for DNAase II

c) Metal Reagent Magnesium sulphate ($MgSO_4 \cdot 7H_2O$) 0.1M was used as a source of magnesium ions for the assay of DNAase I.

Disodium Ethylenediaminotetraacetate dihydrate (Molecular weight 372.25) was used in a 1% (w/v) solution to remove divalent cations in the assay of DNAase II.

d) Precipitant Trichloracetic acid 3.6M (ice-cold)

The reaction mixture consisted of 0.25 ml. substrate, 0.25 ml. of the appropriate metal reagent, 0.75 ml. of the appropriate buffer, and 0.25ml. of test material or a suitable dilution thereof in a stoppered centrifuge tube. After thorough shaking, the tubes were placed in a water-bath at 37° and incubated for 4 hours. The reaction was stopped by addition of 0.4 ml. of precipitant and after shaking, the tubes were allowed to stand for 30 min. in ice. They were then centrifuged at 3,000 r.p.m. in the M.S.E. 'Major' centrifuge for 10 min. after which 0.5 ml. of the supernatant was diluted to 10 ml. with distilled water, and the extinction of the solution at 260 mμ and at 290 mμ was read in the Uvispek spectrophotometer using silica cuvettes and a 1 cm. light path and employing distilled water as a blank. A control was processed in an identical manner except that the enzyme material was incubated separate from the remaining components of the reaction mixture; when the 4 hours had elapsed the precipitant was added to the reaction mixture followed by the test material, after which the various steps described for the assay were carried out for the control.

The values of $E_{260} - E_{290}$ for test and control were calculated and the latter subtracted from the former to give the absorbancy due to the enzymic degradation of the DNA substrate.

Units

One unit of enzyme activity is defined as that amount of enzyme which, under the conditions of the assay, will liberate per ml. of test material sufficient deoxynucleotides to give an absorbancy ($E_{260} - E_{290}$ corrected for that of the control) of 0.004 in the final dilution of supernatant as described above.

In practise, the units per ml. of test material were derived simply by multiplying the absorbancy by 10^3. The specific activity per mg. of protein and the various other parameters of enzyme activity were derived in a manner exactly as described for the ribonucleases in the preceding part of this section.

Diphenylamine Reaction

In preliminary work, this reaction was used to measure the deoxynucleotides released from the substrate by enzymic action. The reaction was carried out exactly as described by Dische (1955) who however is not specific about the time of heating required.

The reagent used consisted of diphenylamine (twice recrystdlised from petroleum ether) 1% (w/v) in glacial acetic acid, with 2.75 ml. of conc. sulphuric acid added to 100 ml. of the final solution. To 1 ml. of the supernatant, derived by centrifugation of the reaction mixture after acid-precipitation of undigested DNA, 2 ml. of the diphenylamine reagent was added. The tube was stoppered, the mixture shaken, and

placed in a water-bath at 100° for exactly 20 min. after which it was removed and placed in a beaker of water at room temperature for exactly 10 min. The extinctions at 595 and at 650 mμ were read in the Uvispek spectrophotometer against a reagent blank prepared by substituting distilled water for the acid-soluble supernatant. The enzyme activity was derived by calculating $E_{595} - E_{650}$ for test and control and subtracting the latter value from the former.

Estimation of Adenosine Deaminase Activity

The principle of the method, which was derived from Solomon (1960), depends upon the reduction of the extinction at 265 mμ of adenosine consequent upon its deamination to inosine, so that the reaction can be followed by simple spectrophotometric measurement. The original author employed a correction in which the extinction at 290 mμ was subtracted from that measured at 265 mμ. This step was presumed to relate to the necessity of correcting for the formation of xanthine derivatives and uric acid from endogenous purines in the chick embryonic liver preparations with which he was working. No such effect was observed with the tissue supernatants employed in the present work, and the reading at 290 mμ was omitted from the procedure.

Technique

The reagents were as follows:

a) Substrate Adenosine (British Drug Houses) was twice recrystallised from hot water and after exhaustive drying was dissolved in hot water to give a 0.6% (w/v) stock solution. This

was stable in the deep-freeze over a period of several years.

b) Buffer 0.1M-phosphate buffer pH 7.0

The stock substrate solution was diluted 1:300 in phosphate buffer to give a working buffer-substrate mixture, 3 ml. of which were placed in a 1 cm. silica cuvette. After addition of 0.05 ml. of test material, the mixture was dispersed by sucking the liquid up and down in a Pasteur pipette. The extinction of the reaction mixture was read at 265 mμ against a control preparation formed by the addition of 0.05 ml. test material to 3 ml. phosphate buffer in a 1 cm. silica cuvette. Test and control were transferred, by means of the same Pasteur pipettes used to ensure mixing, to stoppered tubes and placed in a water-bath at 37° for exactly one hour. They were cooled rapidly in ice, and transferred with clean pasteur pipettes into the same cuvettes as had been used for the initial reading but which had been thoroughly cleaned and dried in the intervening period. The extinction at 265 mμ of the test was once more read against that of the control and the value subtracted from that at zero time to give the fall in extinction at $265_{m\mu}$ of the test solution during the hour's incubation. It was found in preliminary work that the use of detergent or dichromate solutions for cleaning purposes often resulted in erratic results; as a consequence the glassware was washed by immersion in 30% nitric acid for a period of not less than 48 hours, with the exception of the silica cuvettes which, during a working day, were thoroughly cleaned with soap and water between consecutive readings.

Units

One unit of activity is defined as that amount of enzyme which, under

the conditions of the assay, will produce per ml. of material a fall of 0.020 in the extinction of the buffer-substrate mixture. In practise, the units per ml. of test material were derived simply by multiplying the fall in extinction at $265_{m\mu}$ by 10^3. The specific activity per mg. of protein and the various other parameters of enzyme activity were derived in a manner exactly as described for the ribonucleases in an earlier part of this section.

Estimation of Protein

Estimation of Protein

The method used was that of Lowry, Rosebrough, Farr and Randall (1951). The principle involves the use of the Folin-Ciocalteau reagent which reacts with the tyrosine residues of the protein to form a blue coloured complex. The reaction is sensitised by the addition of copper ions in an alkaline medium.

Technique

The following reagents were used:

a) Sodium Carbonate 2% (w/v) anhydrous Na_2CO_3 in 0.1 N-NaOH

b) Copper Sulphate 1% (w/v) $CuSO_4 \cdot 5H_2O$ in water

c) Sodium Tartrate 2% (w/v) Sodium D(+) Tartrate Dihydrate (Mol. Wt. 230.09) in water.

d) Phenol Reagent Folin-Ciocalteau phenol reagent diluted from stock 1:1 in water to give a solution 1 N with respect to acid.

e) Standard Crystalline bovine serum albumin (Armour Pharma-

ceutical Co. Ltd.) was prepared in dilutions ranging from 50-300 ug. per ml.

The solution for assay was diluted to an extent which was expected to bring it within the range of the estimation, namely 50-300 ug. per ml. To 1 ml. of the diluted solution, was added 5 ml. of the alkaline copper solution freshly prepared each day by addition of 1 ml. of a mixture of equal parts of copper sulphate and sodium tartrate solutions to 49 ml. sodium carbonate solution. After shaking, the mixture was left for 10 min. at room temperature. 0.5 ml. of the phenol reagent was then added and the mixture shaken immediately, within 1-2 secs. It was then allowed to stand for 30 min. at room temperature, when the extinction of the mlution was read at 625 mμ in the SP600 (Unicam Instruments Ltd.) against a reagent blank prepared by substituting distilled water for the test material.

From the extinction at 625 mμ, the protein concentration of the solution was derived by reference to a standard curve which was prepared every month, or more frequently if a fresh copper reagent was made up within the four-week period.

Estimation of Deoxyribonucleic Acid Content of Thyroid Homogenates

The principle of the method employed, which is derived from the work of Munro and his associates (Hutchison and Munro 1961; Fleck and Munro 1962; Hallinan, Fleck and Munro 1963) involves precipitation of protein, lipids and nucleic acid by cold perchloric acid with removal of acid soluble components; removal of lipids by extraction with organic solvents; removal

of RNA from the lipid extracted material by alkaline hydrolysis; and determination of the phosphorus content of the residue.

Technique

The reagents used were as follows:

a) Precipitant — Perchloric acid 0.6N (ice-cold)

b) Acid Wash — Perchloric acid 0.2N (ice-cold)

c) Lipid Solvents
1) Potassium Acetate (anhydrous) 1% in absolute ethanol (ice-cold)
2) Chloroform (Reagent Grade) 25% (v/v) in absolute ethanol
3) Diethyl ether (anaesthetic grade) 25% (v/v) in absolute ethanol
4) Diethyl Ether (anaesthetic grade)

d) Alkali — Potassium Hydroxide 0.6N

4 ml. of thyroid homogenate (10% in 0.15M-potassium chloride as described earlier in this section) was added to a centrifuge tube containing 2 ml. perchloric acid 0.6N. The tube was stoppered, shaken thoroughly, and allowed to stand in ice for 10 min. It was then centrifuged at 3,000 r.p.m. for 10 min. in the M.S.E. 'Minor' centrifuge which was placed in the Lightfoot cold-room. The supernatant was discarded and the precipitate washed twice with 6 ml. perchloric acid 0.2N. It was then washed with the potassium acetate solution and centrifuged in the cold as previously, after which the washing with the other lipid solvents in the order listed above was carried out at room temperature, the precipitate being recovered by centrifugation for 3,000 r.p.m. for

5 min., and 5 ml. of each solvent being used.

To the precipitate, 2 ml. of distilled water was added and the mixture dispersed, after which 2 ml. of potassium hydroxide, 0.6N was added, the tube being stoppered and shaken well, after which it was placed in a water-bath at 37° for one hour. The tube was then cooled in ice, 2 ml. of perchloric acid 0.6N was added, and the mixture allowed to stand in ice for 10 min. It was then washed twice with perchloric acid 0.2N as before. The washed precipitate was once again dispersed in 2 ml. water after which 2 ml. potassium hydroxide was added as before, and the tube allowed to stand overnight in a water-bath at 37°.

The contents were transferred quantitatively to a measuring cylinder with the addition of a further 6 ml. 0.6N potassium hydroxide, and made up to 50 ml. with distilled water. This whole procedure was carried out in duplicate, and from each cylinder a suitable aliquot, usually 5 ml. was taken for estimation of phosphorus.

Phosphorus Estimation

The method used was that of Allen (1940) in which the phosphorus - containing material is digested with acid and the inorganic phosphorus liberated is converted to a phosphomolybdic complex which is then reduced to a blue pigment.

The reagents used were as follows:

a) Acid Reagent Sulphuric acid 10 N

b) Ammonium Molybdate 8.3% (w/v) $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in distilled water

c) Reducing Agent 1% (w/v) Amidol (2:4-diamino-phenol hydrochloride) in

20% (w/v) Sodium Metabisulphite, $Na_2S_2O_5$, the solution being filtered before use and stored in the dark at 4° for a period not exceeding 10 days.

To 5 ml. of the extract to be assayed, 0.6 ml. of acid reagent was added together with a glass bead and a few carborundum stones, these additives having been acid washed prior to use. The mixture was digested on a low flame for a period of about 3 hours, the appearance of thick white fumes being taken as the end point of the process. The brown liquor was clarified by careful addition of hydrogen peroxide (100 vols.), after which the flask was cooled and the following additions made in the stated order: 10.83 ml. distilled water; 0.5 ml. ammonium molybdate, and 1 ml. reducing agent. The solution was read at 640 mμ in the SP600 spectrophotometer. A reagent blank was processed in parallel with the test material. The extinction so derived was converted into phosphorus concentration by reference to a standard curve constructed by conducting the entire assay from the stage of digestion onwards on known amounts of anhydrous potassium dihydrogen phosphate.

Estimation of Total Deoxynucleotides in Urine

The method employed was that of Stumpf (1947), modified to the extent of subtracting from the extinction of the reaction mixture, the extinction due to the action of sulphuric acid alone upon the constituents of the urine.

Technique

The following reagents were used:

a) Cysteine — 5% (w/v) Cysteine hydrochloride in distilled water

b) Acid — 75% (v/v) Sulphuric acid, formed by adding 70 vols. of conc. H_2SO_4 to 30 vols. of distilled water under cooling in ice.

To 0.5 ml. of the urine sample, which, on removal from the deep-freeze was warmed to 37° with shaking to redissolve any precipitated material, 0.05 ml. of the cysteine solution was added followed by 5 ml. of acid. The mixture was shaken and allowed to stand at room temperature for 15 min. after which the extinction at 490 mμ was read against a reagent blank prepared by substituting distilled water for urine in the above proceedure. An acidified urine blank was run in parallel with each urine test, substituting 0.05 ml. of distilled water for the cysteine solution, the extinction at 490 mμ being subtracted from that of the corresponding urine test. The corrected extinction for each urine sample was converted to mg. DNA per 100 ml. by reference to a standard curve, prepared anew for each batch of cysteine solution, using the highly polymerised DNA which has already been described as the substrate for assay of deoxyribonuclease activity. By reference to the 24-hour urine output for the patient and the concentration of creatinine in the urine, the deoxynucleotide concentration could be expressed as a 24-hour output and as a concentration relative to urinary creatinine.

Estimation of Urinary Creatinine

The method used was that of Folin (1914) in which the creatinine is allowed to develop a reddish complex with alkaline picrate according to the reaction first described by Jaffe (1886).

Technique

The following reagents were used:

a) Alkali — Sodium Hydroxide 1 N

b) Picrate — Saturated aqueous solution of picric acid (reagent grade) filtered immediately before use.

The urine for analysis was diluted 1:20 with distilled water and 0.5 ml. added to a test-tube followed by 1 ml. of alkali, 1 ml. of picrate, and 7.5 ml. distilled water in that sequence. The contents were thoroughly mixed and allowed to stand for 15 min at room temperature before reading the extinction against distilled water in the Unicam SP300 photoelectric colorimeter using the blue filter. A blank was prepared by substituting distilled water for the urine sample in the above reaction, and the extinction subtracted from that of each test. The corrected extinction for the test was then converted to the appropriate concentration of creatinine by reference to a standard graph prepared from suitable dilutions of a stock solution of creatinine zinc chloride (reagent grade) in 0.1 N-hydrochloric acid.

Statistical Calculations

The formulae for the calculation of the various statistical parameters measured in the course of the present work, and the tables used in the assessment of these parameters, are widely known. The source to which the author has consistently referred is Documenta Geigy: Scientific Tables (6th Edition 1962).

Standard Deviation

This was calculated as follows:

$$S.D. = \sqrt{\frac{1}{N-1}\left[Sx^2 - \frac{(Sx)^2}{N}\right]}$$

Where N = number of observations in group

x = numerical value of each observation

't'-Test

The value of 't' for the difference of the mean between two groups was calculated as follows:

$$'t' = \frac{\bar{x}_a - \bar{x}_b}{\sqrt{\left[Sx^2_a - \frac{(Sx_a)^2}{N_a}\right] + \left[Sx^2_b - \frac{(Sx_b)^2}{N_b}\right]}} \times \sqrt{\frac{N_a \times N_b}{N_a + N_b}}$$

Where N_a = number of observations in first group

N_b = number of observations in second group

x_a = numerical value of each observation in first group

x_b = numerical value of each observation in second group

$\bar{x}_a$ = mean of first group

$\bar{x}_b$ = mean of second group

Null Hypothesis

Analysis of the significance of the difference between the values found in carcinoma tissue from the same patient before and after radiation was by this technique, in which the significance of the difference in the

change occurring from zero change is measured by determining the value of 't_o' according to the following formula:

$$'t_o' = \frac{\bar{x}_o \times \sqrt{N}}{\sqrt{\frac{1}{N-1}\left[Sx^2 - \frac{(Sx)^2}{N}\right]}}$$

Where N = number of pairs of specimens

x = difference between values obtained before and after radiation, expressed as a positive or a negative integer

x_o = mean of differences i.e. $\frac{S(x)}{N}$

Analysis of Variance

This was conducted on the data derived from studies on blood and urine before, and at various intervals during, treatment by radium and supervoltage deep X-ray therapy. The technique is as follows:

The Correction Factor (C.F.) is calculated

$$C.F. = \frac{(\text{Sum of every value in entire data})^2}{\text{number of observations in entire data}}$$

The Sum of Total Squares (T.S.) is calculated

T.S. = (Sum of the square of every value in entire data) - C.F.

The Sum of Subject Squares (S.S.) is calculated

$$S.S. = \frac{(A^2 + B^2 + C^2 + \ldots\ldots)}{N_p} - C.F.$$

Where A, B, C, ... represent the sum of all the observations for a single subject under all procedures and N_p is the number of procedures.

The Sum of Procedure Squares (P.S.) is calculated

$$P.S. = \frac{(a^2 + b^2 + c^2 + \ldots)}{N_s} - C.F.$$

Where a,b,c,... represent the sum of all the observations for all the subjects under a single procedure and N_s is the number of subjects.

The Residual Error (R.E.) is calculated

$$R.E. = T.S. - S.S. - P.S.$$

The mean square for Subjects, Procedures, and Residual Error was calculated by dividing S.S., P.S., and R.E. by the appropriate value for the degrees of freedom; these were as follows:

$N_s - 1$ for Subjects

$N_p - 1$ for Procedures

$N_s \times N_p - N_s - N_p + 1$ for Residual Error

The Variance Ratio (F) for Subjects was obtained by dividing the mean square for Subjects by the mean square for the R.E. and the Variance Ratio (F) for the Procedures was obtained from division of the mean square for Procedures by the mean square for the R.E. The significance of F between Procedures was found by consulting the appropriate tables, the degrees of freedom being those for the R.E.

Finally, the Fiducial Interval (F.I.) was calculated from the formula

$$F.I. = {}'t'_p \times \sqrt{\frac{\text{mean square for R.E.}}{N_s} \times 2}$$

Where $'t'_p$ refers to the value of 't' for that level of probability at

which the value of F- was significant corresponding to the same degrees of freedom as applied to the R.E.

Correlation Coefficient

This was calculated for the data on estimation of deoxyribonuclease activities by two methods, as follows:

$$V_{ab} = S(a \times b) - \frac{(Sa) \times (Sb)}{N}$$

where a refers to each individual value obtained by one method

b refers to each individual value obtained by the second method on the same samples, and

N is the number of samples.

$$V_a = S(a^2) - \frac{(Sa)^2}{N}$$

$$V_b = S(b^2) - \frac{(Sb)^2}{N}$$

The Correlation Coefficient, r , was then given by the formula

$$r = \sqrt{\frac{V_{ab}}{V_a} \times \frac{V_{ab}}{V_b}}$$

RESULTS

EVALUATION OF THE METHODS EMPLOYED

The principles involved in all the methods employed in the present work were fairly well established and did not require detailed appraisal. Nevertheless, a number of preliminary investigations were carried out to ensure, in so far as possible but without the expenditure of an undue period of time, that the conditions chosen were appropriate for the biological materials being examined. For the enzyme assays, it was essential that the methods used should give results linear with time over the period of incubation, and that the activities measured should be proportional to the amount of test material added to the system; at any rate, if these conditions only applied within certain limits, it was necessary to define these limits. It was also desirable to make quite certain that the enzyme preparations, once obtained, remained stable during the conditions of storage applying when assays were not conducted at once. An attempt was also made to distinguish two peaks of RNAase activity in some of the materials. The effect of ultra-sound upon the liberation of enzymes from the particle fractions was also studied in a few instances.

It would have been preferable if these questions had been rigorously examined in all the materials studied. For reasons of time it was not possible to do this. To a certain extent short-cuts were taken, as where the results on assays from different materials have been collated; where the results on

one type of lesion have been extrapolated to all tissues of the same organ; and where the properties of an enzyme assay in one cytoplasmic fraction have, where satisfactory, been taken as approval for the use of an identical technique in all fractions of that tissue. The most serious omission - the failure to check the morphology of all particulate preparations - was unavoidable; the analysis of a single preparation entailed a colleague in one entire week's work and represented a considerable personal favour. For this reason, inverted commas are used to emphasise that the terms "Mitochondrial" and "Microsomal" are only very approximate descriptions of these fractions. Within these limits, the techniques adopted were found adequate for the purposes to which they were put, provided account is taken of certain reservations which are fully explained in the Discussion.

Enzyme Stability

This was tested by taking 10 samples for each enzyme studied drawn from a variety of materials. The studies on alk. and acid RNAases were conducted on 3 sera, 2 urines, 3 supernatants of [illegible] thyroid tissue, and one supernatant each from normal breast and normal cervix. The samples used to study the stability of both DNAases consisted of 5 urines and 5 supernatants of various tissues. Tissue supernatants only were used for the experiments on adenosine deaminase stability. Estimations on each sample were carried out in duplicate as

ENZYME	24 hours at 20°	24 hours at 4°	48 hours at 4°	1 week at -20°	4 weeks at -20°
Alk. RNAase	98.8	97.5	104.2	106.1	100.2
Acid RNAase	100.6	101.5	96.6	99.8	105.0
DNAase I	97.1	106.6	96.7	103.2	97.8
DNAase II	98.0	98.4	95.2	102.3	104.3
Adenosine Deaminase	91.4*	94.9	90.2*	97.3	94.6

TABLE I

EFFECT OF STORAGE ON

ENZYME ACTIVITY

Each value is the mean of 10 duplicate assays in which the activity at the intervals stated above is expressed as a percentage of the initial activity for that sample. * Denotes a result significantly lower than mean initial value ($P < 0.05$).

TABLE I.

soon as practicable; in one or two instances this meant on the following day, in which case they were kept at 4^0 overnight. Thereafter assays were carried out in duplicate in all samples after a further 24 hours at 20^0 (room temperature), 24 hours at 4^0 (domestic refrigerator), 48 hours at 4^0, 1 week at -20^0, and 4 weeks at -20^0. To carry out this scheme, each specimen was divided into five portions treated in one or other of the above manners immediately after the preliminary assay.

The data obtained are summarised in Table 1 where the mean activity after each method of storage is expressed as a percentage of the initial value. To obtain these means, the individual values for both RNAases and adenosine deaminase were expressed as a percentage of the initial value for that specimen and the average at each time interval was calculated. A different method was adopted for both DNAases; this was necessitated by the low values for urinary DNAase II and supernatant DNAase I, whereby a small numerical change in activity was reflected as an enormous change in percentage. To overcome this difficulty, the total number of units in all ten samples were added, and the sum of the activities at each interval expressed as a percentage of the initial figure.

Some changes are indeed apparent, but in the main these were erratic. Analysis of the data was carried out by applying the Null Hypothesis in all instances where the mean value after a certain interval differed by more than 5% from the initial mean. The only differences which proved to be significant

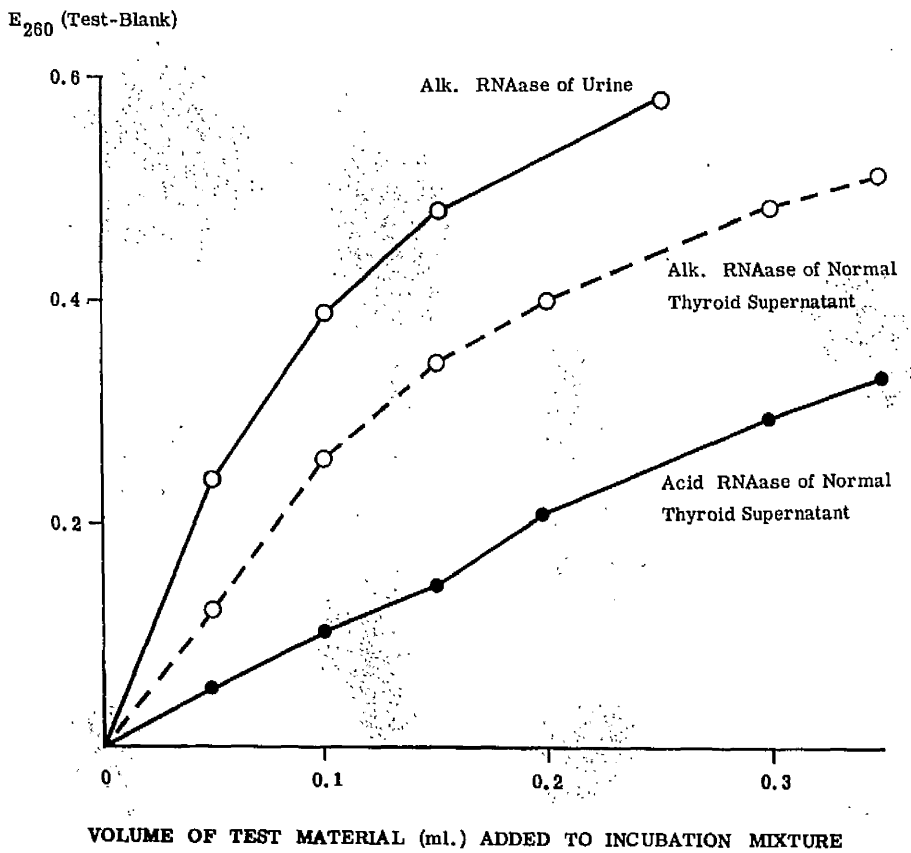


FIGURE 9

RELATIONSHIP BETWEEN ACTIVITY OF RIBONUCLEASES MEASURED AND AMOUNT OF MATERIAL ADDED

were decreased activity of adenosine deaminase after 24 hours at 20^0 and after 48 hours at 4^0. Storage of samples for assay of adenosine deaminase activity was always at -20^0 in the work to be reported. For the other enzymes, no condition of storage was used which was not one or other of the examples listed in Table 1.

Assay of RNAases

It must be clearly understood that the estimation of RNAase activity, and also of DNAase activity, as used in this work depends upon an empirical and abitrary phenomenon: the ability of the enzyme to degrade its macromolecular substrate to fragments small enough to remain soluble in the presence of strong acids which precipitate oligonucleotides above a certain chain length. It is in no sense a direct measurement of the number of bonds in the substrate ruptured by the enzyme, though there must of course be some connection between the two processes. The relationship between the extinction at 260 mμ of the acid-soluble products released by RNAase action, and the amount of enzyme solution added to the reaction mixture was examined under acid and alkaline conditions in 2 sera, 2 urines and 2 supernatants, using quadruplicate assays at each point. The volume of the mixture and the concentration of all reagentswere kept constant throughout. Representative of the curves obtained are those shown in Figure 9. . The salient point which emerged from this study is that, irrespective of the material assayed or the

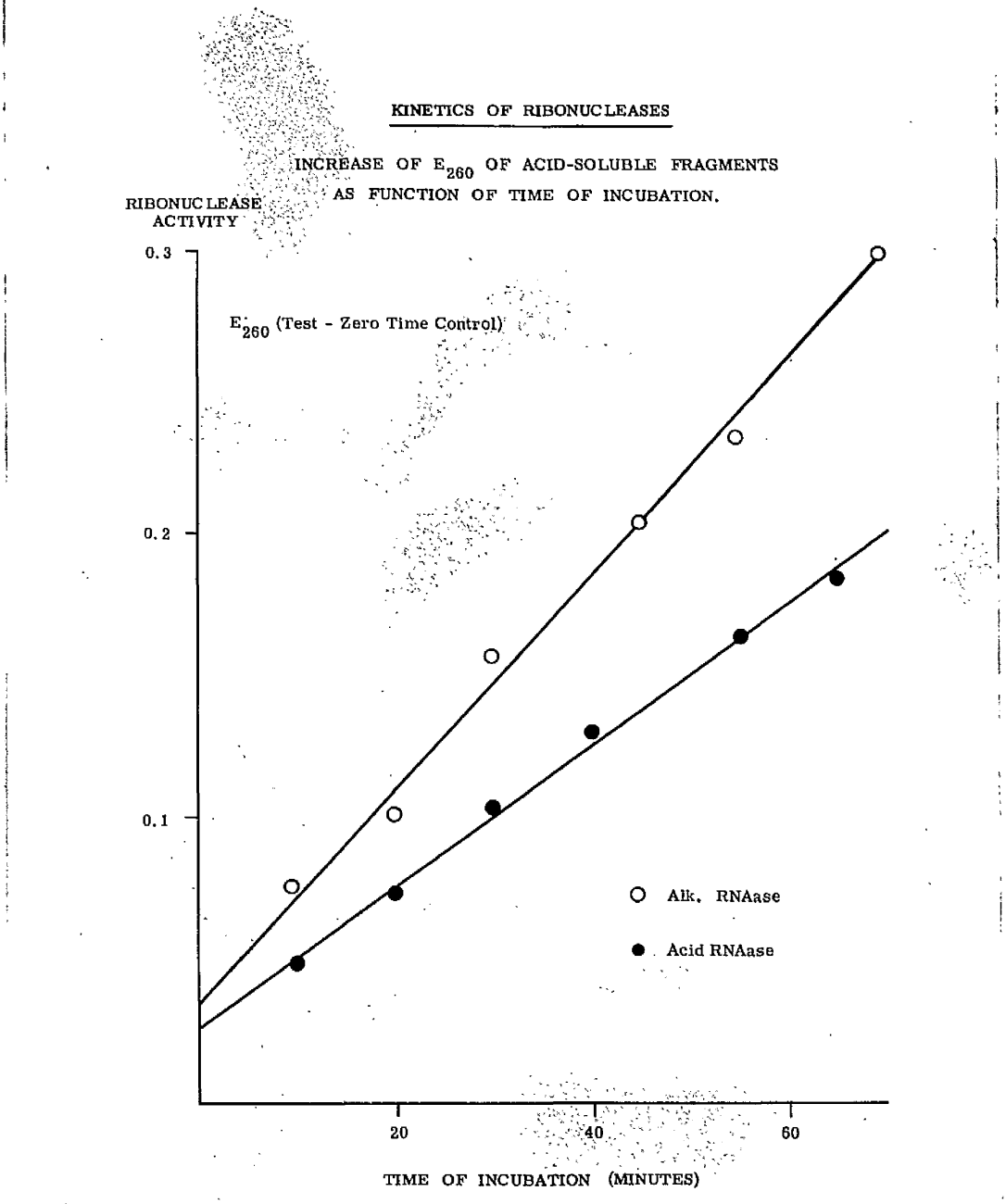


FIGURE 10

KINETICS OF RIBONUCLEASES

amount added, linearity does not hold above an E_{260} of 0.4. Another approach which yielded a similar conclusion was a study of the release of acid-soluble products with time. This was conducted on one sample each of serum, urine and breast supernatant. A large volume of the reaction mixture was incubated at 37°; at zero time and at various time intervals thereafter, aliquots of 3 ml. were withdrawn in duplicate and added to the uranyl acetate/perchloric acid reagent and processed in the usual manner. Typical results obtained are shown in Figure 10 from which it may be seen that the reaction is linear with time up to an extinction of 0.3. By chance rather than by design, it happened that no result in this group of experiments exceeded an extinction of 0.3. It was concluded that up to this level, all results could be accepted as directly proportional to enzyme concentration. Values above 0.3 were treated as an indication to repeat the assay using more dilute material, and this principle was adhered to during all subsequent experimental work. One other feature deserves comment. It is noteworthy that the lines as drawn in Figure 10 do not pass through the origin despite the fact that a zero-time blank has been deducted from each point. The explanation of this curious feature almost certainly lies in a very fast initial phase of RNAase activity before the reaction settles down to follow a linear rate. An identical observation was recorded by Kalnitsky et al. (1959) using a very similar method for assay of crystalline pancreatic RNAase.

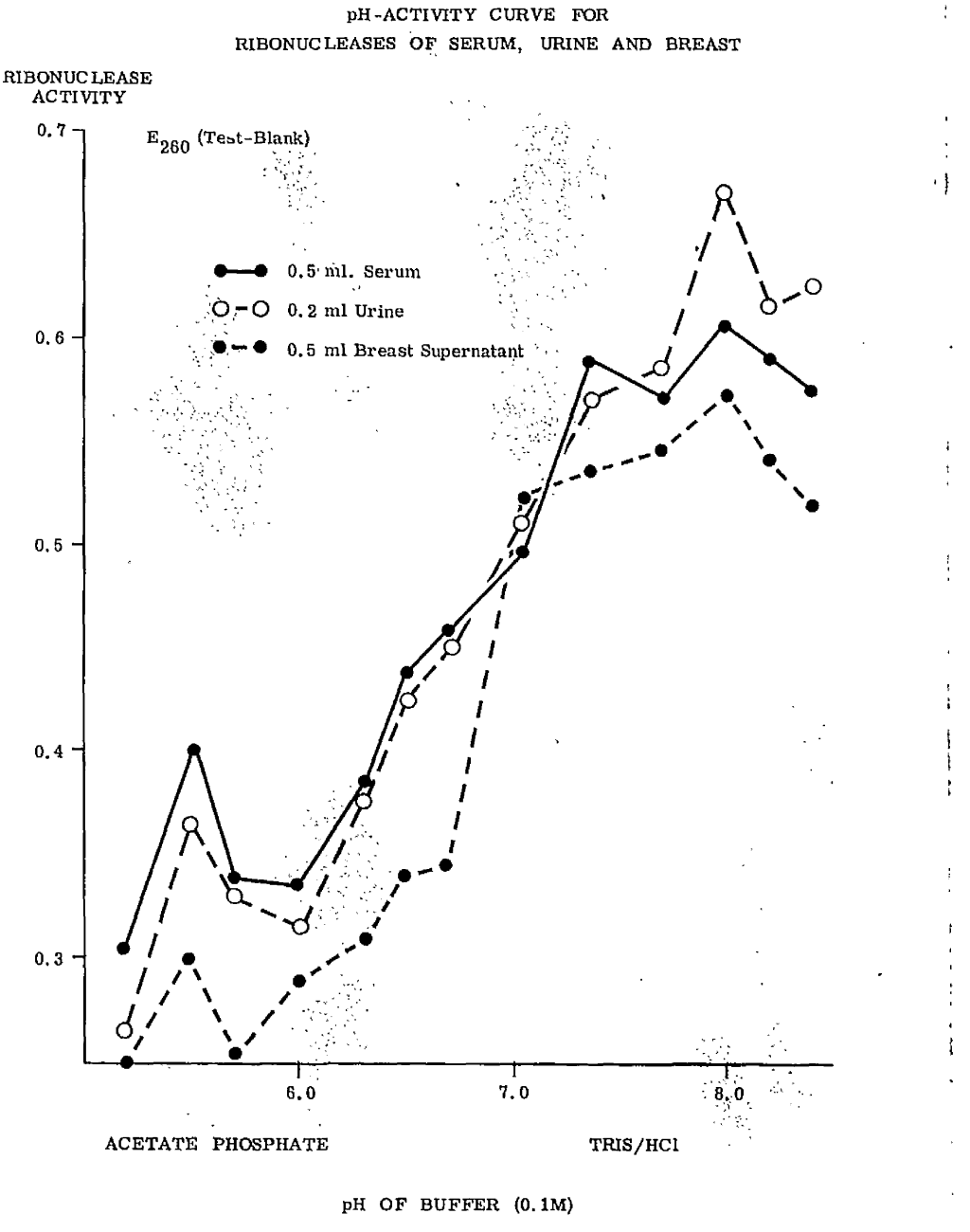


FIGURE 11

RELATIONSHIP BETWEEN RIBONUCLEASE ACTIVITY AND pH OF INCUBATION

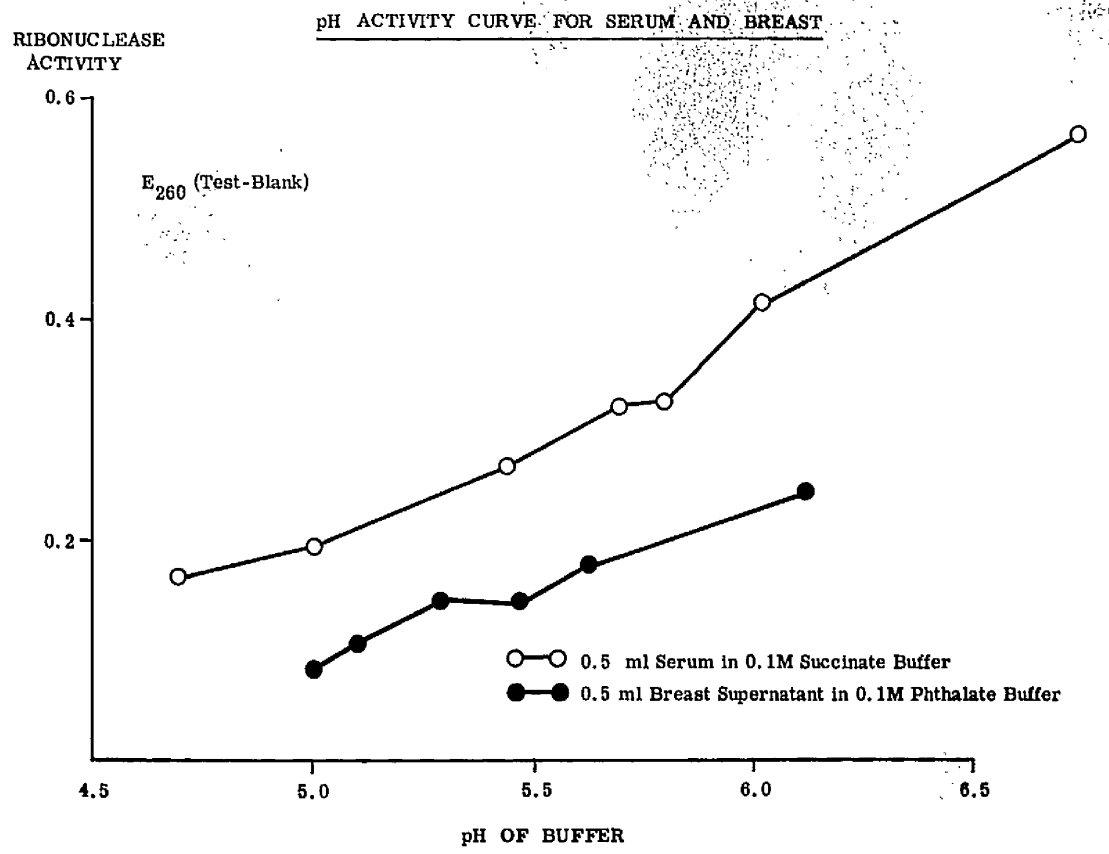


FIGURE 12

RELATIONSHIP BETWEEN RIBONUCLEASE ACTIVITY

AND pH OF INCUBATION

The next question to be examined was the relationship between those activities designated as 'alk. RNAase' and 'acid RNAase' in some of the materials to be studied. This work was restricted to serum, urine, and the supernatant fractions of breast (one case each of fibroadenoma and cystic mastopathy) and cervix (one case each of normal and carcinoma). The behaviour of all the samples was remarkably similar from a qualitative point of view. The change of activity with pH is shown for serum, urine, and breast supernatant in Figure 11. A broad peak is apparent between pH 7 and pH 8.5 probably maximal at pH 8. There was evidence of an additional peak at pH 5.5 in all three materials, but as this coincided with a change of buffer, it could not be accepted without further examination. Accordingly, further experiments were carried out to cover the critical range. The first set, using phthalate buffers, produced very high blanks because of the high extinction in the ultraviolet associated with the organic anion. The second set, using succinate buffers was more satisfactory and agreed reasonably well with the previous set. One example from each set is shown in Figure 12 from which it will be apparent that no clear evidence of a peak in the acid range was obtained.

Nevertheless further work was undertaken in an attempt to confirm this by studying the effects of different treatments on the activity at two pH values, one in the alkaline and the other in the acid range. The results of such an experiment

Treatment	E_{260} pH7.4	E_{260} pH5.6
60^{0} 1 hour	42	62
100^{0} 2 min	20	32
EDTA 1×10^{-6} M	89	78
PCMB 1×10^{-6} M	103	125
PCMB 2×10^{-6} M	102	129
Mg^{++} 1×10^{-5} M	110	127
Mg^{++} 2×10^{-5} M	103	132
Mg^{++} 5×10^{-5} M	106	150
Mg^{++} 8×10^{-5} M	90	139
Mg^{++} 1×10^{-6} M	84	123

TABLE 2.

EFFECT OF VARIOUS TREATMENTS ON SERUM RNAase ACTIVITY AT ALKALINE AND ACID pH

Buffers: Tris pH 7.4 (0.1M) Acetate pH 5.6 (0.1M)
Mg^{++} : as $MgCl_2 \cdot 7H_2O$
PCMB : para Chloromercuribenzoic Acid
EDTA : Ethylenediametetra-acetic acid, disodium salt

First column gives conditions of heating or final concentration of reagent added to standard incubation mixture.
Second and third columns give results of duplicate assays as percentage of value for untreated control.

with normal serum are shown in Table 2 where the results of quadruplicate analyses on the same specimen under various conditions are given as a percentage of the untreated control for that specimen. Some differences are apparent, especially in the effect of magnesium chloride upon the two activities. However one complication arose which should be mentioned at this stage and which prevented a clear-cut conclusion being reached. In all samples containing protein, and especially with serum which of course had the highest protein concentration of all, turbidity developed on adding the test material to the acid buffer-substrate mixture. An identical occurrence was observed with DNAase II estimations. This turbidity was not found when the nucleic acid alone was added to the buffer or when the test material alone was added except in so far as a slight haze was seen in some instances; nor was this phenomenon ever observed with nuclease assays conducted in an alkaline medium. The explanation almost certainly lies in the formation of complexes between nucleic acids and acidic protein, since such complexes have been frequently described and indeed form the basis for several methods of estimating nuclease activity (Houck, 1958a; 1959; Fiers and Moller, 1960). In view of this, it was possible that the effect of magnesium chloride was upon the substrate rather than upon the enzyme. One or two attempts to resolve this question were initiated, such as a study of the activating effect of a fixed concentration of magnesium chloride in the presence of increasing concentrations of substrate, but

these were soon abandoned as being peripheral to the real purpose of this investigation. It is necessary simply to make it clear that definite evidence for the existence of separate RNAases in the materials studied has not been obtained. These points will be considered later when the results on the various tissues and fluids are discussed. In passing, it should be stated that neither of the cervix supernatants examined showed any indication of a peak of RNAase activity in the acid range; the peak in the alkaline range was further to the left than those of the samples shown in Figure 11 and worked out at approximately pH 7.4.

Assay of DNAases

Once again, the principle of the methods was well-established, and involved measurement of the acid soluble products released from the substrate by enzyme action. However, in this case, two methods were examined and compared before a definite choice was made. This stemmed, in fact, from the unsatisfactory nature of the first method employed. In this, the acid soluble products of digestion were measured by the diphenylamine reaction of Dische for deoxyribose-containing compounds (Dische, 1955). Although this colour reaction occupies a hallowed place in nucleic acid chemistry, and has been used by several investigators for assay of DNAase under conditions identical to those used by the present author, several features caused concern. Contrary to Dische, the reaction was found to be time dependent;

moreover, factors such as the position of the tube in the boiling water-bath - whether or not it rested on the bottom and was therefore subjected to direct heat - influenced the intensity of the blue pigment produced. More serious difficulties arose, however, in the form of interfering substances. In most specimens, some acid-soluble peptide material was present along with the digestion products of DNAase action; in thyroid, material other than protein - most probably carbohydrate in view of the known content of mucoprotein in this tissue which is especially high in the 'Colloid' pool (Dempsey, 1955) - interfered with the estimation; in urine, the values for the blanks were remarkably high - partly due to the presence of nucleic acid derivatives, but probably also due to carbohydrate derivatives such as glycoproteins which are present in human urine (Dische, Kawasaki, Rothschild, Danilchenko and Zinsser, 1964). Dische has himself given certain correction factors for dealing with these difficulties, but the greatest difficulty of all arose in an entirely different respect. It was simply non-specific charring of the test material as a result of boiling up organic matter with the concentration of sulphuric acid used in the diphenylamine reagent. Of this difficulty, Dische makes no mention. The only possible way of correcting for this, would have been to run sulphuric acid blanks on both test and control for each enzyme assay.

Because of these difficulties, it was thought preferable to measure the extinction of the test solution after addition

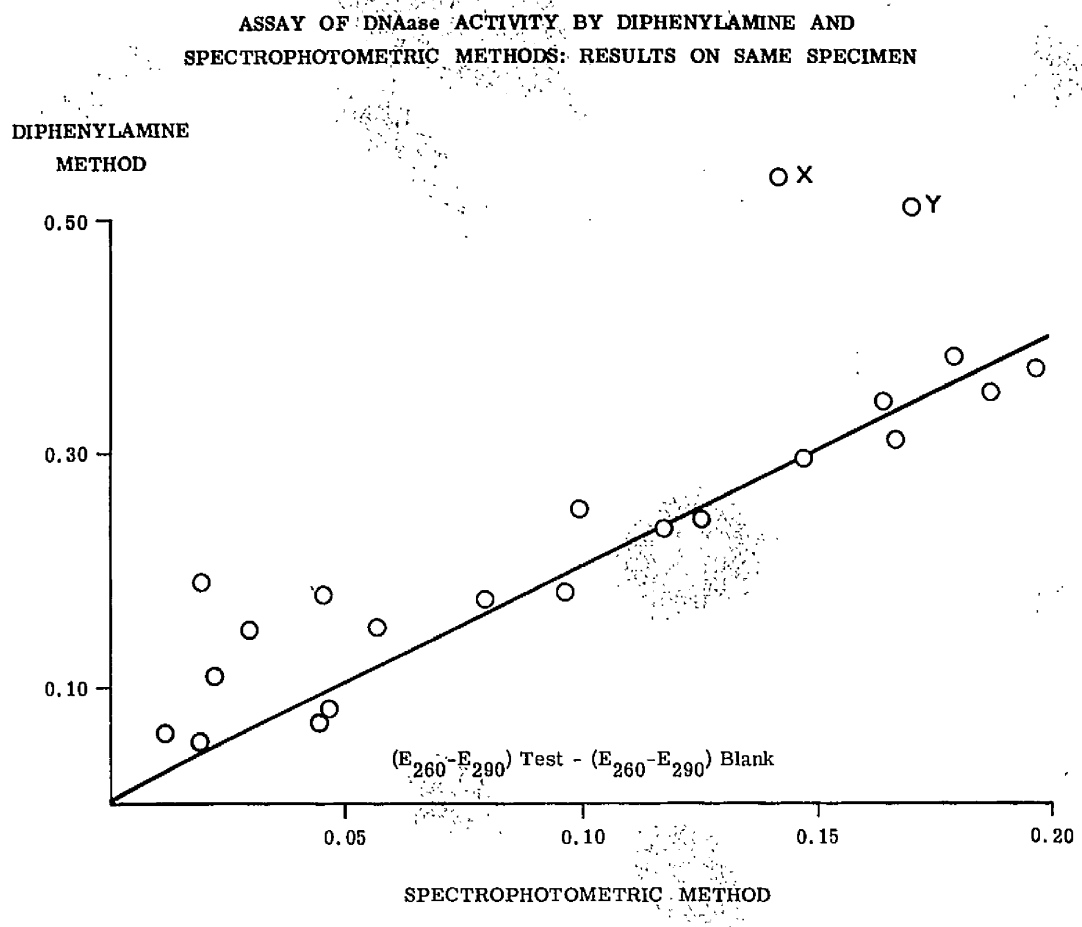


FIGURE 13

COMPARISON OF DEOXYRIBONUCLEASE ASSAY BY

DIPHENYLAMINE AND SPECTROPHOTOMETRIC METHODS

of tri-chloroacetic acid at 260 mμ and to employ this as an index of enzyme activity. Although this precipitant is unpopular in estimations involving measurements in the ultra-violet, its extinction was not a problem in the dilutions which were made for measuring E_{260} of test and control solutions. The value for the controls was as a rule only about 80% above the values given by the RNAase controls, and it was not considered necessary to alter the precipitant on these grounds. It was still felt necessary to correct for acid soluble peptide material, and in many samples of cell particle fractions, a slight turbidity persisted after addition of the acid which was probably due to lipid, and could not be precipitated even by addition of carrier albumin. To correct for both these hazards, the extinction of the solution at 290 mμ was subtracted from that at 260 in both test and control.

Comparison of the two methods was made by carrying out duplicate analyses of 20 samples of various biological samples by both methods. The results are plotted in Figure 13 and give a correlation coefficient $r = 0.933$; $t = 13.06$; $p < 0.001$. The most noteworthy feature, apart from the good agreement between the two methods, is that at low values, the Dische reaction tended to give high results, whereas with very active samples the agreement was extremely satisfactory. Although the factor given by Dische (1955) for correction of interference due to peptides was used, it is probable that this was inadequate and that the extinction measured was increased by background

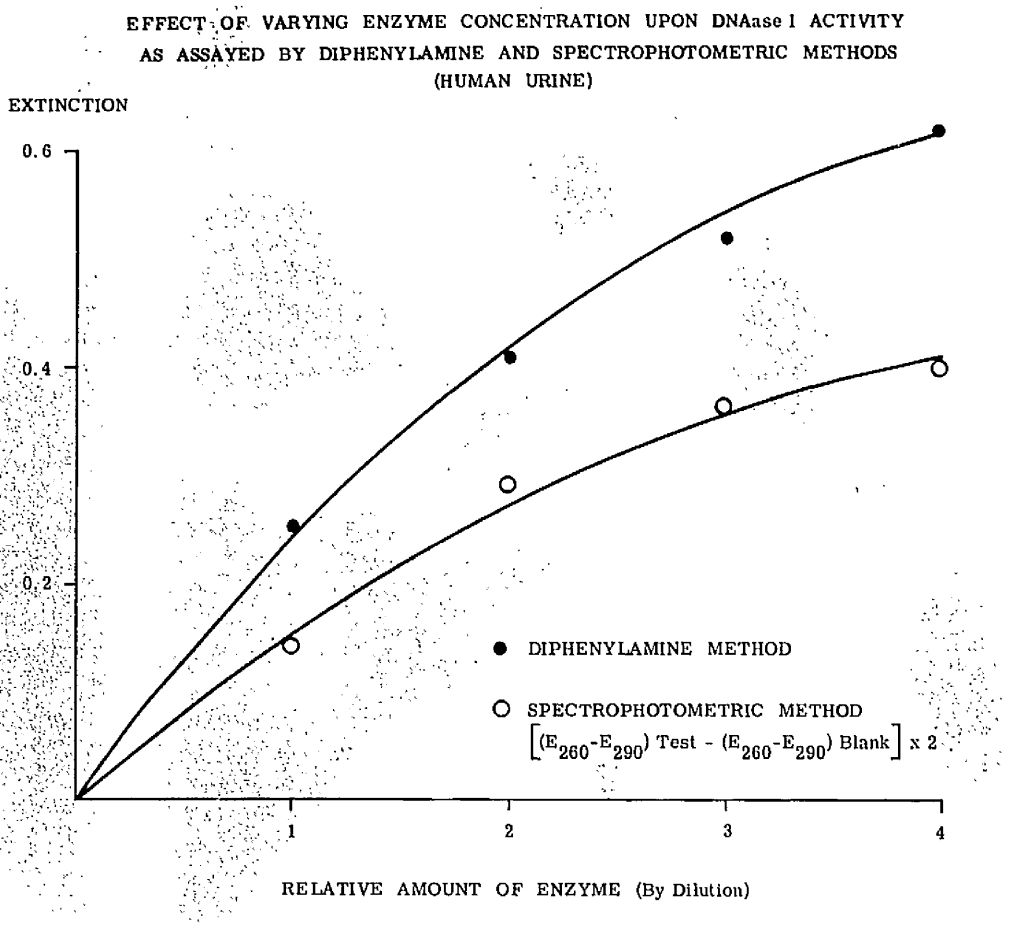


FIGURE 14

EFFECT OF VARYING ENZYME CONCENTRATION

UPON DNAase I ACTIVITY

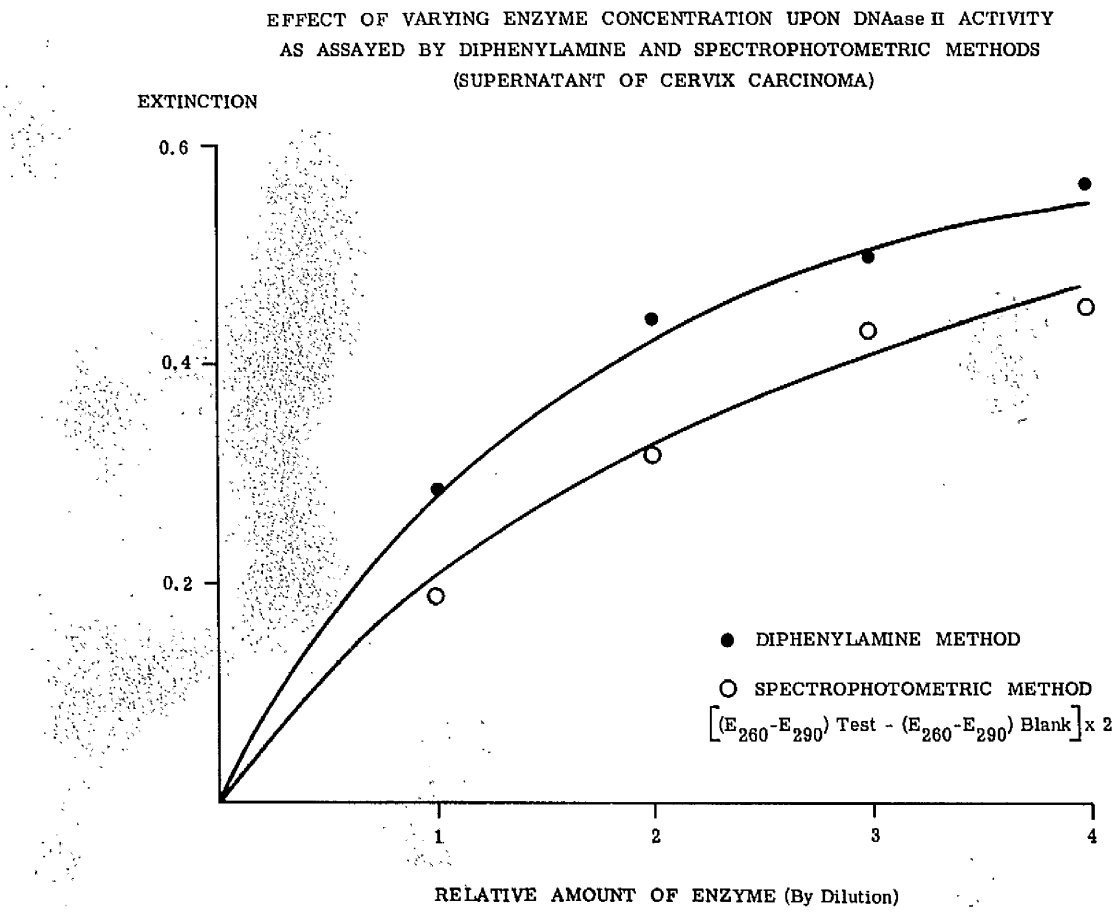


FIGURE 15

EFFECT OF VARYING ENZYME CONCENTRATION

UPON DNAase 11 ACTIVITY

charring which at low activities made a considerable difference to the actual measurement, whereas this interference was not so important at high activity. The other feature of interest is that two points shown in the Figure as X and Y were excluded from the calculation of the correlation coefficient since they were wildly at variance with the rest of the data. These were in fact the only two samples of thyroid supernatant studied and came from normal glands with a high 'Colloid' content. It is almost certain that interference in the estimation by carbohydrate substances contained in 'Colloid' contributed to the disproportionately high extinction given by the Dische reaction, since in a study of the suitability of a similar reaction for DNA as applied to thyroid tissues - the indole/hydrochloric acid method of Ceriotti (1952), a similar difficulty was encountered and the method had to be abandoned (Ayre, 1965).

The relationship between the activity as measured by the two methods and the amount of enzyme added was studied with three samples of urine for DNAase I and one supernatant from each of the three tissues for DNAase II. Typical of the results obtained are those depicted in Figures 14 and 15 . It is interesting that the ratio between the extinction of the blue pigment measured as $E_{595} - E_{650}$ produced by enzyme action and the UV adsorbancy of the products of the same enzyme material measured as $E_{260} - E_{290}$ was higher in the case of urine as shown in Figure 14 than in the case of cervix supernatant as shown in Figure 15 . This bears out what has already been

KINETICS OF DEOXYRIBONUCLEASES

INCREASE IN ACID-SOLUBLE FRAGMENTS AS FUNCTION OF TIME OF INCUBATION.

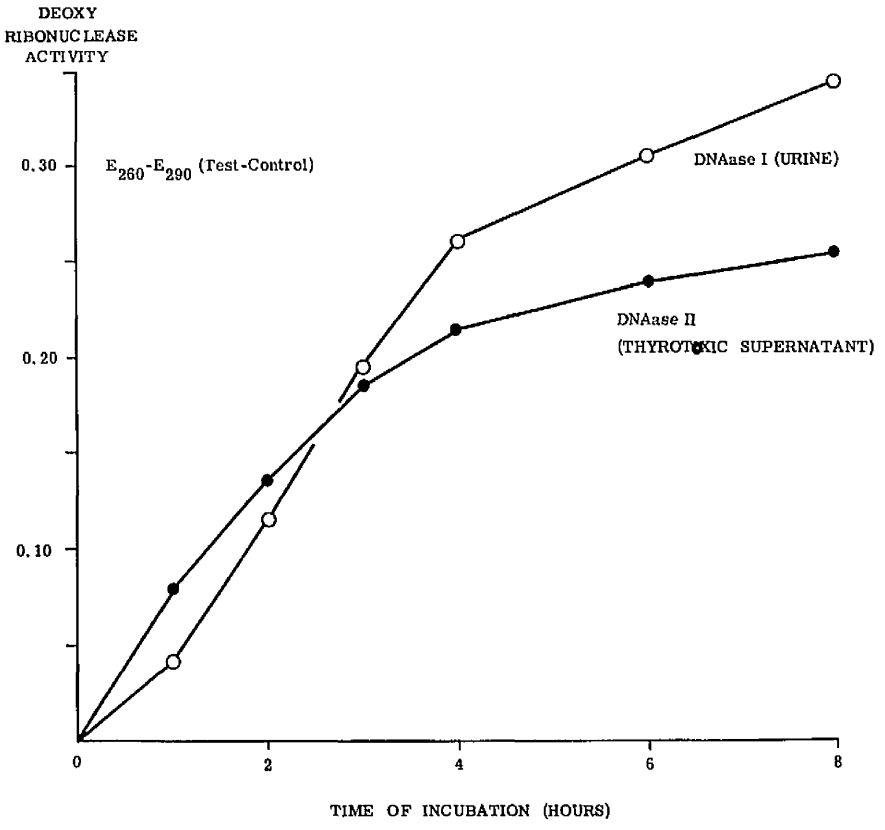


FIGURE 16

KINETICS OF DEOXYRIBONUCLEASES

stated regarding high background colour in the urine samples which cannot be corrected by a simple enzyme control. A similar phenomenon was found in the sample of thyroid supernatant used for DNAase II assay in this set of experiments.

The relationship between increasing amounts of enzyme and the activity recorded are not strictly linear with either method, though the two are roughly comparable in the extent to which they deviate from true linearity. Another feature which should be pointed out as apparent in these results, since it was found with the other samples studied in this set of experiments, is the fact that the activity of DNAase I in urine measured spectrophotometrically at 4 hours is 273% of that measured in the same fashion at 1 hour of incubation; the supernatant DNAase II activity at 4 hours was 240% of that measured at 1 hour. The relevance of this observation is a little clearer when Figure 16 is examined. This is one of two experiments in which the kinetics of enzyme action were studied for both DNAases. It appears that over the first hour, the rate of release of acid-soluble fragments is faster for DNAase II and then tails off. In contrast, DNAase I seems to show a lag during this initial period and then operates at a much faster rate. This lag period for DNAase I is in agreement with a similar observation made by several workers employing a variety of techniques (Reichmann, 1956 ; Schumaker et al., 1956 ; Thomas, 1956 ; Williams et al., 1961) and contrasts with the observation made

by Houck (1959), using a turbidimetric assay procedure, that activity tails off after 30 min. This would support the suggestion that DNAase I operates by double-hit cleavage, i.e. it splits only one of the twin stranded helices of DNA at a time (Fredericq, 1958). It must be stressed, however, that all these workers were using crystaline pancreatic DNAase. The apparent kinetics of DNAase II in the tissue supernatants are in agreement with those workers who have proposed that splenic DNAase II operates by single-hit cleavage at least in the early stages, i.e., it splits both strands of DNA simultaneously at the same level (Bernardi and Sadron, 1961 ; Desreux et al., 1962 ; Bernardi, 1964).

The main practical conclusion drawn from these studies was that the assay method chosen was probably as close as one could get to a truly linear and strictly proportional assay procedure which could be applied to assay of both DNAases in a wide range of materials. Individual variations would undoubtedly have produced improvements for certain methods but would not have been generally applicable in view of the wide spectrum of activity to be measured in the prospective work schedule. Accordingly, an incubation time of 4 hours was chosen, since this was necessary for low activities such as those found in serum; but all specimens in which the extinction as measured by $E_{260} - E_{290}$ exceeded a value of 0.20 were diluted, and so far as possible this situation was anticipated

by diluting specimens routinely where high activity was suspected.

Adenosine Deaminase

The principle of the method used was first established by Kalckar (1947) and had been employed by many subsequent investigators. The conditions utilised in this study had been developed by Schneider and Hogeboom (1952) and by Solomon (1960). These authors were working with liver homogenates, and had found it necessary to deduct the extinction at 290 mμ from that at 265 mμ in the enzyme-substrate mixture at zero-time and at the completion of the incubation period. The reasons for this modification are not elaborated. In the author's experience such values as were obtained for E_{290} were frequently negative and served only to confuse the issue and delay the assay.

Since none of the authors using this spectrophotometric technique have checked for the formation of compounds other than inosine from the substrate, one or two experiments were undertaken with this purpose in mind. In the first set, an active supernatant preparation was allowed to act with substrate for 1, 3 and 6 hours after which each reaction mixture was placed in a boiling water bath to heat-coagulate the protein, and to evaporate the water. When the volume was reduced to about 0.5 ml, the mixture was centrifuged and the supernatant

CHROMATOGRAPHY OF DEPROTENIZED SUPERNATANT FROM ADENOSINE DEAMINASE INCUBATION MIXTURES, ENZYME BLANK, AND MARKERS.

(n-Butanol - NH_3; descending; Overnight run)

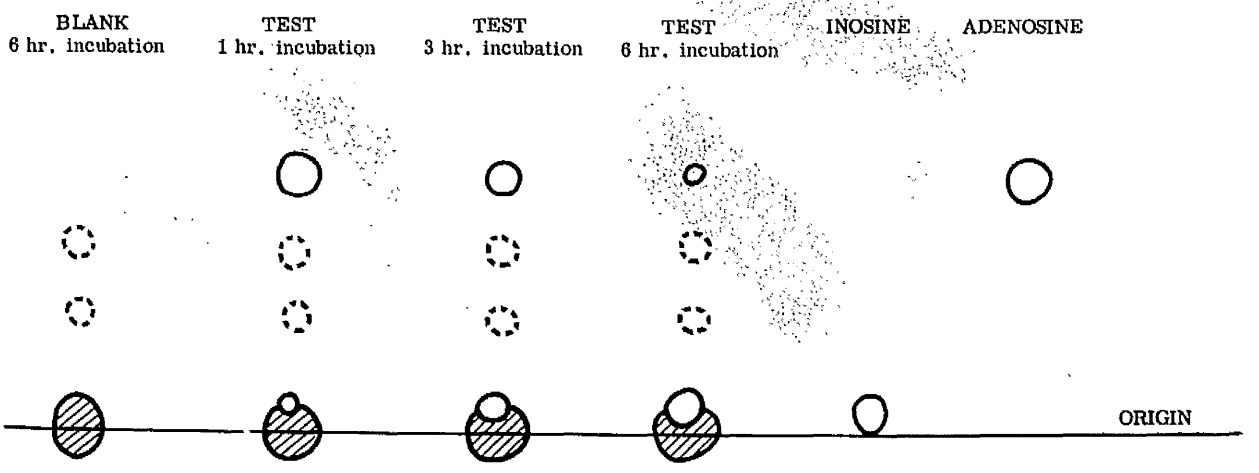


FIGURE 17

The shaded area at the origin represents chromogenic material, as do the broken circles. None of these areas were associated with quenching of UV-light, and probably represent products of haem degradation.

removed as completely as possible with a Pasteur pipette and the entire volume applied to Whatman No. 1 filter-paper as a discrete spot. The chromatogram was developed by a descending run in the n-Butanol and ammonia solvent system of Markham and Smith (1949), consisting of the following reagents: n-butanol 430 ml, distilled water 45 ml, ammonia (S.G. 0.88) 25 ml. The results of two such experiments are summarised in the form of a diagram in Figure 17 since it did not prove possible to take a permanent photographic record of the spots which were only visible on ultraviolet light. The spots in the positions corresponding to inosine and adenosine markers were the only ones to show quenching on ultraviolet illumination. Unfortunately, attempts at elution and identification by means of their spectral properties were unsuccessful because of the low concentration of the nucleosides and the relatively high background absorption of the paper. There was no doubt, however, that by visual inspection, a decrease in the spot corresponding to adenosine with an increase in that corresponding to inosine became progressive with time.

In this particular solvent system, it is not possible to distinguish between inosine and xanthine derivatives. Two specimens were therefore screened by developing a chromatogram of protein free concentrate, obtained in the manner just described, in water adjusted to pH 10.0 with N. NH_4OH according to Wyatt (1955). Only inosine and adenosine markers were applied, and they did not display the R_f values published by Wyatt (1955).

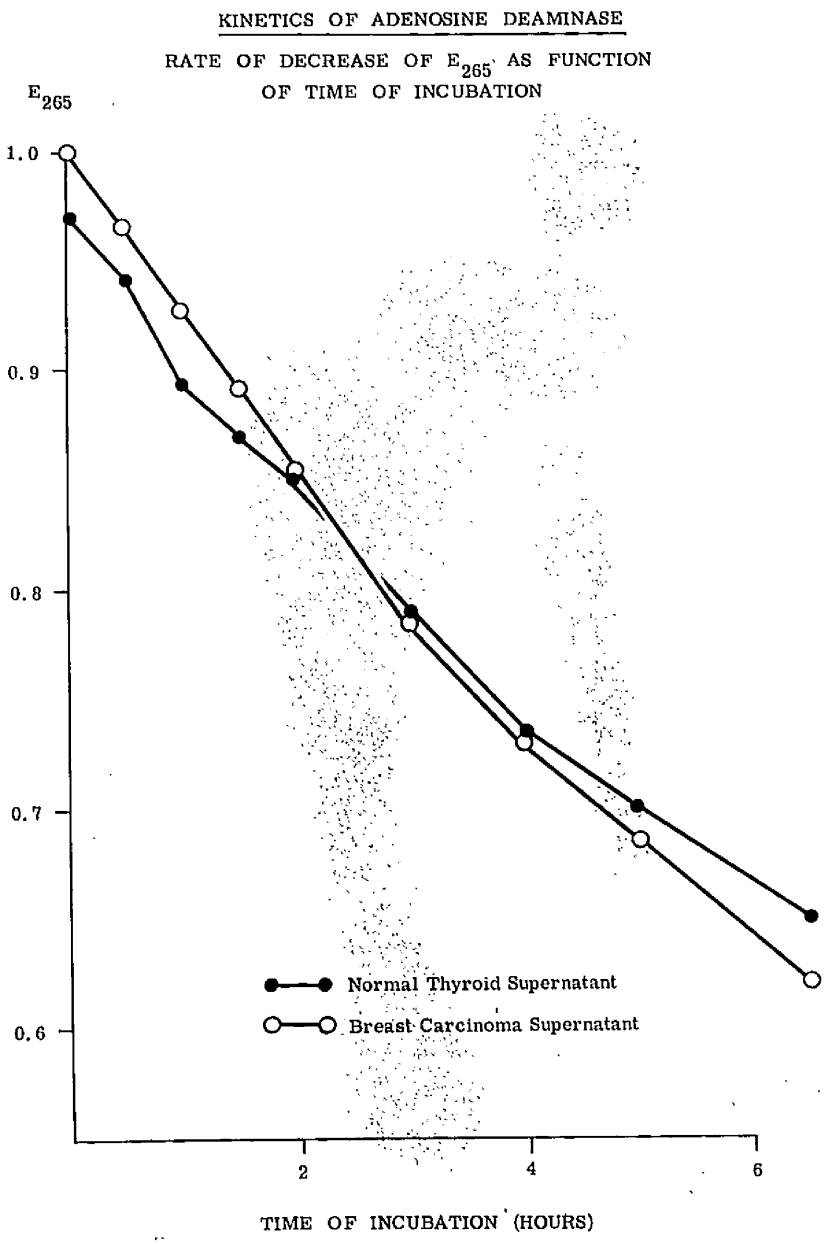


FIGURE 16

KINETICS OF ADENOSINE DEAMINASE

It was subsequently realised that this was occasioned by failure to adjust to pH 10 the solutions to be tested and those of the markers prior to their application to the paper. The main point however was that spots corresponding to both markers were present on the test strips and there were no other spots demonstrating quenching in ultraviolet light. It was concluded that the assay was indeed a measure of the deamination of adenosine, and that no side-reaction or sequential reactions were occurring to an extent likely to disturb the validity of the method.

In this type of enzyme assay, it is important to make quite sure that the concentration of substrate present throughout the period of incubation is in all cases adequate to sustain a linear reaction rate (Dixon and Webb, 1964). Accordingly, the kinetics of the reaction were studied on several samples and typical results are presented in Figure 18 . It is apparent that the reaction was linear with time over several hours, and down to an extinction at 265 mμ of 0.75. A number of active samples were diluted, and the relationship between enzyme concentration and activity was established, exactly as in the case of the other enzymes, over a range that included a fall in the E_{265} of 0.25. This was taken as the upper limit of sensitivity of the method, although there was a wide margin of safety, and specimens which produced a greater fall in E_{265} of the substrate were diluted and the assay repeated.

EFFECT UPON ENZYME ACTIVITY OF EXPOSURE TO ULTRASONIC VIBRATIONS AS FUNCTION OF TIME (MITOCHONDRIA FROM CYSTIC MASTOPATHY)

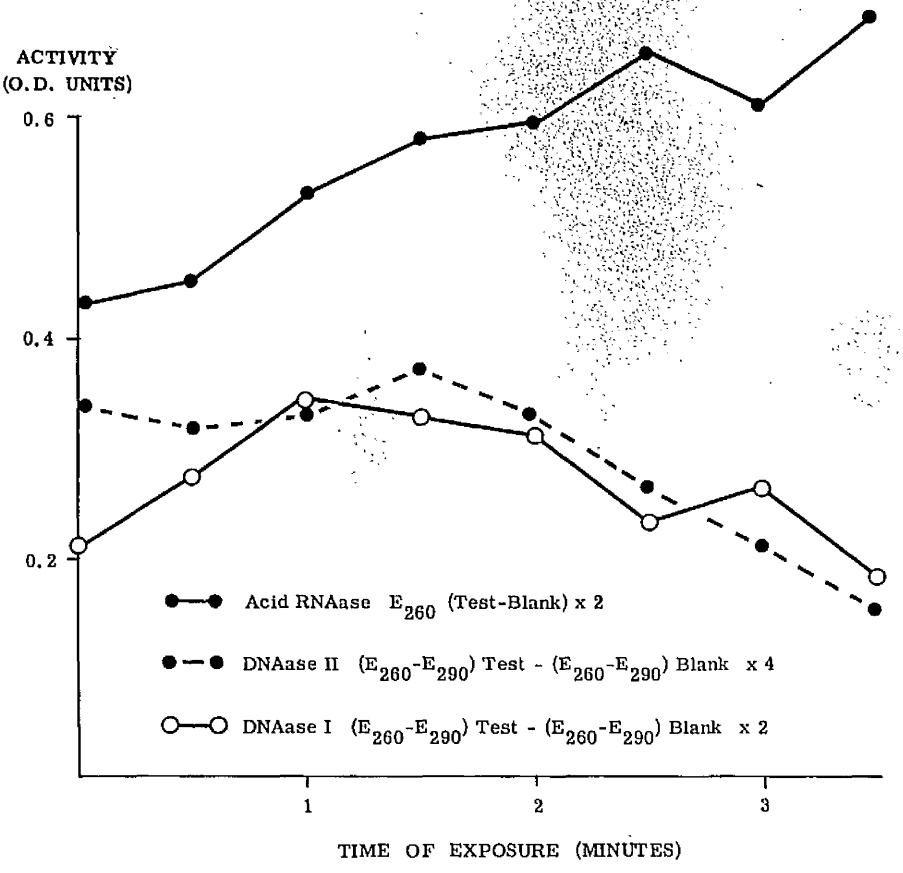


FIGURE 19

Effect of Ultrasound Upon Particle Preparations

It was not an easy matter to examine this question systematically. To do so required a very large yield of particles, and it was seldom that these could be obtained in sufficient quantities in the early experiments. However, it became possible to do so in two cases of thyrotoxicosis and one case of cystic mastopathy. The preparations were suspended in a fairly generous volume of distilled water and subjected to ultrasonic disintegration under the identical conditions it was proposed to employ in the subsequent experimental work. Before disruption, and at various time intervals, aliquots sufficient to allow duplicate analyses of most or all enzymes were withdrawn and chilled immediately. As the glass receptacle containing the suspension to be ruptured could not comfortably hold more than 10 ml, the disruption of a single preparation had to be carried out in a number of stages each covering several time intervals. For this reason, it is probable that some inhomogeneity in the starting material would contribute variability to the results. The results obtained in such an experiment are shown for the "Mitochondrial" fraction of breast in Figure 19 . Because of a technical disaster, the alk. RNAase assays were entirely vitiated. The other estimtions were satisfactory and show that acid RNAase increased by more than 50% over the 3½ minute period; DNAase I increased by two-thirds over 1½ minutes and then declined;

EFFECT OF TIME OF EXPOSURE OF THYROXIC MITOCHONDRIA TO ULTRASONIC VIBRATIONS UPON ENZYME ACTIVITY

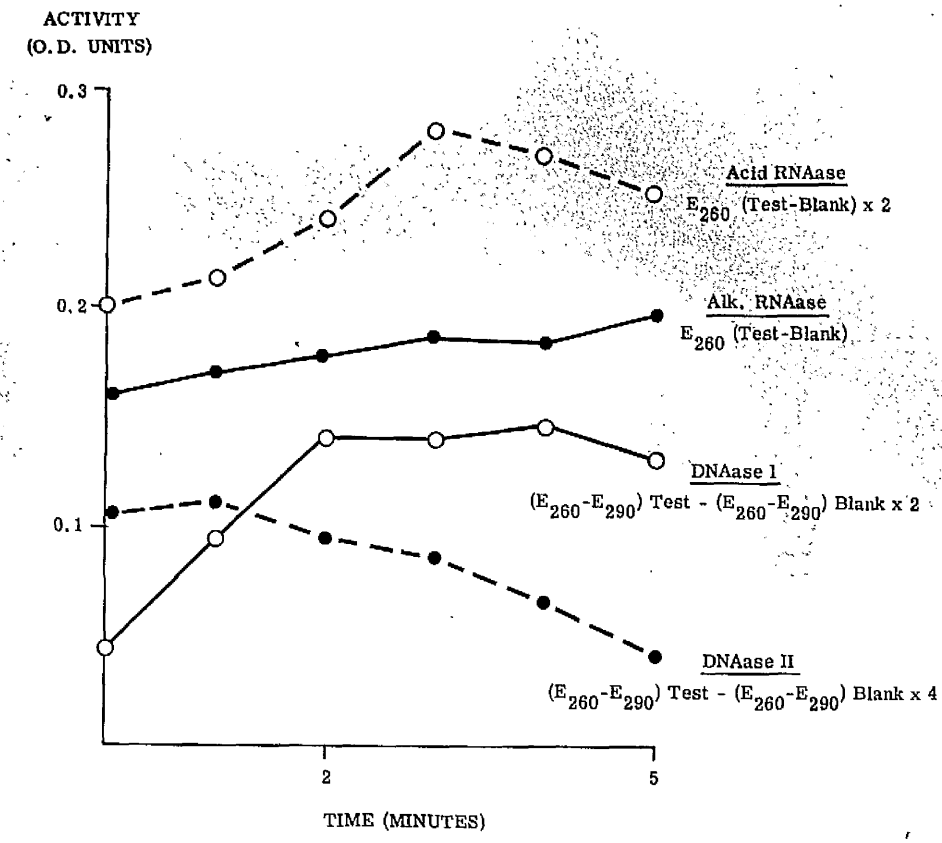


FIGURE 20

EFFECT UPON ENZYME ACTIVITY OF EXPOSURE TO ULTRASONIC VIBRATIONS AS A FUNCTION OF TIME (THYROTOXIC MICROSOMES)

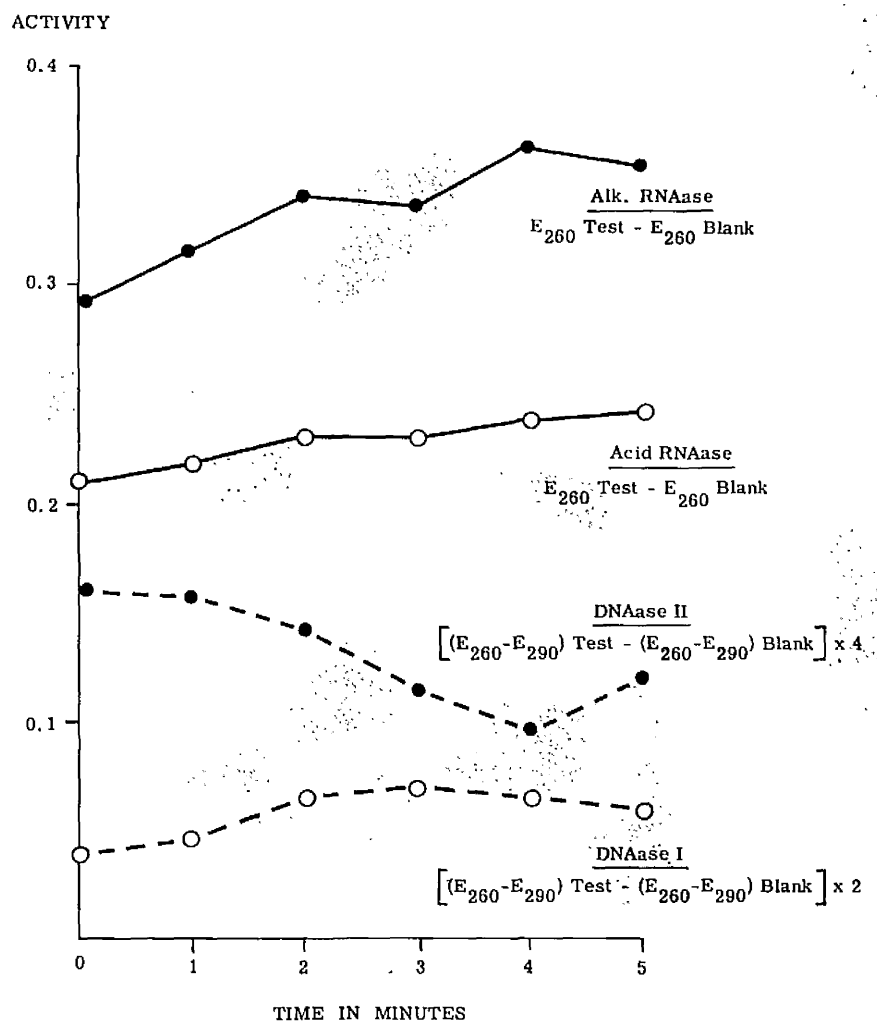


FIGURE 21

DNAase II showed hardly any change until 2 minutes when it started to decline. Only half the number of points could be obtained with the "Microsomal" fraction from this tissue. The results have not been shown; they were qualitatively similar, acid RNAase and DNAase I showing an increase but not to the same extent as occurred in the "Mitochondrial" fraction; DNAase II showed a gradual fall; and alk. RNAase fell victim to the same technical disaster.

The results obtained with one of the thyrotoxic samples are shown in Figures 20 and 21 . In the "Mitochondrial" fraction, acid RNAase increased by 40% over 3 minutes, DNAase I by 300% over 2 minutes; alk. RNAase showed a very slight and very gradual rise; and DNAase II a gradual decline. In the "Microsomal" fraction, alk. RNAase increased by 20% over 5 minutes; acid RNAase showed little change; DNAase I an increase of 75% over 2 mimutes; and DNAase II a steady decline. The results with the second sample are not shown in detail, and are summarised here instead: DNAase II showed a steady fall in both fractions and DNAase I increased by about 150% in both at 2 minutes; however alk. RNAase was increased by 50% after 5 minutes in the "Mitochondrial" fraction with little change in acid RNAase, and in the "Microsomal" fraction acid RNAase was increased by 30% at the end of 5 mimutes and alk. RNAase by only 10%. It is clear that many discrepancies remain to be clarified, since the results of these two experiments were rather contradictory. However, the main purpose was to decide

the optimum time of exposure to ultrasound which would permit the assay of all four enzymes in the same material. The critical enzyme was considered to be DNAase I which, as will become apparent later, was the only one of the four to have a consistently higher specific activity in the particles than in the supernatant. It was also considered more important to ensure that inactivation of an enzyme did not take place, even though this might perhaps mean that another enzyme was not fully activated. With these points in mind, a compromise was made by selecting 90 seconds as the period during which all subsequent particle preparations were routinely exposed to ultrasound.

Measurement of Deoxymucleotides in Urine

The cysteine/sulphuric acid reaction according to Stumpf (1947) has already been described in the Methods, Page 98, and has been used by many investigators in the past. The method was not without its problems when applied to urine, As in the case of the diphenylamine reaction, a colour which on naked-eye appearance could not be distinguished from that of the test solution was formed when sulphuric acid alone was added to the urine samples. It was feared that cysteine might be excreted in some of these urines in amounts sufficient to interfere with the analysis, since a very small concentration is required.

This problem was solved by preparing various solutions

SPECTRUM OF PIGMENT FORMED BETWEEN URINE AND SULPHURIC ACID IN PRESENCE AND ABSENCE OF CYSTEINE

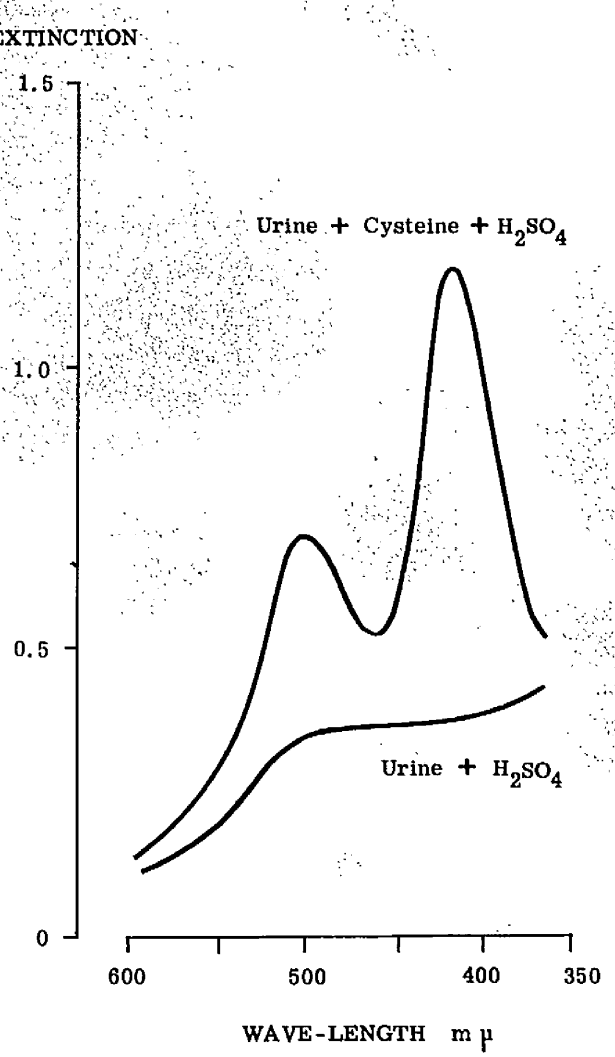


FIGURE 22

SPECTRUM OF PIGMENTS FORMED IN STUMPF REACTION
BY DEOXYRIBOSE, DNA, AND URINE WITH ADDED DNA

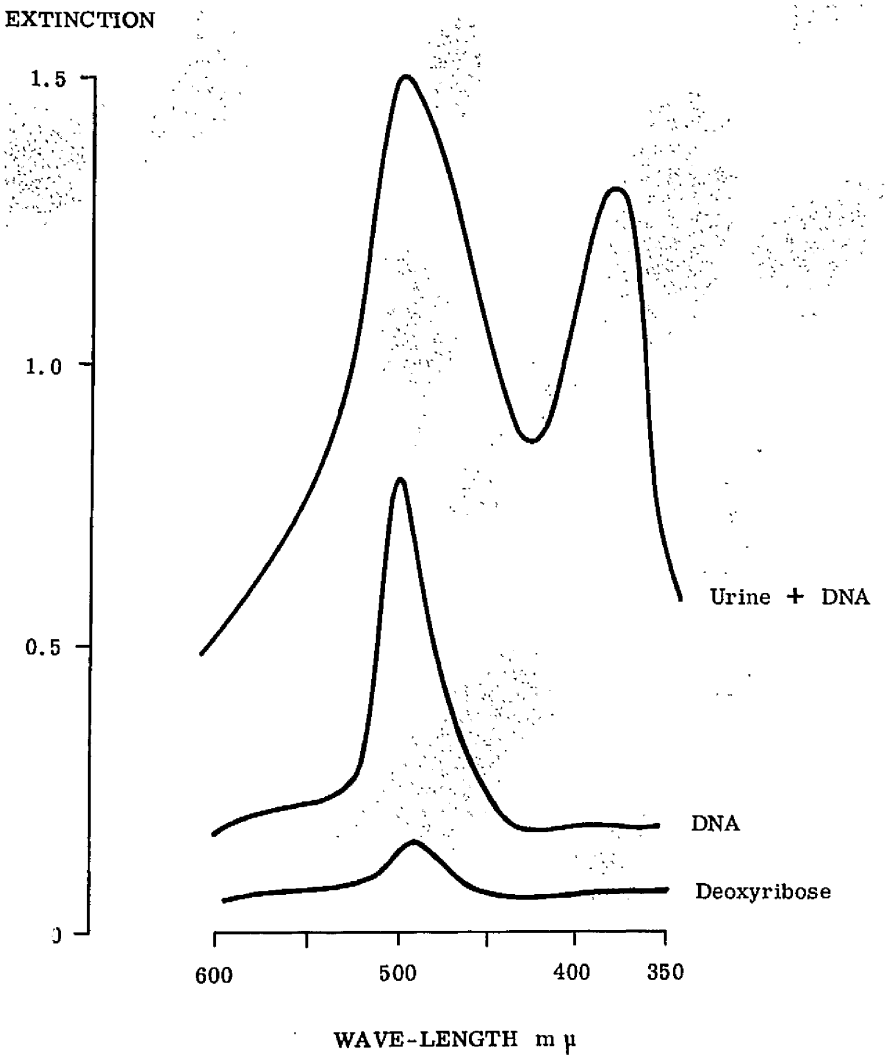


FIGURE 23

of urine, of deoxyribose, of DNA, and of urine with added DNA. To these were added cysteine and sulphuric acid, and sulphuric acid alone. The spectra of the various pigments formed were examined in the Carey Automatic Recording Spectrophotometer Model 11-50 (courtesy of Prof. J.N. Davidson). Some representative tracings obtained are shown in Figures 22 and 23 . It will be seen in Figure 22 that two peaks are given by the cysteine/sulphuric acid reagent with human urine, only one of which corresponds to that given with pure DNA. Furthermore, almost half the extinction given by the test solution at 495 mμ is given by a sulphuric acid blank. Figure 23 demonstrates that the E_{495} of a solution of pure DNA is five times as great as that given by an equivalent amount of deoxyribose. It also shows that one peak only is given by pure DNA with a peak at 495 mμ. Finally, it is apparent that the extinctions of the urine and the pure DNA are additive, since the urine to which the DNA was added is the same as that studied in the previous Figure. Virtually no reaction was given when sulphuric acid was added to pure DNA.

It was therefore decided to go ahead with the estimation as described by Stumpf (1947), subtracting from each urine specimen a blank derived by omitting cysteine from the complete reaction mixture. It is gratifying that some time after reaching this conclusion, a study of deoxynucleotide excretion in human urine was published by Berry et al. (1963) who reported similar difficulties with the Stumpf reaction and

adopted a similar strategy to cope with this situation.

The Nature of the Particle Fractions

A single thyrotoxic gland was collected, stored, homogenised, and separated into fractions following the procedure identical to that used in the preparations to be reported. Each of the three sediments collected after centrifugation at 500 g, 5000 g, and 35,000 g were taken for electron microscopy which was carried out by Dr. R. F. Macadam, Dept., of Pathology, Western Infirmary, Glasgow. The top of the first sediment, and samples from top and bottom of the next two sediments were examined. Many fields were scanned and several sections cut from each block. Some of the photographs obtained are shown in Plates 1 to 5, and it should be emphasised that these were chosen to demonstrate the separation in the most unfavourable light possible so that no illusions would be conveyed regarding the extent of separation obtained. In fact, many fields in each fraction showed homogeneity of the particles present. The overall picture is however best summarised by reporting the impression derived by Dr. Macadam from a detailed examination of the material. This showed that the initial sediment contained the nuclei, most of which were intact; an occasional ruptured nucleus was seen. This sediment was almost entirely free of mitochondria, the only other contaminant being strands of endoplasmic reticulum attached in places to the nuclear membrane. The second sediment consisted

PLATES 1-5 : GENERAL LEGEND.

TE 1. NUCLEAR FRACTION x 5,000.

Electron micrograph of sediment obtained by centrifugation of whole homogenate at 500g. Very little particulate material is present, apart from endoplasmic reticulum attached to nuclear membrane which in most nuclei is intact.

TE 2. BOTTOM OF MITOCHONDRIAL FRACTION x 22,000.

Electron micrograph of sediment obtained by centrifugation between 500 - 5,000g showing large, poorly-preserved and small, well-preserved mitochondria, several strands of endoplasmic reticulum, and a single nucleus.

TE 3. TOP OF MITOCHONDRIAL FRACTION x 22,000.

The heterogeneous nature of this fraction is apparent from this field showing several large mitochondria and occasional dense spherical bodies akin to lysosomes. The remainder of the material is composed of microsomes and small mitochondria.

TE 4. BOTTOM OF MICROSOMAL FRACTION x 22,000.

This field is very similar to that shown in the previous figure although mitochondria are less abundant. The material shown here was sedimented between 5,000 - 35,000g.

TE 5. TOP OF MICROSOMAL FRACTION x 22,000.

The bulk of the material visible in this field is microsomal. About a dozen each of mitochondria and lysosomal-like bodies are also present.

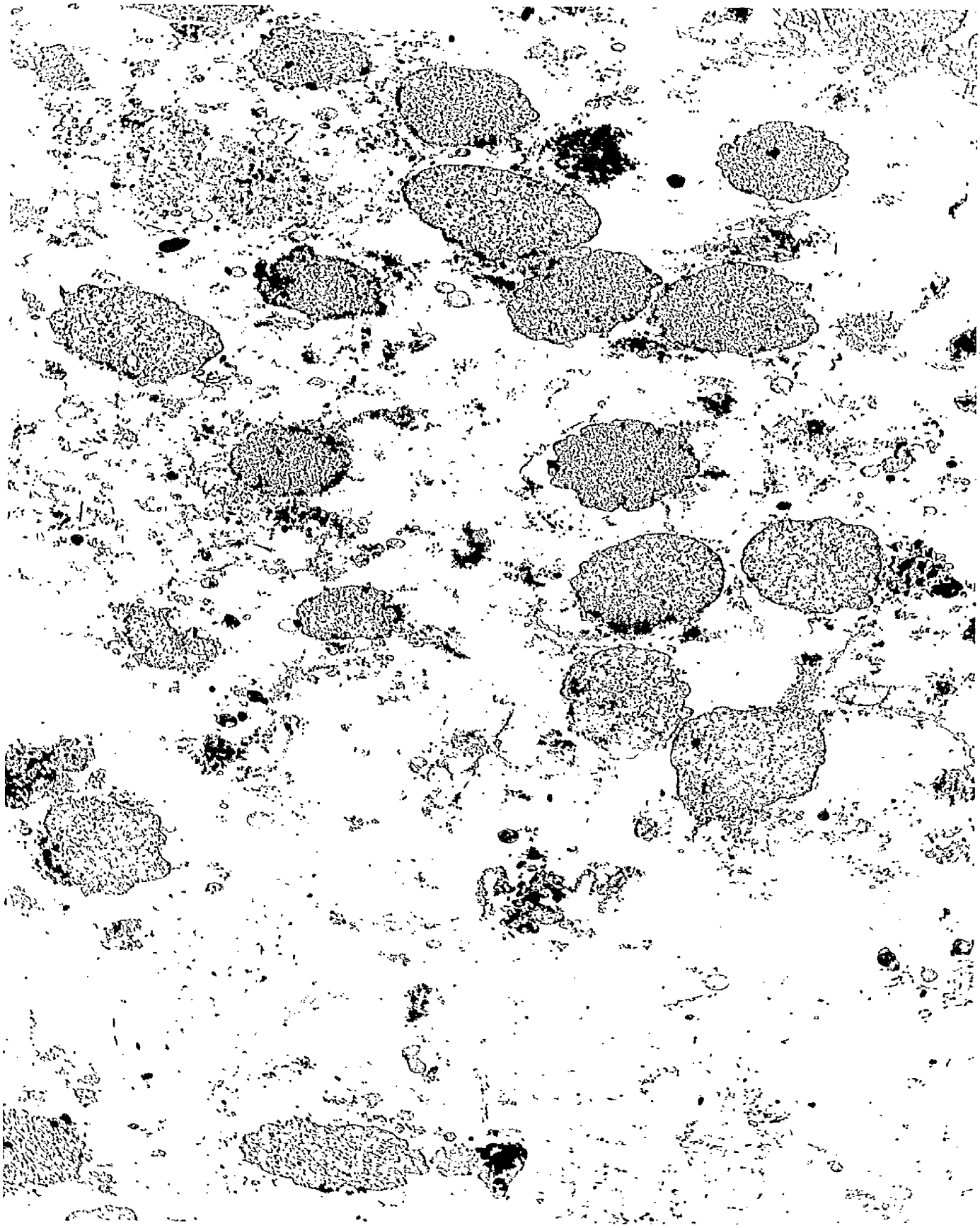


PLATE 1. NUCLEAR FRACTION x 5,000.

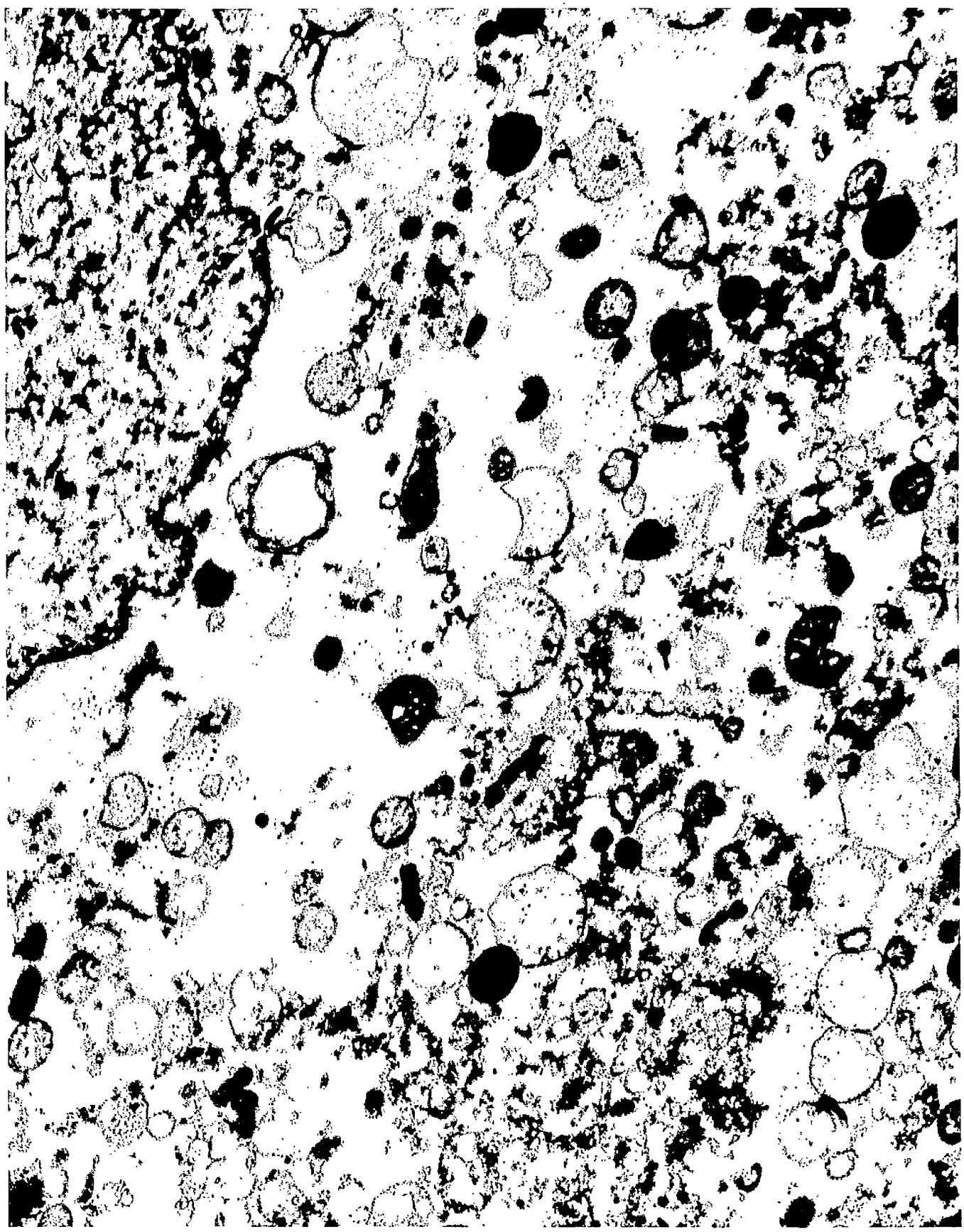


PLATE 2. BOTTOM OF MITOCHONDRIAL FRACTION x 22,000.

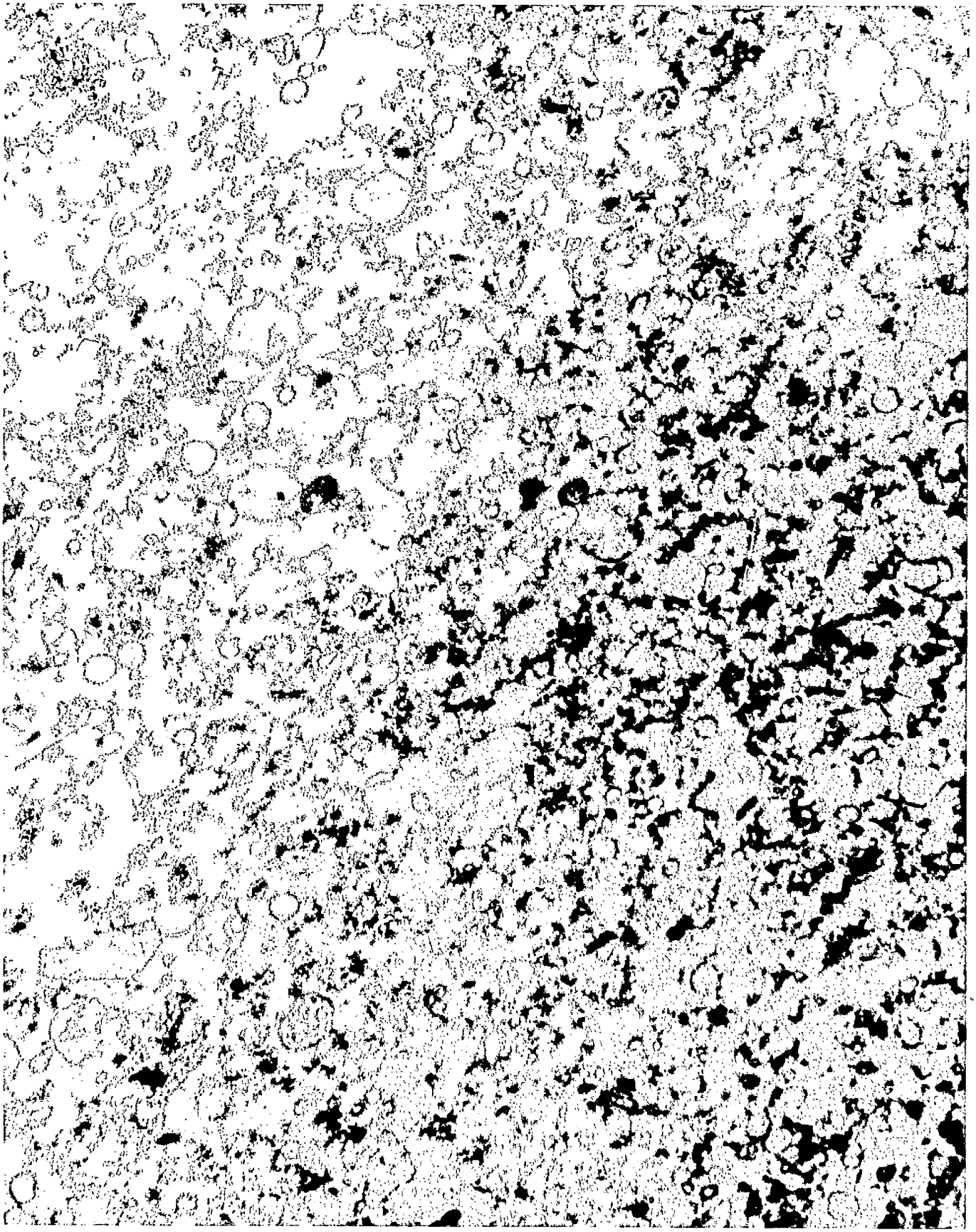


PLATE 3. TOP OF MITOCHONDRIAL FRACTION x 22,000.

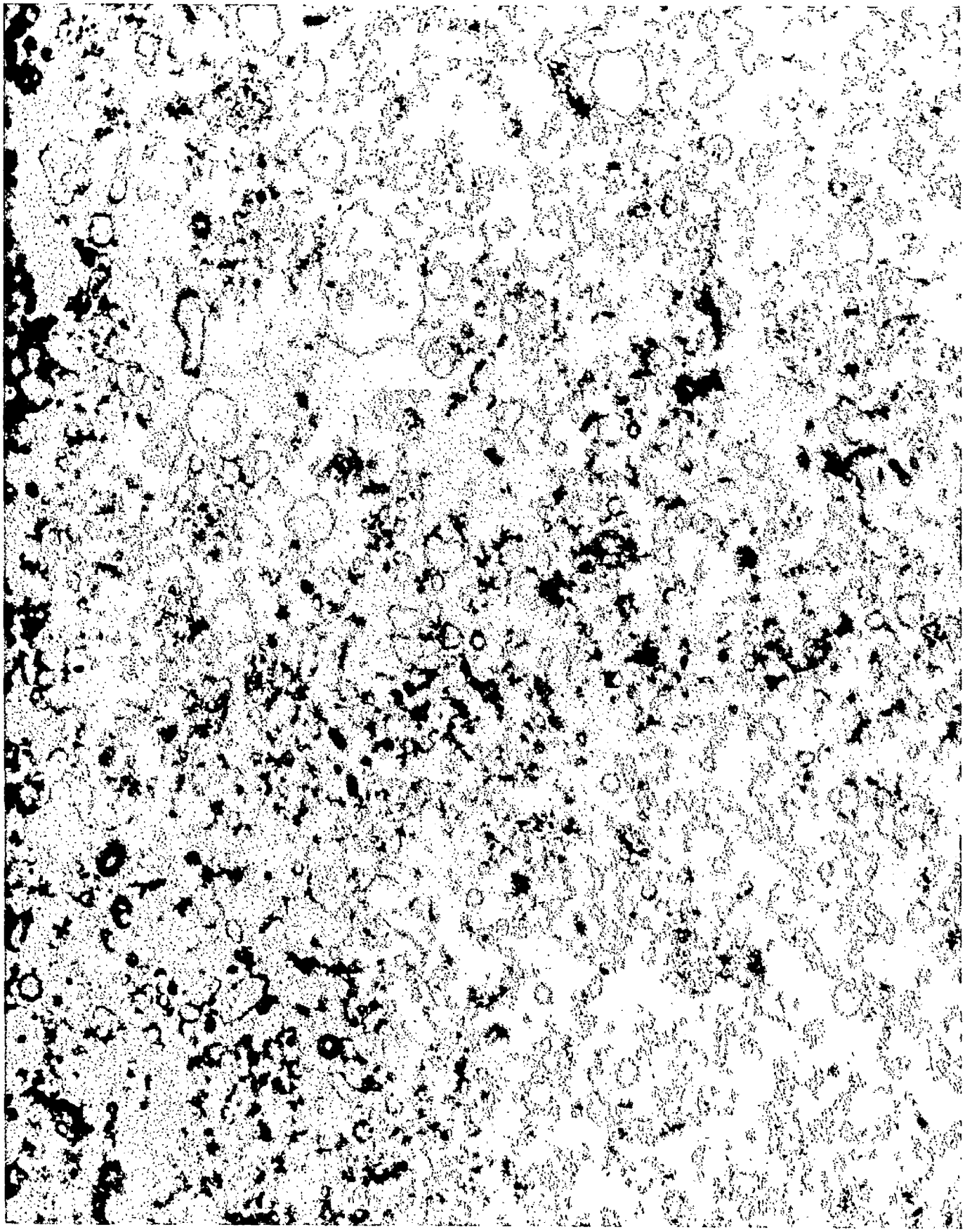


PLATE 4. BUTTON OF MICROSOMAL FRACTION x 22,000.

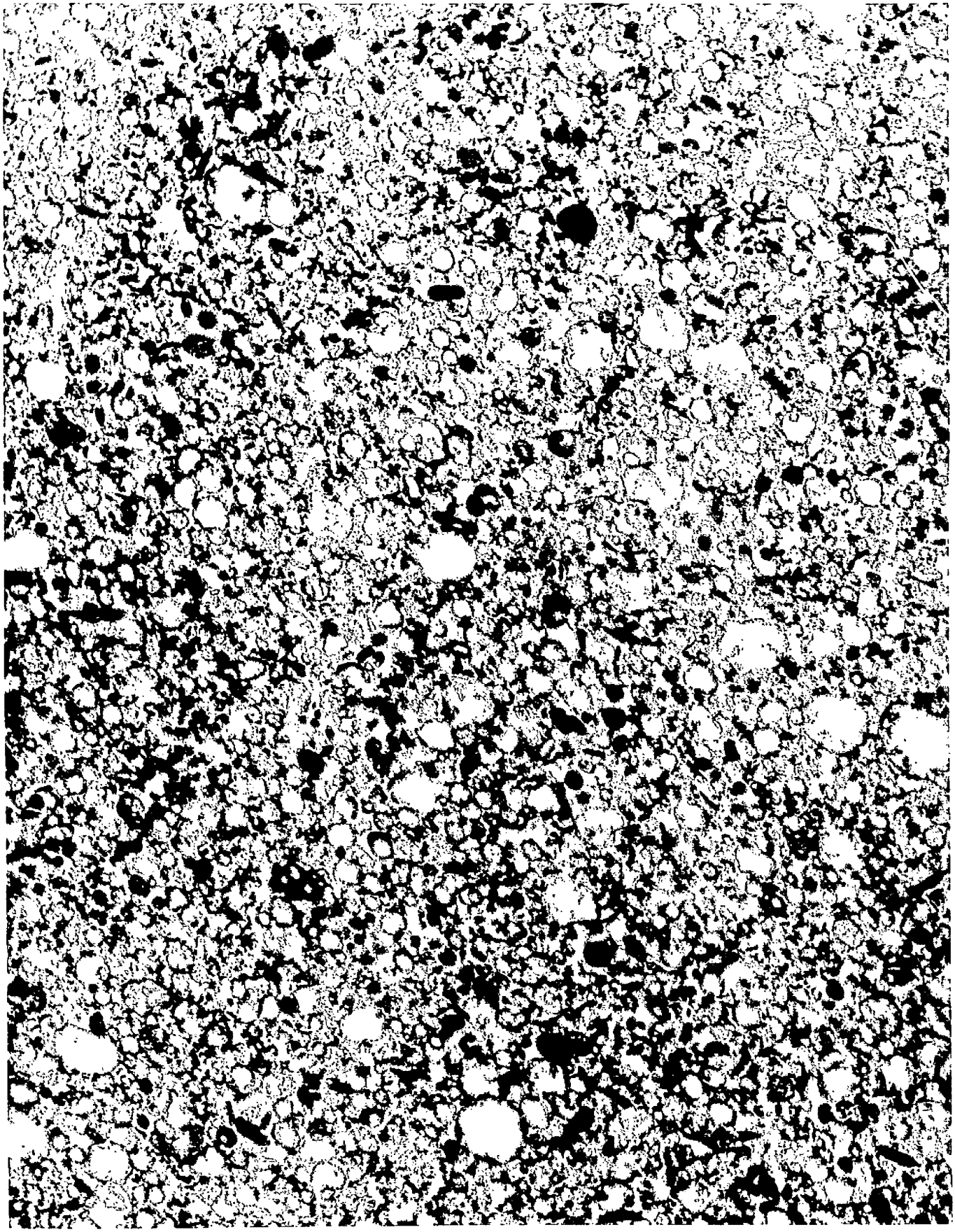


PLATE 5. TOP OF MICROSOMAL FRACTION x 22,000.

in the bottom portion of large mitochondria, many of which had lost their contents although the membrane was in most cases intact, and small mitochondria in a fairly good state of preservation. In all the fields examined, only two nuclei were seen. Strands of endoplasmic reticulum with microsomes attached were occasionally seen. The top portion which to the naked eye had a fluffy appearance was more heterogeneous. Free microsomes were commonly present, and were estimated to compose between one quarter and one-third of the field. The mitochondria were predominantly of the smaller variety, although large mitochondria were by no means uncommon. Approximately three or four particles having the morphological features of lysosomes were seen in each field.

The bottom of the third pellet was not unlike the top of the second, except that the ratio of microsomes to mitochondria was more than reversed and was now of the order of 5:1. The top portion of this pellet was densely packed with microsomes, but approximately 6-12 mitochondria and 4-8 lysosome-like structures were seen in each field.

Clearly, the separation of mitochondria from microsomes was far from complete. It did seem however that in this one preparation, the bulk of the mitochondria were in the second pellet, and the majority of the microsomes in the third pellet. The lysosomes were distributed between the two, and although it would be very difficult to obtain quantitative proof of this, it is likely that more were present in the third pellet

than in the second. Further evidence for overlap between the two fractions has been obtained by Ayre (1965) who studied the distribution of succinate dehydrogenase in several samples of these preparations.

THYROID

Series I

Alkaline Ribonuclease

The activity of alk. RNAase in the supernatant of normal thyroid showed a mean value of 0.041 units per mg. protein and 4.32 units per g. wet weight for the tissues of the First Series (Table 3). The corresponding means for the thyrotoxic glands in this series were very little different from these values, being 0.045 units per mg. protein and 4.43 units per g. wet weight respectively. Similar values were found for the supernatant of thyroid adenomata, which gave means of 0.046 units per mg. protein and 3.33 units per g. wet weight. (Table 4). By contrast, the carcinomas of thyroid gave a mean activity of 0.070 units per mg. protein, this value differing significantly from that of the normals at the 2% level; on the other hand, the activity per g. wet weight was a little lower than that of the normals, though not significantly so (Table 5). By far the greatest activity of alk. RNAase in the supernatant was found in the samples taken from patients with Hashimoto's thyroiditis, the value per mg. protein being 0.338 units and per g. wet weight 12.75 units; both these values are significantly different from normal at the 0.1% level, and far exceed those measured in other abnormalities of the gland.

The activity of alk. RNAase in the "Mitochondrial" fraction of normal thyroid tissue showed a mean value of 0.053 units per mg. protein and 0.22 units per g. wet weight (Table 6). The activity of this enzyme in the corresponding fraction of thyrotoxic tissue was considerably higher than normal, but the differences were not significant whether expressed per mg. protein or per g. wet weight. Thyroid adenomata showed slightly

TABLE 3.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	10	10	6
	Mean	0.041	4.32	87.9
	S.D.	0.015	1.97	7.1
	Range	0.022-.069	1.9-8.4	77.0-95.7
THYROTOXICS	No. of tissues	20	20	20
	Mean	0.045	4.43	87.0
	S.D.	0.016	1.31	8.3
	Range	0.027-.076	2.75-7.60	60.5-94.6
	t	0.68	0.18	

TABLE 3.

ALKALINE RIBONUCLEASE ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic In Series I

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
SUPERNATANT	Mean	0.046	3.33	84.8
	S.D.	0.026	2.08	12.9
	Range	0.012-0.119	0.80-8.10	50.5-96.4
"MITOCHONDRIAL" FRACTION	Mean	0.064	0.265	8.4
	S.D.	0.045	0.200	9.5
	Range	0.015-0.156	0.069-0.551	1.8-34.8
"MICROSOMAL" FRACTION	Mean	0.068	0.255	6.8
	S.D.	0.046	0.243	4.1
	Range	0.031-0.163	0.075-0.837	1.6-14.7

TABLE 4.

ALKALINE RIBONUCLEASE ACTIVITY

IN ADENOMATA OF

HUMAN THYROID GLAND

Results in Ten Tissues:

Three Cytoplasmic Fractions of Each

TABLE 5.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	No. of tissues	5	5	4
	Mean	0.070	3.94	82.2
	S.D.	0.026	1.22	5.3
	Range	0.050-.112	2.30-5.65	75.2-86.5
	t	2.78	0.39	1.37
	P	$< 0.02 > .01$		
HASHIMOTOS	No. of tissues	4	4	4
	Mean	0.338	12.75	82.4
	S.D.	0.146	4.11	3.7
	Range	0.172-.494	8.60-18.38	79.1-86.1
	t	6.82	5.37	1.41
	P	< 0.001	< 0.001	

TABLE 5.

ALKALINE RIBONUCLEASE ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Results in Cases of Carcinoma and Hashimoto's Thyroiditis with Statistical Evaluation against Normal Thyroid Supernatant

higher activity than normal for this enzyme in the "Mitochondrial" fraction (Table 4). The alk. RNAase activity of the "Mitochondrial" fraction of thyroid carcinomas was 50% higher than that of normal expressed per mg. protein, and twice the normal value expressed per g. wet weight; however only the latter difference is significant at the 5% level (Table 7). As with the supernatant, the "Mitochondrial" fraction of glands affected by Hashimoto's thyroiditis yielded activity far in excess of that shown by the corresponding fraction in the other glands examined, being about five times the activity of the normal gland whether expressed per mg. protein or per g. wet weight; these differences are once again significant at the 0.1% level.

Turning to the "Microsomal" fraction, it will be seen that the mean alk. RNAase activity of the normal gland does not differ greatly from that found in the first two cytoplasmic fractions, being 0.053 units per mg. protein and 0.334 units per g. wet weight (Table 8). As was the case with the "Mitochondrial" fraction, the "Microsomal" fraction of the thyrotoxic group shows activity for this enzyme per mg. protein almost double that of the corresponding fraction from normal tissue without this result achieving significance in the present series; the activity per g. wet weight shows a slightly lower value than that found in the normals. As with the other fractions, the activity obtained from thyroid adenomata is not greatly different from normal (Table 4). Curiously, the group of thyroid carcinomata show only slight increase above normal in this fraction when alk. RNAase activity is expressed per mg. protein; and though the activity per g. wet weight is about 50% above normal in this fraction, the difference is not significant (Table 9). The "Microsomal" fraction

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	6	6	6
	Mean	0.053	0.22	5.0
	S.D.	0.022	0.10	2.6
	Range	0.013-0.075	0.06-0.32	1.3-9.1
THYROTOXICS	No. of tissues	20	20	20
	Mean	0.095	0.34	6.8
	S.D.	0.055	0.25	5.0
	Range	0.042-0.176	0.08-1.11	2.0-23.4
	t	1.82	1.18	

TABLE 6.

ALKALINE RIBONUCLEASE ACTIVITY IN "MITOCHONDRIAL" FRACTION OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic

TABLE 7.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	Mean	0.079	0.46	8.8
	S.D.	0.031	0.21	3.7
	Range	0.038-0.114	0.29-0.76	7.0-14.3
	t	1.55	2.50	1.96
	P		0.05	
HASHIMOTOS	Mean	0.296	1.38	9.3
	S.D.	0.148	0.28	2.3
	Range	0.128-0.489	1.06-1.65	7.2-11.5
	t	13.0	9.47	1.98
	P	< 0.001	< 0.001	

TABLE 7.

ALKALINE RIBONUCLEASE ACTIVITY IN "MITOCHONDRIAL" FRACTION OF HUMAN THYROID GLAND

Results in Four Cases of Carcinoma and Four Cases of Hashimoto's Thyroiditis with Statistical Evaluation against Normal Thyroid "Mitochondrial" Fraction

TABLE 8.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	7	7	6
	Mean	0.053	0.334	7.1
	S.D.	0.021	0.341	5.6
	Range	0.027-0.091	0.062-1.039	2.5-14.4
THYROTOXICS	No. of tissues	20	20	20
	Mean	0.095	0.307	6.1
	S.D.	0.053	0.189	3.7
	Range	0.041-0.236	0.120-0.766	2.7-16.1
	t	1.99	0.26	

TABLE 8.

ALKALINE RIBONUCLEASE ACTIVITY IN "MICROSOMAL" FRACTION OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	Mean	0.060	0.473	9.0
	S.D.	0.026	0.157	2.9
	Range	0.027-0.086	0.262-0.617	6.4-12.3
	t		0.70	1.34
HASHIMOTOS	Mean	0.263	1.24	8.2
	S.D.	0.152	0.28	1.5
	Range	0.112-0.473	0.99-1.54	6.7-9.8
	t	3.75	4.52	1.00
	P	< 0.005	< 0.005	

TABLE 9.

ALKALINE RIBONUCLEASE ACTIVITY IN "MICROSOMAL" FRACTION OF HUMAN THYROID GLAND

Results in Four Cases of Carcinoma and Four Cases of Hashimoto's Thyroiditis with Statistical Evaluation against Normal Thyroid "Microsomal" fraction

of those glands affected by Hashimoto's thyroiditis once again showed very much greater activity than did the corresponding fraction of the other groups studied, the activity whether expressed per mg. protein or per g. wet weight being four to five times the activity of the normal gland; these differences are significant at the 0.5% level.

In the particulate fractions of thyrotoxic glands, the specific activity per mg. protein of alk. RNAase was double that of the supernatant. In the normal gland the specific activity in the particle fractions was a little above that in the supernatant. The adenomata showed an elevation in the particulate specific activity that was intermediate between those of normal and thyrotoxic glands. Of the carcinoma and Hashimoto thyroids, only the "Mitochondrial" fraction of the former displayed activity per mg. protein that was greater than was found in the corresponding supernatant.

In all types of thyroid tissue, the bulk of the alk. RNAase activity was located in the supernatant, the mean percentage of the total cytoplasmic activity found in this fraction ranging from 82.2% in the carcinomas to 87.9% in the normals. In all tissues, the remaining cytoplasmic activity was almost evenly distributed between "Mitochondrial" and "Microsomal" fractions although wide variations were found from one specimen to another. The normal gland tended to have less activity located in the particle fractions than did the other types of thyroid tissue, but none of the differences from normal proved to be significant in the present series, although the percentage of cytoplasmic enzyme in the "Mitochondrial" fractions of carcinomata and Hashimoto tissue was almost double that found in the corresponding fraction of normal thyroid and came nearest to reaching statistical significance.

Acid Ribonuclease

The distribution of this enzyme in the tissues examined showed features that differed from those displayed by alk. RNAase in certain respects. Thus, while the activity in the supernatant of normal thyroid was lower than that of the alk. RNAase, the mean values being 0.031 units per mg. protein and 3.51 units per g. wet weight (Table 10), the activity in the supernatant of thyrotoxic glands was a little higher than that of the alk. RNAase, the mean values being 0.046 units per mg. protein and 4.58 units per g. wet weight. Thus, in their content of acid RNAase, the thyrotoxic glands differ from the normal, the difference being significant at the 2.5% level when expressed per mg. protein, and at the 5% level when expressed per g. wet weight. The adenomata, in respect of the activity of acid RNAase in the supernatant, behaved in a similar fashion to the thyrotoxic glands, the mean value expressed per mg. protein being 50% higher than that found in the normal gland and approximating to the value for alk. RNAase in the supernatant fraction of thyroid adenomata (Table 11); however, because of the large variance of the data, this difference was not significant in the present series. Compared with the activity of alk. RNAase in the supernatant, that of acid RNAase is roughly halved in the carcinomata and Hashimoto glands (Table 12). While the activity per mg. protein in the carcinoma is still considerably above that found in the normal gland, the activity per g. wet weight is much lower than normal, though neither of these differences are statistically significant; and though the activity per mg. protein in the supernatant of Hashimoto tissue is 5 times that of the normal ($P<0.02$), the activity per g. wet weight is only 60% above normal and the difference is not significant

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	10	10	6
	Mean	0.031	3.51	91.3
	S.D.	0.017	1.69	3.2
	Range	0.009-.059	0.4-5.8	87.6-95.9
THYROTOXICS	No. of tissues	20	20	20
	Mean	0.046	4.58	90.4
	S.D.	0.015	1.08	6.1
	Range	0.028-.072	2.55-6.90	75.5-96.3
	t	2.40	2.09	
	P	< 0.025	< 0.05	

TABLE 10.

ACID RIBONUCLEASE ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic In Series I

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
SUPERNATANT	Mean	0.044	3.48	89.5
	S.D.	0.024	2.02	7.0
	Range	0.015-0.084	1.38-7.50	75.4-97.1
"MITOCHONDRIAL" FRACTION	Mean	0.044	0.221	5.6
	S.D.	0.032	0.193	4.1
	Range	0.003-0.111	0.007-0.525	1.1-13.8
"MICROSOMAL" FRACTION	Mean	0.046	0.187	4.7
	S.D.	0.028	0.165	3.3
	Range	0.010-0.106	0.033-0.458	1.4-10.9

TABLE 11.

ACID RIBONUCLEASE ACTIVITY

IN ADENOMATA OF

HUMAN THYROID GLAND

Results in Ten Tissues:

Three Cytoplasmic Fractions of Each

TABLE 12.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	No. of tissues	5	5	4
	Mean	0.047	2.62	83.7
	S.D.	0.025	1.13	6.9
	Range	0.026-.073	1.10-3.71	77.4-91.6
	t	1.53	1.05	2.31
	P			< 0.05
HASHIMOTOS	No. of tissues	4	4	4
	Mean	0.150	5.80	74.5
	S.D.	0.141	5.16	8.1
	Range	0.061-362	2.40-13.45	66.4-83.8
	t	2.81	1.32	4.69
	P	< 0.02-> .01		< 0.005-> .001

TABLE 12.

ACID RIBONUCLEASE ACTIVITY IN

SUPERNATANT OF HUMAN

THYROID GLAND

Results in Cases of Carcinoma and Hashimoto's Thyroiditis

with Statistical Evaluation against

Normal Thyroid Supernatant

because of the variance of the sample.

Comparison between acid RNAase in the "Mitochondrial" fraction of normal and thyrotoxic glands shows that the activity of the latter exceeds that of the former whatever method of expression is used, but due to the variance being large, these differences do not reach statistical significance (Table 13). In contrast to the supernatant, the acid RNAase activity of the "Mitochondrial" fraction of thyrotoxic glands was only two-thirds of that shown by the alk. RNAase in this fraction. The adenomata (Table 11) and carcinomata (Table 14) of thyroid display the same activity per mg. protein in the "Mitochondrial" fraction as does this fraction of the normal gland; the increase in weight of this fraction per g. of tissue in these conditions raises the activity per g. wet weight above that of the normal but not significantly so. On the other hand, the "Mitochondrial" fraction of Hashimoto tissue has much greater acid RNAase activity than the normal whether measured per mg. protein ($P < 0.05$) or per g. wet weight ($P < 0.005$).

The acid RNAase activity of the "Microsomal" fraction of the normal thyroid per mg. protein is less than that of the normal "Mitochondrial" fraction while the acid RNAase activity of the "Microsomal" fraction of the thyrotoxic gland is greater than that of the "Mitochondrial" fraction of thyrotoxic tissue (Table 15). As a result, the difference between normal and toxic is significant at the 5% level. The activity of this fraction per g. wet weight compared with normal is not elevated to the same degree, and the difference is not significant. Again, the position of adenomata with respect to the acid RNAase activity of the "Microsomal" fraction is intermediate between that of the normal and toxic gland, the

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	6	6	6
	Mean	0.043	0.18	4.5
	S.D.	0.018	0.08	3.4
	Range	0.010-0.062	0.05-0.27	1.3-9.1
THYROTOXICS	No. of tissues	20	20	20
	Mean	0.066	0.24	4.7
	S.D.	0.038	0.16	3.0
	Range	0.017-0.148	0.06-0.57	1.3-10.9
	t	1.42	0.86	

TABLE 13.

ACID RIBONUCLEASE ACTIVITY IN

"MITOCHONDRIAL" FRACTION

OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	Mean	0.043	0.27	7.3
	S.D.	0.021	0.21	4.0
	Range	0.023-0.061	0.15-0.58	3.6-12.9
	t	[illegible]	1.05	1.37
HASHIMOTOS	Mean	0.185	0.82	13.1
	S.D.	0.145	0.35	4.3
	Range	0.076-0.398	0.59-1.34	8.3-16.8
	t	2.45	4.31	3.60
	P	< 0.05	< 0.005	< 0.01

TABLE 14.

ACID RIBONUCLEASE ACTIVITY IN
"MITOCHONDRIAL" FRACTION
OF HUMAN THYROID GLAND

Results in Four Cases of Carcinoma and
Four Cases of Hashimoto's Thyroiditis
with Statistical Evaluation against
Normal Thyroid "Mitochondrial" Fraction

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	7	7	6
	Mean	0.037	0.185	4.2
	S.D.	0.025	0.134	1.9
	Range	0.011-0.088	0.044-0.440	2.8-7.7
THYROTOXICS	No. of tissues	20	20	20
	Mean	0.072	0.249	4.8
	S.D.	0.039	0.169	3.1
	Range	0.010-0.162	0.051-0.425	1.0-11.6
	t	2.15	0.90	
	P	$< 0.05 > 0.025$		

TABLE 15.

ACID RIBONUCLEASE ACTIVITY IN "MICROSOMAL" FRACTION OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic

differences from the normal levels of activity not being significant (Table 11). The activity of acid RNAase in the "Microsomal" fraction of thyroid carcinomata (Table 16) is much the same as that of the normal when measured per mg. protein, and though it is almost double that of normal when expressed per g. wet weight, this difference is not significant. The "Microsomal" fraction of Hashimoto tissue has virtually the same activity as the "Mitochondrial" fraction of this tissue, so that once again its activity is greater than the normal at a level of significance of 5% when measured per mg. protein and 0.5% when measured per g. wet weight; in this connection, it should be observed (Tables 14 and 16) that while in both particulate fractions of Hashimoto tissue the activity on both scales is more than four times that of the corresponding fractions of the normal gland, the variance obtaining when the activity is expressed per mg. protein reduces the level of significance much below that calculated when the results are expressed per g. wet weight, when the variance becomes smaller.

Examination of the distribution of acid RNAase activity within the three cytoplasmic fractions studied showed some interesting features. Relative to the distribution of alk. RNAase, a higher percentage of the cytoplasmic activity of this enzyme is located in the supernatant of normal, thyrotoxic and adenomatous thyroid tissue; the distribution of the two activities is similar in thyroid carcinomata; but in the Hashimoto tissue, the percentage located in the supernatant is much less than that of the alk. RNAase. This in turn means that more of the cytoplasmic acid RNAase activity is located in the particulate fractions of Hashimoto tissue and less in these fractions of the normal, thyrotoxic, and

adenomatous glands. Certain of these differences from the normal in the distribution of the enzyme are statistically significant: the percentage activity of the Hashimoto supernatant being lower than normal ($P < 0.005$), that of the "Mitochondrial" fraction of the same tissue being higher than normal (P 0.01), as is the "Microsomal" fraction ($P < 0.005$); and the percentage activity of acid RNAase in the "Microsomal" fraction of thyroid carcinoma is also higher than normal ($P < 0.05$). No remarkable differences in percentage of cytoplasmic activity of acid RNAase were found between the two particle fractions in any of the groups of thyroid tissue examined.

Deoxyribonuclease I

The activity of RNAase I in the supernatant of normal thyroid gave a mean value of 0.66 units per mg. protein and 64.5 units per g. wet weight (Table 17). The corresponding values for the thyrotoxic tissue are only slightly higher, and although the mean values for the adenomata are about 30% higher than normal (Table 18), the differences are not significant. The mean activity per mg. protein in supernatant of thyroid carcinomata is three times that of the normal ($P < 0.02$) and the activity per g. wet weight almost twice that of the normal, but the latter difference is not statistically significant because of the large variance of the data. The activity of the Hashimoto supernatant exceeded that of the normal five-fold when measured per mg. protein ($P < 0.001$) and two-fold when measured per g. wet weight, but as was the case with the carcinomata, the latter increase is just outside the 5% probability level (Table 19).

The results obtained in the particle fractions of normal and toxic thyroid and thyroid adenomata show many interesting features, not least

TABLE 17.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	10	10	5
	Mean	0.66	64.5	62.3
	S.D.	0.47	33.9	11.1
	Range	0.16-1.56	10.0-130.2	46.7-77.9
THYROTOXICS	No. of tissues	20	20	20
	Mean	0.76	76.1	60.4
	S.D.	0.38	45.6	13.5
	Range	0.12-1.37	15.0-167.1	34.6-82.7
	t	0.62	0.71	

TABLE 17.

DEOXYRIBONUCLEASE I ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic in Series I

TABLE 19.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	No. of tissues	5	5	4
	Mean	1.81	109	49.8
	S.D.	1.04	83	9.8
	Range	0.98-3.59	50-255	37.5-61.3
	t	2.99	1.48	1.76
	P	$< 0.02 > .01$		
HASHIMOTOS	No. of tissues	4	4	4
	Mean	2.98	125	61.2
	S.D.	1.55	82	12.4
	Range	1.48-4.00	50-230	46.0-76.4
	t	4.48	2.04	
	P	< 0.001	$< 0.1 > .05$	

TABLE 19.

DEOXYRIBONUCLEASE I ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Results in Cases of Carcinoma and Hashimoto's Thyroiditis with Statistical Evaluation against Normal Thyroid Supernatant

TABLE 18.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
SUPERNATANT	Mean	0.98	84	57.4
	S.D.	0.85	96	23.0
	Range	0.25-3.15	20-350	13.2-81.7
"MITOCHONDRIAL" FRACTION	Mean	5.95	30.8	20.6
	S.D.	1.95	28.9	11.0
	Range	2.27-8.51	4.7-102.4	12.4-44.6
"MICROSOMAL" FRACTION	Mean	7.46	30.5	22.0
	S.D.	3.58	29.9	17.4
	Range	4.63-14.12	4.5-99.8	5.8-66.3

TABLE 18.

DEOXYRIBONUCLEASE I ACTIVITY

IN ADENOMATA OF

HUMAN THYROID GLAND

Results in Ten Tissues:

Three Cytoplasmic Fractions of Each

being the fact that the activity per mg. protein in these fractions is almost ten times that found in the respective supernatants, in contrast to the situation applying with alk. and acid RNAases (Tables 20 , 22 and 18). Another feature to be noted is that the specific activity of the "Microsomal" fraction exceeds that of the "Mitochondrial" fraction in all groups, although the differences are not large. As in the supernatant fraction, so in both particle fractions the specific activity of the thyrotoxic tissue is greater than that of the normal, while that of the thyroid adenomata is greater than both the thyrotoxic and the normal. However, the only difference to achieve statistical significance is that between the "Mitochondrial" fractions of normal and thyrotoxic glands; in this connection, it must be borne in mind that the relatively large number of samples in the thyrotoxic group will tend to exaggerate the statistical importance of deviations from normal displayed by this group although larger deviations are present in other types of tissue which are not so well represented and therefore fail to make a full impact when expressed in statistical terms. Little need be said concerning the activities per g. wet weight of the particle fractions in these tissues, since no significant differences between the normal gland and the other two types occur, except to point out that once again the particle fractions of the adenomata are more active than those of the normal and thyrotoxic glands.

Turning to the particle fractions of thyroid carcinomata and glands affected by Hashimoto's thyroiditis (Tables 21 and 23) it will be seen that, while the increase in specific activity over that found in the supernatants of the respective tissues is only two- to four-fold, the yield of particles per g. of tissue, particularly so with the carcinomata,

TABLE 20.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	5	5	5
	Mean	4.14	16.0	14.8
	S.D.	2.46	11.9	9.8
	Range	1.53-7.74	6.1-34.7	6.4-30.6
THYROTOXICS	No. of tissues	20	20	20
	Mean	5.83	22.0	19.0
	S.D.	1.25	13.0	7.7
	Range	4.38-8.94	9.8-52.8	8.4-35.8
	t	2.12 *	0.94	1.04
	P	(0.05->0.025		

TABLE 20.

DEOXYRIBONUCLEASE I ACTIVITY IN

"MITOCHONDRIAL" FRACTION

OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic

TABLE 21.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	Mean	6.81	43.4	18.5
	S.D.	1.53	21.4	4.2
	Range	5.09-8.43	22.7-64.8	15.6-24.3
	t	1.89	2.46	0.70
	P		< 0.05	
HASHIMOTOS	Mean	4.73	29.1	16.9
	S.D.	1.71	23.8	5.8
	Range	2.44-6.49	9.0-63.3	9.4-23.7
	t	0.41	1.08	1.69

TABLE 21.

DEOXYRIBONUCLEASE I ACTIVITY IN

"MITOCHONDRIAL" FRACTION

OF HUMAN THYROID GLAND

Results in Four Cases of Carcinoma and
Four Cases of Hashimoto's Thyroiditis
with Statistical Evaluation against
Normal Thyroid "Mitochondrial" Fraction

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	6	6	5
	Mean	5.90	23.4	22.9
	S.D.	3.36	12.7	12.4
	Range	2.08-9.90	6.4-38.8	9.6-30.7
THYROTOXICS	No. of tissues	20	20	20
	Mean	6.45	21.2	19.9
	S.D.	1.34	8.65	7.3
	Range	3.24-9.08	11.1-37.8	6.4-33.1
	t	0.61	0.49	0.82

TABLE 22.

DEOXYRIBONUCLEASE I ACTIVITY IN

"MICROSOMAL" FRACTION

OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	Mean	8.44	71.5	31.8
	S.D.	1.20	26.6	6.7
	Range	7.31-9.94	43.7-96.2	23.1-38.2
	t	1.43	3.90	1.28
	P		< 0.005	
HASHIMOTOS	Mean	7.90	45.3	24.3
	S.D.	1.67	27.5	7.6
	Range	5.88-9.80	27.8-86.3	14.2-30.3
	t	1.09	1.73	0.21

TABLE 23.

DEOXYRIBONUCLEASE I ACTIVITY IN "MICROSOMAL" FRACTION OF HUMAN THYROID GLAND

Results in Four Cases of Carcinoma and Four Cases of Hashimoto's Thyroiditis with Statistical Evaluation against Normal Thyroid "Microsomal" Fraction

is relatively very much greater than that obtained in the first three groups. Consequently, none of the particle fractions in these two tissues has specific activity of DNAase I per mg. protein significantly higher than that of its counterpart in normal thyroid in this series. On the other hand, the activity of the "Mitochondrial" and "Microsomal" DNAase I activity of the carcinoma group per g. wet weight is significantly higher than that of the corresponding fraction of the normal gland when expressed in this fashion, the values of P being less than 0.05 and 0.005 respectively. It is also worthy of mention that in both Hashimoto and carcinoma groups, the activity found in the "Microsomal" fraction, whether expressed per mg. protein or per g. wet weight, exceeded that of the "Mitochondrial" fraction.

It follows from what has been described that the percentage of the cytoplasmic activity of DNAase I present in the supernatant fraction ought to be less than the percentage of cytoplasmic alk. and acid RNAase activities located in this fraction. Such indeed is the case, the mean value being in the region of 60% for all groups, except the carcinomata where the value is as low as 49.6%. By the same token, a greatly increased share of the cytoplasmic activity of this enzyme is distributed between the particle fractions, the "Microsomal" fractions containing a rather higher percentage than the "Mitochondrial" fraction; this is especially the case with the carcinoma and thyroiditis groups.

Deoxyribonuclease II

In all types of thyroid tissue, the activity of this enzyme in the supernatant is 15-30 times greater than that of DNAase I. The mean activity per mg. protein of the normal thyroid supernatant is 17.5 units

TABLE 24.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	10	10	5
	Mean	17.5	1758	93.6
	S.D.	8.8	703	3.4
	Range	6.99-36.3	1250-3630	88.2-96.4
THYROTOXICS	No. of tissues	20	20	20
	Mean	26.3	2366	88.3
	S.D.	10.7	674	6.9
	Range	8.7-48.9	1165-4299	72.9-99.3
	t	2.23	2.28	1.64
	P	< 0.05	< 0.05	

TABLE 24.

DEOXYRIBONUCLEASE II ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic in Series I

TABLE 25.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
SUPERNATANT	Mean	25.6	1970	88.3
	S.D.	14.2	911	10.4
	Range	9.8-52.9	655-3600	62.2-97.2
"MITOCHONDRIAL" FRACTION	Mean	21.7	111	4.9
	S.D.	14.6	103	4.2
	Range	6.7-60.3	8-338	1.2-13.9
"MICROSOMAL" FRACTION	Mean	36.2	155	6.8
	S.D.	23.1	143	6.6
	Range	13.2-92.0	11-418	1.6-23.9

TABLE 25.

DEOXYRIBONUCLEASE II ACTIVITY

IN ADENOMATA OF

HUMAN THYROID GLAND

Results in Ten Tissues:

Three Cytoplasmic Fractions of Each

TABLE 26.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	No. of tissues	5	5	4
	Mean	32.8	1885	83.3
	S.D.	5.0	458	5.0
	Range	29.8-38.8	1505-2840	78.0-90.1
	t	3.53	0.36	3.68
	P	$<0.005->.001$		$<0.01->.005$
HASHIMOTOS	No. of tissues	4	4	4
	Mean	46.7	1886	85.5
	S.D.	10.9	740	6.3
	Range	32.9-59.4	1320-2970	77.7-92.8
	t	5.28	0.31	2.57
	P	<0.001		$<0.05->.025$

TABLE 26.

DEOXYRIBONUCLEASE II ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Results in Cases of Carcinoma and Hashimoto's Thyroiditis with Statistical Evaluation against Normal Thyroid Supernatant

and that of the thyrotoxic supernatant 26.3 units, the difference being statistically significant ($P < 0.05$), as is the case when these activities are related to g. wet weight of the tissue when the mean values are 1758 and 2366 units respectively (Table 24). It will be seen (Table 25) that the supernatant of thyroid adenomata is less active than that of the thyrotoxic gland but more active than that of the normal, with mean activities of 25.6 units per mg. protein and 1970 units per g. wet weight, although these differences from normality are not significant. The mean activity of the supernatant fraction of the carcinoma group is almost twice that of the normals per mg. protein ($P < 0.005$) while that of the Hashimoto group (Table 26) is almost three times that of the normal per mg. protein ($P < 0.001$). On the other hand, when the supernatant activity of these tissues is expressed per g. wet weight, they differ little from that of the normal gland, a situation for which the low percentage of the wet weight of these tissues accounted for by the supernatant is responsible. In essence, the differences between these five types of thyroid tissue are qualitatively similar to those reflected in the activity of supernatant DNAase I; thus, in increasing order of activity, the groups can be arranged in the order normal, toxic, carcinoma, and thyroiditis in respect of both enzymes; but quantitatively these differences have been magnified by virtue of the great increase in activity of DNAase II over that of DNAase I in the supernatant so that statistical treatment yields more convincing conclusions.

The activity of DNAase II in the "Mitochondrial" fractions of all five groups of thyroid tissue per mg. protein is considerably lower than that found in the supernatant fraction, with the exception of the thyrotoxic

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	5	5	5
	Mean	14.3	53	3.1
	S.D.	8.0	31	1.8
	Range	7.9-27.9	20-103	1.4-5.7
THYROTOXICS	No. of tissues	20	20	20
	Mean	39.2	146	5.0
	S.D.	26.2	120	3.8
	Range	12.2-96.5	36-508	0.3-12.9
	t	2.08	1.70	1.23
	P	$< 0.05 > 0.025$		

TABLE 27.

DEOXYRIBONUCLEASE II ACTIVITY IN "MITOCHONDRIAL" FRACTION OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic

TABLE 28.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	Mean	25.1	156	7.3
	S.D.	2.3	57.8	3.4
	Range	22.2-27.1	81-213	2.6-10.7
	t	2.55	10.96	2.41
	P	< 0.05	< 0.001	< 0.05
HASHIMOTOS	Mean	26.4	172	6.5
	S.D.	12.2	173	3.3
	Range	17.3-44.2	66-431	3.6-11.3
	t	1.80	1.53	2.81
	P			< 0.05

TABLE 28.

DEOXYRIBONUCLEASE II ACTIVITY IN

"MITOCHONDRIAL" FRACTION

OF HUMAN THYROID GLAND

Results in Four Cases of Carcinoma and
Four Cases of Hashimoto's Thyroiditis
with Statistical Evaluation against
Normal Thyroid "Mitochondrial" Fraction

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	6	6	5
	Mean	17.1	60	3.3
	S.D.	15.1	32	1.7
	Range	2.0-27.9	23-111	1.6-6.1
THYROTOXICS	No. of tissues	20	20	20
	Mean	55.5	193	6.6
	S.D.	30.3	139	3.6
	Range	27.0-128.8	56-518	0.4-14.4
	t	2.98	2.31	2.12
	P	< 0.01->0.005	< 0.05->0.025	< 0.05->0.025

TABLE 29.

DEOXYRIBONUCLEASE II ACTIVITY IN "MICROSOMAL" FRACTION OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
CARCINOMAS	Mean	25.9	211	9.3
	S.D.	4.9	27.0	1.6
	Range	21.0-31.8	171-228	7.3-11.3
	t	1.11	7.80	5.26
	P		< 0.001	< 0.001
HASHIMOTOS	Mean	31.0	261	8.0
	S.D.	11.6	289	3.3
	Range	20.2-47.4	66-691	3.6-11.0
	t	1.55	1.74	2.80
	P			< 0.025

TABLE 30.

DEOXYRIBONUCLEASE II ACTIVITY IN "MICROSOMAL" FRACTION OF HUMAN THYROID GLAND

Results in Four Cases of Carcinoma and Four Cases of Hashimoto's Thyroiditis with Statistical Evaluation against Normal Thyroid "Microsomal" Fraction

group where it is one and a half times as great as that of the supernatant. Consequently the mean activity of the thyrotoxic group, 39.2 units per mg. protein, is nearly three times that of the normal group, 14.3 units (Table 27), and this difference is statistically significant ($P < 0.05$). Indeed the activity of thyrotoxic tissue per mg. protein is much higher than that of the other groups, and while the activities of the adenomata (Table 25), of the carcinomata (Table 28) and of the thyroiditis groups are higher than that of the normal "Mitochondrial" fraction, the large variance and the small numbers involved prevent these differences attaining statistical significance in all but the carcinoma group. Much the same considerations apply when the activity of the "Mitochondrial" fraction is expressed per g. wet weight; for the mean values range from 53 units in the normal group to 172 in the thyroiditis group; yet the S.D. of the latter group is as high as its mean so that significance cannot be demonstrated statistically. Intermediate between these two extremes come the adenomata with 111 units per g. wet weight (Table 25), the thyrotoxic group with 146 units per g. wet weight (Table 27), and the carcinomata with 156 units per g. wet weight.

In all five groups of thyroid tissue, the activity of the "Microsomal" fraction is higher than that of the "Mitochondrial" fraction. The mean value for the normal group is 17.1 units per mg. protein (Table 29), while that of the thyrotoxic group is greatly increased to a mean of 55.5 units per mg. protein ($P < 0.01$). The mean value for "Microsomal" DNAase II per mg. protein in the adenomata is 36.2 units (Table 25), a value which is higher than those of the other two cytoplasmic fractions of this tissue, but the difference from that of the normal group is not statistically

significant in the present series. The specific activities of the "Microsomal" fraction of the carcinoma and thyroiditis series (Table 30) are intermediate between those of the other two fractions of these tissues, and though the values of 25.9 units and 31.0 units respectively are well above that of the corresponding fraction of the normal gland, the differences are not significant. When the activity of this fraction is expressed per g. wet weight of tissue, the mean value for the normal gland is 60 units (Table 29). This value is greatly exceeded by that of the thyrotoxic series, 193 units ($P < 0.05$), and by the carcinoma series, 211 ($P < 0.001$); but the mean values for the adenomata and thyroiditis groups, while they are much higher than that of the normal at 155 and 261 units per g. wet weight respectively, are associated with a marked degree of variance within the groups so that statistical significance is not obtained.

The distribution of DNAase II within the various fractions of the cytoplasm shows a relatively higher percentage of the activity located in the supernatant of the normal gland in comparison with the other types of tissue. In turn, these have a higher percentage of the activity located in the particulate fractions. Thus in the normal, 93.6% of the total cytoplasmic activity measured is found in the supernatant, a higher percentage than that of any other nuclease studied (Table 24). In the toxic and adenoma groups, the mean percentage is lower, the value being 88.3% in both (Tables 24 and 25). In the carcinoma and thyroiditis series, it is very much lower still, the mean percentage of the cytoplasmic activity of DNAase II found in the supernatant falling to 83.3% ($P < 0.01$) and 85.5% ($P < 0.05$) respectively (Table 26). Conversely, in comparison with the low particulate share of the cytoplasmic activity of this enzyme in the normal tissue of around 3% in both fractions, the thyrotoxic tissue

had 5% of the cytoplasmic activity in the "Mitochondrial" fraction and 6.6% in the "Microsomal" fraction ($P < 0.05$) as recorded in Tables 27 and 29 . The corresponding values for the adenomata were 4.9% and 6.8%, although both these means are associated with very large variance (Table 25). The mean values found in the carcinoma group are higher still, that for the percentage of cytoplasmic DNAase II in the "Mitochondrial" fraction being 7.3% ($P < 0.05$) while the corresponding figure for the "Microsomal" fraction is 9.3% ($P < 0.001$) as recorded in Tables 28 and 30 ; in the same Tables, it may be observed that the distribution of the enzyme in the particulate fractions of the thyroiditis series is less than in the carcinoma group but significantly higher than the normal group, the mean for the "Mitochondrial" fraction being 6.5% ($P < 0.05$) and the mean for the "Microsomal" fraction being 8.0% ($P < 0.025$).

Adenosine Deaminase

The activity of this enzyme in normal thyroid supernatant showed a mean value of 14.2 units per mg. protein (Table 31). The activity of the supernatant of the thyrotoxic glands showed a mean value of 23.0 units per mg. protein, which is significantly higher than that of the normal group ($P < 0.05$). The mean activity of the adenomata is intermediate between these two values (Table 32). The mean activity of the thyroiditis group per mg. protein is more than twelve times greater than that of the normals ($P < 0.001$) as can be seen from the data presented in Table 33 . When the results are expressed per g. wet weight, the relative positions of these four groups of thyroid tissue are unaltered; thus the

		Activity per mg. protein	Activity per g. wet weight
NORMALS	Mean	14.2	1292
	S.D.	9.6	748
	Range	3.5-30.3	540-2410
THYROTOXICS	Mean	23.0	1976
	S.D.	11.0	745
	Range	5.8-48.7	870-3165
	t	2.13	2.35
	P	< 0.05	< 0.05

TABLE 31.

ADENOSINE DEAMINASE ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic in Series I

Mean of 10 Normal and 20Thyrotoxic Tissues

	Activity per mg. protein	Activity per g. wet weight
Mean	20.8	1524
S.D.	13.2	728
Range	6.2-43.0	413-2700

TABLE 32.

ADENOSINE DEAMINASE ACTIVITY

IN ADENOMATA OF

HUMAN THYROID GLAND

Results in Supernatant of Ten Tissues

TABLE 33.

		Activity per mg. protein	Activity per g. wet weight
CARCINOMAS	Mean	86.6	5580
	S.D.	109.1	6073
	Range	28.1-281.3	1960-19951
	t	2.16	1.74
	P	< 0.05	
HASHIMOTO'S	Mean	167.0	7533
	S.D.	27.2	1931
	Range	149.3-205.9	5640-10200
	t	10.5	6.27
	P	< 0.001	< 0.001

TABLE 33.

ADENOSINE DEAMINASE ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Results in Cases of Carcinoma (5 cases) and Hashimoto's Thyroiditis (4cases) with Statistical Evaluation against Normal Thyroid Supernatant

thyrotoxic glands have significantly more activity than the normals ($P < 0.05$) with the adenomata intermediate between the two, and the thyroiditis group contain far more activity than the normals ($P < 0.001$).

Special consideration must be given to the carcinomata. From Table 33 it will be seen that the mean activity per mg. protein is five times that of the normal supernatant ($P < 0.05$), while the mean activity per g. wet weight is also five times that of the normal although the variance is such as to prevent this difference being statistically significant. It must be pointed out that these mean values are distorted by the inclusion of the anaplastic thyroid carcinoma, the activity of which is seven times the mean of the other four carcinomata. When this sample is removed from the group, the means become 37.5 units per mg. protein and 1987 units per g. wet weight. The reduction in variance brought about by this step raises the values of 't' for the statistical comparison with the normal thyroid to 4.12 and 1.88 respectively.

Protein

Although the protein determinations in the various cytoplasmic fractions of these tissues were carried out primarily for the provision of a parameter against which changes in enzyme activity could be assessed, analysis of the data is necessary for an understanding of other aspects of this work, especially for a true appreciation of the meaningfulness attaching to the measurement of enzyme activity per g. wet weight and as a percentage of the total cytoplasmic content.

In the normal thyroid gland (Table 34), it appears that the cytoplasm contains about 90% of the soluble protein of the cytoplasm, the two

TABLE 34.

		Protein mg. per g. wet weight	Protein % Total Cytoplasm
SUPERNATANT	Number	10	6
	Mean	121.6	91.1
	S.D.	43.7	3.87
	Range	52.4-166.0	84.4-96.1
"MITOCHONDRIAL" FRACTION	Number	6	6
	Mean	4.3	3.9
	S.D.	1.5	1.5
	Range	2.6-7.1	2.0-6.0
"MICROSOMAL" FRACTION	Number	7	6
	Mean	5.8	4.9
	S.D.	4.1	2.8
	Range	1.6-11.6	1.8-9.7

TABLE 34.

PROTEIN CONTENT OF THE CYTOPLASMIC FRACTIONS

OF NORMAL HUMAN THYROID

		Protein mg. per g. wet weight	Protein % Total Cytoplasm
SUPERNATANT	Mean	106.1	93.2
	S.D.	23.6	3.3
	Range	65.0-153.0	84.3-96.7
	t	1.28	1.58
"MITOCHONDRIAL" FRACTION	Mean	3.6	3.4
	S.D.	1.6	1.8
	Range	1.9-7.5	1.6-9.1
	t	0.99	0.71
"MICROSOMAL" FRACTION	Mean	3.3	3.2
	S.D.	1.4	1.7
	Range	1.7-7.1	1.3-6.9
	t	2.46	1.93
	P	< 0.025	-

TABLE 35.

PROTEIN CONTENT OF THE CYTOPLASMIC FRACTION OF THYROTOXIC HUMAN THYROID GLANDS:

Statistical Comparison with Corresponding Fraction of Results of Twenty Samples

		Protein mg. per g. wet weight	Protein % Total Cytoplasm
SUPERNATANT	Mean	84.6	89.0
	S.D.	35.2	10.5
	Range	41.9-158.0	62.5-97.5
"MITOCHONDRIAL" FRACTION	Mean	5.2	6.4
	S.D.	4.9	7.3
	Range	1.2-17.6	1.1-26.2
"MICROSOMAL" FRACTION	Mean	3.8	4.6
	S.D.	2.2	3.2
	Range	0.8-7.6	1.3-11.3

TABLE 36.

PROTEIN CONTENT OF THE CYTOPLASMIC FRACTION

OF HUMAN THYROID ADENOMATA

Results of Ten Samples

(No significant Differences from Normal found)

		Protein mg. per g. wet weight	TABLE 37. Protein % Total Cytoplasm
SUPERNATANT	Number	5	4
	Mean	57.9	80.2
	S.D.	14.9	7.1
	Range	42.0-76.3	70.9-88.1
	t	3.12	3.15
	P	< 0.01	< 0.01
"MITOCHONDRIAL" FRACTION	Number	4	4
	Mean	6.4	8.6
	S.D.	2.9	4.2
	Range	3.0-9.6	3.5-13.7
	t	1.53	2.57
	P	-	< 0.05
"MICROSOMAL" FRACTION	Number	4	4
	Mean	8.4	11.1
	S.D.	2.2	3.1
	Range	6.0-10.8	8.4-15.4
	t	1.15	3.27
	P	-	< 0.02

TABLE 37.

PROTEIN CONTENT OF CYTOPLASMIC FRACTIONS OF HUMAN THYROID CARCINOMATA:

Statistical Comparison with Corresponding Fraction of Normal Human Thyroid

TABLE 38.

		Protein mg. per g. wet weight	Protein % Total Cytoplasm
SUPERNATANT	Mean	40.4	83.2
	S.D.	10.5	7.3
	Range	27.0-50.0	72.8-90.0
	t	3.55	2.24
	P	< 0.005	< 0.10
"MITOCHONDRIAL" FRACTION	Mean	4.6	8.5
	S.D.	3.6	3.9
	Range	1.6-9.8	5.3-14.2
	t	0.18	2.61
	P	-	< 0.05
"MICROSOMAL" FRACTION	Mean	4.6	8.4
	S.D.	3.2	3.5
	Range	1.4-8.9	4.7-13.0
	t	0.51	3.16
	P	-	< 0.02

TABLE 38.

PROTEIN CONTENT OF THE CYTOPLASMIC FRACTIONS OF THE HUMAN THYROID GLAND IN HASHIMOTO'S THYROIDITIS

Statistical Comparison with Corresponding Fraction of Normal Human Thyroid

Results of Four Samples

particulate fractions sharing the remainder in the ratio of 5:4 in favour of the "Microsomal" fraction. In the thyrotoxic gland (Table 35), the amount of soluble protein per g. wet weight is reduced in all three fractions compared with the normal gland; furthermore, the supernatant increases its share of the total cytoplasmic protein to 93% with a consequent reduction in that of the particulate fractions; in addition, the ratio of the percentage of the cytoplasmic protein found in the particulate fractions is reversed, so that the "Mitochondrial" fraction has more protein than the "Microsomal" fraction per g. wet weight of tissue. Indeed, the most notable change in the thyrotoxic gland compared with the normal is the reduction in "Microsomal" protein per g. wet weight, and it is the only one which is statistically significant ($P < 0.025$).

The protein content of the supernatant fraction of thyroid adenomata is only two-thirds of that found in the normal gland. The protein content of the "Mitochondrial" fraction is a little higher than that of the normal gland and the protein content of the "Microsomal" fraction considerably lower. The supernatant accounts for 89% of the total cytoplasmic protein of thyroid adenomata and the ratio of the percentages attributable to the particulate fractions, as in the thyrotoxic gland, favours the "Mitochondrial" fraction; but none of these differences from normality are statistically significant. (Table 36)

The carcinomata show a drastic reduction in the protein content of the supernatant per g. wet weight of tissue to a mean value of 57.9 mg. (Table 37). This is only half the mean normal value and the difference is significant ($P < 0.01$). On the other hand, the protein content of the particulate fractions was 50% higher than in the corresponding fractions

of the normal glands, although the differences were not significant because of the small numbers involved in the study. When the percentage of the total cytoplasmic protein found in each fraction of the carcinomata was calculated from the data, the difference in the distribution from that operating in the normal gland is greatly enhanced. Thus the percentage of the cytoplasmic protein found in the supernatant of thyroid carcinomata is only 80.2% ($P < 0.01$) while that of the "Mitochondrial" fraction is significantly increased to 8.6% ($P < 0.05$) and that of the "Microsomal" fraction to 11.1% ($P < 0.02$); it should be noted that the ratio of the protein in the two particulate fractions of the carcinomata is approximately the same as that in the normal thyroid tissue.

The thyroiditis group is interesting in showing (Table 38) an even greater reduction in the supernatant content of soluble protein to 40.4 mg. per g. wet weight, a value which is significantly different from that of the normal gland ($P < 0.005$). On the other hand, there is no increase in the mean protein content of the particulate fractions which show little difference from the mean values found in the normal gland. Nevertheless the distribution of protein in these fractions is increased percentage-wise because of the low supernatant content, so that in addition to the latter fraction containing a lower percentage of the total cytoplasmic protein than the normal gland ($P < 0.1$; not significant) the percentage of the cytoplasmic protein found in the "Mitochondrial" fraction is significantly higher than that of the corresponding fraction of the normal gland ($P < 0.05$), and the elevation in the percentage of protein found in the "Microsomal" fraction above that of its normal counterpart is yet more significant ($P < 0.02$).

THYROID

Series II

The purpose of the study described under this section was to discover whether in the human thyroid gland, the differences in the activity of various enzymes detected when the activity was measured per mg. of protein and per g. of wet weight was a true reflection of the activity per cell. For this reason, DNA-phosphorus was chosen as the parameter of cellularity of the fifteen specimens analysed. These were restricted to three types of tissue - normal thyroid, thyroid adenomata, and thyrotoxic thyroid tissue-for reasons that will be discussed later, the enzyme activity of the supernatant being measured with reference to three parameters: soluble protein content of supernatant, wet weight of tissue, and DNA-P content of the whole homogenate from which the supernatant was prepared.

In comparing the normal thyroid tissues with those removed from thyrotoxic subjects, the two ribonucleases and DNAase I can be considered together. The first feature of note is that in both groups, the activity of all three enzymes whether expressed per mg. protein or per g. wet weight is greater than that found for the supernatant fraction of the same group in Series I. Thus, the mean activity of alk. RNAase per mg. protein is 0.054 units in the normal and 0.061 units in the thyrotoxic groups respectively (Table 39), values which are 30-35% above the corresponding values for these groups in the first series; the mean activity of acid RNAase per mg. protein is 0.042 units in the normal and 0.056 units in the thyrotoxic groups respectively (Table 40), these values being 25-30% above those found for these groups in Series I; the activity of DNAase I per mg. protein in the normal group is 0.94 (Table 41) which

		Activity per mg. protein	Activity per g. wet weight	Activity per mg. DNA-P
NORMALS	Mean	0.054	6.13	27.4
	S.D.	0.022	2.60	8.9
	Range	0.029-.082	3.48-9.80	11.6-35.0
THYROTOXIC	Mean	0.061	6.66	22.4
	S.D.	0.008	2.35	8.7
	Range	0.053-.075	5.35-10.61	17.7-37.9
	t	0 .69	0.33	1.13

TABLE 39.

ALKALINE RIBONUCLEASE ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic in Series II - Five Cases of Each

TABLE 40.

		Activity per mg. protein	Activity per g. wet weight	Activity per mg. DNA-P
NORMALS	Mean	0.042	4.77	21.6
	S.D.	0.013	1.57	6.9
	Range	0.027-.058	3.38-6.85	11.4-24.7
THYROTOXICS	Mean	0.056	6.23	21.0
	S.D.	0.013	2.99	10.9
	Range	0.039-.076	4.10-10.82	12.4-38.6
	t	1.56	1.08	0.11

TABLE 40.

ACID RIBONUCLEASE ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic in Series II - Five Cases of Each

		Activity per mg. protein	Activity per g. wet weight	Activity per mg. DNA-P
NORMALS	Mean	0.94	106	494
	S.D.	0.48	51.8	259
	Range	0.52-1.53	70-180	200-822
THYROTOXICS	Mean	1.21	128	423
	S.D.	0.81	80.4	266
	Range	0.54-2.14	50-220	169-737
	t	0.63	0.51	0.42

TABLE 41.

DEOXYRIBONUCLEASE I ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic in Series II - Five Cases of Each

		Activity per mg. protein	Activity per g. wet weight	Activity per mg. DNA-P
NORMALS	Mean	22.4	2520	11865
	S.D.	6.7	479	4483
	Range	16.1-30.6	1920-3060	6857-20136
THYROTOXICS	Mean	44.3	4702	15654
	S.D.	11.8	992	3606
	Range	30.6-57.1	3582-5820	10855-20421
	t	3.57	4.38	1.35
	P	< 0.01	< 0.005	

TABLE 42.

DEOXYRIBONUCLEASE II ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic in Series II - Five Cases of Each

is 30% higher than the corresponding value for the normal group in the first series, while the value in the toxic group is 1.21, compared with the corresponding value of 0.76 units found for the toxic group of Series I. The elevation of activity found in the supernatants of the present series is even greater when expressed per g. wet weight, the mean alk. RNAase activities for the two groups being 45-50% higher, the mean acid RNAase activities 30-40% higher, and the mean DNAase I activities being 65-70% higher than the corresponding values found in the first series. It will be recalled (Table 10) that the only difference between the normal and toxic groups in Series I found to be statistically significant with respect to these three enzymes was the activity of acid RNAase. Although in Series II with its smaller numbers these differences are not significant, the value of 't' for the comparison of acid RNAase activity in the normal and thyrotoxic tissues is 1.56 when the activity is measured per mg. protein and 1.08 when measured per g. wet weight; and these values are much higher than those calculated for alk. RNAase and DNAase I.

When the enzyme activities are measured relative to the DNA-P content of the tissues, any superiority in enzyme content held by the thyrotoxic tissue over the normal gland is lost; indeed, the alk. RNAase activity of the normal is 20-25% higher than that of the thyrotoxic when so measured ('t'=1.13) and it is arguable whether in a large series this difference might attain statistical significance.

It is surprising that in this small series, the differences between normal and thyrotoxic tissues with respect to DNAase II and adenosine deaminase which were found to be statistically significant in the previous series are also significant once again . . . . Thus, although the

TABLE 43.

		Activity per mg. protein	Activity per g. wet weight	Activity per mg. DNA-P
NORMALS	Mean	15.6	1736	8233
	S.D.	4.5	475	3536
	Range	11.6-25.4	1380-2540	4600-12390
THYROTOXICS	Mean	25.1	2617	8662
	S.D.	7.7	495	1673
	Range	12.7-33.6	1802-3090	6438-10475
	t	2.36	2.83	0.24
	P	< 0.05	< 0.025	

TABLE 43.

ADENOSINE DEAMINASE ACTIVITY IN SUPERNATANT OF HUMAN THYROID GLAND

Comparison of Normal with Thyrotoxic in Series II - Five Cases of Each

mean activities as measured per mg. protein and per g. wet weight are increased in the normal group by about one-third compared with the corresponding values found in Series I, the mean activities per mg. protein and per g. wet weight in the thyrotoxic group of Series II are double the corresponding values found in Series I, and these higher means are associated with much the same degree of variance as was found to apply to the data of Series I. Consequently the difference between normal and thyrotoxic with respect to DNAase II activity per mg. protein is significant at the 1% level in the present series, while the difference between these two tissues with respect to DNAase II activity per g. wet weight is significant at the 0.5% level (Table 42). On the other hand, when the enzyme activity is measured per mg. of DNA-P, the difference between normal and thyrotoxic tissue is not significant. None the less, the mean activity of the latter when so expressed is 50% higher than that of the former, and the value of 't' is 1.35; this is the highest value of 't' found for the comparison between normal and thyrotoxic of the activity of any enzyme per mg. DNA-P.

specific
The /... activity of adenosine deaminase in the supernatant of the normal and thyrotoxic tissues of Series II is in good agreement with the values found for these groups in Series I. Curiously, the variance is very much less in the present series, so that the difference between normal and thyrotoxic groups is significant ($P < 0.05$). When the results are measured per g. wet weight of tissue, the mean values are 30% higher than those found for the corresponding groups of Series I, and once again the variance is reduced; thus the differences between normal and thyrotoxic groups is now significant at the 0.25% level. However, measured against

DNA-P, there is very little difference between the adenosine deaminase activity of the two groups, and it seems unlikely that a significant difference could be established by extending the present work.(Table 43)

The differences in the mean enzyme activities found in the adenomata of Series II (Tables 44-5) from those of the first series are very variable. A striking increase in alk. RNAase is apparent (Table 44), the activity per mg. protein being increased by 40% and per g. wet weight by more than 100% of the values found for this group in Series I. The increase in acid RNAase is not so dramatic, the mean values for Series II being 15% higher than in Series I when measured per mg. protein, and 50% higher when measured per g. wet weight. With DNAase I, the mean activity per mg. protein is actually lower than that found for the adenomata of Series I, though the activity per g. wet weight is 50% higher. The mean activity of DNAase II is 20% lower per mg. protein and 10% higher per g. wet weight than the corresponding means for the adenomata of Series I; while the activity of adenosine deaminase shows a reduction of 30% per mg. protein and 10% per g. wet weight compared with the mean values for the previous series.

It would be unwise to make too much of these differences in material so obviously heterogeneous and associated with such wide variance as thyroid adenomata appear to be, and it remains to point out that relative to the other two tissues of this series, the adenomata are richest in alk. RNAase, intermediate in acid RNAase and DNAase I, and poorest in DNAase II and adenosine deaminase as assessed per mg. protein and per g. wet weight. When the activities are however assessed relative to the DNA-P content of the tissue, the picture changes; for the adenomata are very much richer

		Activity per mg. protein	Activity per g. wet weight	Activity per mg. DNA-P
ALKALINE RIBONUCLEASE	Mean	0.076	7.49	45.2
	S.D.	0.052	4.62	24.7
	Range	0.030-0.142	4.00-15.30	21.2-100.0
ACID RIBONUCLEASE	Mean	0.051	5.27	31.6
	S.D.	0.034	3.69	25.8
	Range	0.020-0.094	2.50-11.70	16.3-76.5

TABLE 44.

ACTIVITY OF RIBONUCLEASES

IN SUPERNATANT OF HUMAN THYROID GLAND

Results of Five Thyroid Adenomata
in Series II

		Activity per mg. protein	Activity per g. wet weight	Activity per mg. DNA-P
DNAase I	Mean	0.82	126	542
	S.D.	0.36	59	282
	Range	0.10-1.04	10-160	67-777
DNAase II	Mean	21.5	2152	12311
	S.D.	11.0	567	3975
	Range	13.8-40.4	1180-2670	7866-17450
ADENOSINE DEAMINASE	Mean	14.2	1366	7928
	S.D.	7.9	387	3100
	Range	8.6-26.1	1050-2010	5822-13400

TABLE 45.

ACTIVITY OF DEOXYRIBONUCLEASES AND

ADENOSINE DEAMINASE

IN SUPERNATANT OF HUMAN THYROID GLAND

Results of Five Thyroid Adenomata

in Series II

in both ribonucleases than the other two groups, and with the three remaining enzymes, the activity is of the same order as that of the normal and thyrotoxic tissues. Indeed, the difference between the alk. RNAase activity per mg. DNA-P of the normal and adenoma groups is statistically significant ('t'=2.41; $P<0.05$) and it is the only respect in which the adenomata differed significantly from the normals in this series.

BREAST

Ribonucleases

The mean activity of alk. RNAase is 0.028 units per mg. protein in the normal breast, 0.037 units in the fibroadenomata, 0.041 units in the cystic mastopathy group, and 0.096 units in the carcinomata (Tables 46 and 47). The carcinomata thus show a mean value which is significantly higher than the normals ($P < 0.02$) and the mastopathy group ($P < 0.05$). When the results are expressed per g. wet weight, the same order prevails, except in so far as the fibroadenomata displace the mastopathy group as the second most active tissue of the four. Furthermore, the carcinomata, with a mean activity of 2.27 units per g. wet weight, are significantly higher than the normals and the mastopathy group, the level of significance being as high as 0.5% with respect to both (Tables 46 , 47 and 52).

The activity of acid RNAase in these four groups of breast tissue shows some interesting divergence from the pattern prevailing with the alk. RNAase. The most notable feature, apart from the fact that the enzyme activity is lower than that of the alk. RNAase in all tissues, is that the drop in activity is most drastic in the mastopathy group, so that the activity of this group which shows a mean of 0.013 units per mg. protein, is lower than that of the normals, of which the mean activity per mg. protein is 0.018 units per mg. protein. The fibroadenomata contain activity a little higher than that of normal breast (mean 0.021 units per mg. protein) while that of the carcinomata is as high as 0.064 units per mg. protein, a result which is significantly higher than that of both the normal and mastopathy group at the 1% level. The mean activities per g.

TABLE 46.

		ALKALINE RIBONUCLEASE		ACID RIBONUCLEASE	
		Activity per mg. protein	Activity per g. wet weight	Activity per mg. protein	Activity per g. wet weight
NORMALS	Mean	0.028	0.59	0.018	0.38
	S.D.	0.020	0.42	0.010	0.22
	Range	0.006-0.051	0.16-1.05	0.004-0.027	0.10-0.65
CARCINOMAS	Mean	0.096	2.27	0.064	1.44
	S.D.	0.046	0.85	0.028	0.41
	Range	0.060-0.164	1.20-3.25	0.026-0.089	0.98-2.10
	t	2.96	3.92	3.42	4.86
	P	< 0.02	< 0.005	< 0.01	< 0.005

TABLE 46.

ALKALINE AND ACID RIBONUCLEASE ACTIVITIES

OF HUMAN BREAST SUPERNATANT

Comparison of Normal with Carcinoma:

Five Cases of Each

TABLE 47.

	ALKALINE RIBONUCLEASE		ACID RIBONUCLEASE	
	Activity per mg.protein	Activity per g.wet weight	Activity per mg.protein	Activity per g. wet weight
FIBROADENOMAS				
Mean	0.037	0.74	0.021	0.44
S.D.	0.016	0.44	0.009	0.31
Range	0.017-0.050	0.20-1.37	0.014-0.351	0.17-0.97
CYSTIC MASTOPATHY				
Mean	0.041	0.63	0.013	0.37
S.D.	0.019	0.33	0.007	0.35
Range	0.027-0.072	0.33-1.12	0.006-0.024	0.14-0.96

TABLE 47.

ALKALINE AND ACID RIBONUCLEASE ACTIVITIES

OF HUMAN BREAST SUPERNATANT

Results for Five Fibroadenomas and

Five Cases of Cystic Mastopathy

wet weight of the acid DNAase content of normal breast, fibroadenomata, and mastopathy groups, are similar and range from 0.37 to 0.44 units; the activity per g. wet weight of the carcinomata gave a mean of 1.44 units which is significantly higher than that of the other groups ($P < 0.005$).

Deoxyribonucleases

The DNAase I content of these tissues shows no significant differences between any of the groups whether expressed per mg. protein or per g. wet weight (Tables 48 and 49). Curiously, the mastopathy group appears to be richer in this enzyme than the other three groups, the mean values being 3.98 units per mg. protein and 58 units per g. wet weight, while the fibroadenomata are the poorest of the four with mean activities of 1.47 units per mg. protein and 26.2 units per g. wet weight.

In all four tissues, the content of DNAase II is very much higher than that of DNAase I, the increase varying from four-fold in the normals to almost twenty-fold in the carcinomata. The tissues are thus arranged in ascending order of activity (Tables 48 and 49): normals, 8.2 units per mg. protein and 176 units per g. wet weight; fibroadenomata, 9.4 units per mg. protein and 187 units per g. wet weight; cystic mastopathy group, 23.6 units per mg. protein and 372 units per g. wet weight; carcinomata, 39.3 units per mg. protein and 1075 units per g. wet weight. The difference between the mastopathy group and the normals is significant when the results are expressed per mg. protein ($P < 0.01$) but just miss being significant when the activity is expressed per g. wet weight ($P < 0.1$). Relative to its wet weight, the carcinoma group is significantly higher than the normals ($P < 0.01$) and the mastopathy group ($P < 0.05$), but relative

		DEOXYRIBONUCLEASE I		DEOXYRIBONUCLEASE II	
		Activity per mg.protein	Activity per g. wet weight	Activity per mg.protein	Activity per g. wet weight
NORMALS	Mean	2.20	47.8	8.2	176
	S.D.	0.91	21.1	5.7	113
	Range	1.17-3.33	29.8-79.6	3.1-16.9	60-322
CARCINOMAS	Mean	2.30	56.8	39.3	1075
	S.D.	1.53	39.7	22.6	559
	Range	0.80-4.55	18-115	10.0-67.6	110-1520
	t		0.44	2.95	3.50
	P			< 0.02	< 0.01

TABLE 48.

DEOXYRIBONUCLEASES I AND II ACTIVITIES

OF HUMAN BREAST SUPERNATANT

Comparison of Normal with Carcinoma:

Five Cases of Each

TABLE 49.

	DEOXYRIBONUCLEASE I		DEOXYRIBONUCLEASE II	
	Activity per mg.protein	Activity per g. wet weight	Activity per mg.protein	Activity per g. wet weight
FIBROADENOMAS				
Mean	1.47	26.2	9.4	187
S.D.	2.10	42.7	9.6	176
Range	0.20-4.80	2.1-100.0	3.0-24.7	33-550
CYSTIC MASTOPATHY				
Mean	3.98	58	23.6	372
S.D.	2.03	26	7.3	183
Range	1.63-6.45	30-90	14.4-34.2	214-630
t	1.77	0.68	3.70	2.25
P	-	-	0.01	0.1

TABLE 49.

DEOXYRIBONUCLEASE I AND II ACTIVITIES

OF HUMAN BREAST SUPERNATANT

Results for Five Fibroadenomas and
Five Cases of Cystic Mastopathy:
Statistical Evaluation Refers to Comparison Between
Normals and Cystic Mastopathy

to its protein content, significance attaches only to the difference in activity from that of the former group ($P < 0.02$).

Adenosine Deaminase

As with DNAase II, the activity of adenosine deaminase in the supernatant permits the tissues to be arranged in ascending order of activity whether measured per mg. protein or per g. wet weight: normal, fibroadenomata, mastopathy group, and finally carcinomata (Tables 50 and 51). Considering first of all the results as expressed per mg. protein, the mean for the mastopathy group of 39.4 units is more than double the mean for the normals of 17.9 units ($P < 0.05$), while the mean of the fibroadenomata is intermediate between these two values and not significantly different from either; the carcinomata have twice the activity of the mastopathy group ($P < 0.01$) and are significantly different from the normals at the 0.1% level - indeed this is the most striking difference between normals and cancers of all the enzymes of breast tissue studied in the present work. When the results are measured with reference to the wet weight of the tissue, the carcinomata are still very much more active than the mastopathy group ($P < 0.025$) and the normals ($P < 0.005$), but the differences between the other three tissues are diminished and the range of spread reduced so that, although the relative order is still the same, the mastopathy group has only one and a half times the activity of the normal group and this difference is not significant.

Particle Fractions

As was indicated in the section on "Materials and Methods", great

		Activity per mg. protein	Activity per g. wet weight
NORMALS	Mean	17.9	406
	S.D.	6.3	157
	Range	10.5-26.3	211-627
CARCINOMAS	Mean	74.3	2011
	S.D.	9.4	995
	Range	67.3-83.7	825-3375
	t	10.08	3.94
	P	< 0.001	< 0.005

TABLE 50.

ADENOSINE DEAMINASE ACTIVITY

OF HUMAN BREAST SUPERNATANT

Comparison of Normal with Carcinoma:

Five Cases of Each

		Activity per mg. protein	Activity per g. wet weight
FIBROADENOMAS	Mean	25.5	505
	S.D.	14.8	287
	Range	5.8-47.1	65-800
CYSTIC MASTOPATHY	Mean	39.4	642
	S.D.	19.0	417
	Range	24.2-66.3	300-1220
	t	2.39	1.16
	P	< 0.05	

TABLE 51.

ADENOSINE DEAMINASE ACTIVITY

OF HUMAN BREAST SUPERNATANT

Results for Five Fibroadenomas and
Five Cases of Cystic Mastopathy:
Statistical Evaluation Refers to Comparison Between
Normals and Cystic Mastopathy

	Mean of Cystic Mastopathy Series	Mean of Carcinoma Series	t	P
Alk. RNAse/mg.Protein	0.041	0.096	2.46	< 0.05
Alk. RNAse/g. wet wt.	0.63	2.27	3.94	< 0.005
Acid RNAse/mg.Protein	0.013	0.064	3.79	< 0.01
Acid RNAse/g. wet wt.	0.37	1.44	4.29	< 0.005
DNAse I/mg.Protein	3.98	2.30	1.46	-
DNAse I/g. wet wt.	58	56.8	-	-
DNAse II/mg.Protein	23.6	39.3	1.46	-
DNAse II/g. wet wt.	372	1075	2.64	< 0.05
A.D./mg.Protein	39.4	74.3	3.61	< 0.01
A.D./g. wet wt.	642	2011	2.81	< 0.025

TABLE 52.

ENZYME ACTIVITIES IN SUPERNATANT OF HUMAN BREAST TISSUE

Statistical Comparison of Results in
Carcinoma Series (50ases)
with those in
Cystic Mastopathy Series (5 Cases)

difficulty was encountered in preparing particulate fractions from human breast tissue. Apart from the technical difficulties, the impression was firmly derived that this tissue is poorly endowed with subcellular particles. Thus, even when it was possible to exclude all technical factors militating against the quantitative collection of these fractions from the whole homogenate, the yield was very small, particularly in the non-malignant group, and especially was this so with respect to the "Microsomal" fraction. Indeed, in most cases, the clarity of the fluid expressed through muslin and spun at 500g for 10 min. was such as to belie the possibility of subcellular particles being suspended in it; experience with many types of tissue in the course of several years has enabled the investigator to predict in a qualitative manner the likely yield of particles in a nuclear-free preparation of cytoplasm by examination of the opacity of the fluid.

Such results as were obtained from preparations that could be relied upon are presented in Tables 53 and 54 . Since less material is required for estimation of ribonucleases, these assays were usually preferred where the amount of material available did not permit the estimation of all four nucleases. The non-malignant tissues have been grouped together in these tables for the purpose of statistical treatment, which in any case is only possible with the activity of ribonucleases in the "Mitochondrial" fraction. It is noteworthy that from 3 out of 5 carcinomata, particulate fractions could be prepared, while from the 15 non-malignant tissue samples, only 5 "Mitochondrial" fractions and only 2 "Microsomal" fractions could be prepared; one of the latter did not satisfy the investigator that the yield of particles collected represented

Enzyme Activities per mg. Protein

		Alk. RNAse	Acid RNAse	DNAse I	DNAse II
NON-CANCER	Mean	0.026	0.010	3.51	8.8
	S.D.	0.018	0.009		
	No. of Cases	5	5	1	1
CANCER	Mean	0.142	0.067	6.19	24.77
	S.D.	0.159	0.059		
	No. of Cases	3	3	2	2
	t	1.71	2.29		

Enzyme Activities per g. wet weight

		Alk. RNAse	Acid RNAse	DNAse I	DNAse II
NON-CANCER	Mean	0.042	0.011	0.75	1.88
	S.D.	0.060	0.008		
	No. of Cases	4	4	1	1
CANCER	Mean	0.385	0.231	57.2	210
	S.D.	0.057	0.071		
	No. of Cases	3	3	2	2
	t	7.59	6.41		
	P	< 0.001	< 0.005		

TABLE 53.

ENZYME ACTIVITIES IN "MITOCHONDRIAL" FRACTION OF HUMAN BREAST TISSUE

Comparison of Carcinoma with Non-Carcinomatous Samples

Enzyme Activities per mg. Protein

		Alk. RNAse	Acid RNAse	DNAse I	DNAse II
NON-CANCER	Mean	0.029	0.007		
	No. of Cases	2	2		
CANCER	Mean	0.117	0.046	10.86	34.6
	No. of Cases	3	3	2	2

Enzyme Activities per g. wet weight

		Alk. RNAse	Acid RNAse	DNAse I	DNAse II
NON-CANCER	Mean	0.011	0.005		
	No. of Cases	1	1		
CANCER	Mean	0.182	0.066	12.8	40.5
	No. of Cases	3	3	2	2

TABLE 54.

ENZYME ACTIVITIES IN "MICROSOMAL" FRACTION OF HUMAN BREAST TISSUE

Comparison of Carcinoma with Non-Carcinomatous Samples

all of these fractions in the original homogenate, and only the activity per mg. protein is included in the data.

Sparse as the results are, they are not without some interest in showing that the particulate fractions prepared from carcinomata of breast are likely to contain far greater activity for all four nucleases than the corresponding fractions prepared from non-malignant samples of breast tissue. In this small series, the activities of alk. and acid RNAase of the particulate fractions of carcinomata are 4 to 7 times greater per mg. of protein than the corresponding fractions prepared from non-malignant breast tissue; because of the high variance, statistical significance could not be demonstrated where numbers permitted the analysis to be attempted. When consideration is given to the activities of these enzymes expressed per g. wet weight, the carcinomata have an advantage because their yield of particles was higher than with the non-malignant tissues. The difference between cancer and non-malignant groups is thus magnified, and ranges from a nine-fold to a twenty-fold increase in the activity of the former group over that of the latter depending upon the fraction and the enzyme in question, and statistical treatment reveals that the differences in alk. and acid RNAases in the "Mitochondrial" fraction are significant at the 0.1% and 0.5% levels respectively. The DNAase results are too few to permit serious comment.

In so far as it is possible to draw any comparison on the basis of these rudimentary results between the relative activities of supernatant and particulate fractions in human breast, it seems unlikely that major differences exist in the non-malignant group when the results are expressed per mg. protein; while in the carcinomata, the only suspicions aroused

point to a possible increase in DNAase I together with a decrease in DNAase II activity in the particulate fractions, while even more remote is the possibility that the alk. RNAase activity may be somewhat increased in the particulate fractions compared with the supernatant.

Protein

The protein content of the supernatant fraction of the various breast tissues studied is shown in Table 55 . The first point that emerges is that these results are very much lower than those obtained in the thyroid tissues. Indeed, with the exception of one carcinoma, one adenoma, and three samples of thyroiditis tissue, none of the thyroid specimens examined have a lower concentration of protein in the supernatant than that breast carcinoma which, with a protein content of 49.6 mg. per g. wet weight, gave the highest concentration of the twenty breast tissues studied in this work.

The difference between the normals and the fibroadenomata is small, and although that between the former and the carcinomata is greater, it is not significant. On the other hand, the cystic mastopathy group display a protein content which is reduced below that of normal breast to a significant level ($t=3.67$; $P< 0.01$).

Such data as were obtained on the particulate fractions are shown in Table 56 . It would be unwise to lay too much emphasis on data as scanty as that presented, but the suspicion is aroused that relative to the non-malignant tissues, the carcinomata may be more richly endowed with subcellular particles, and that in both the malignant and the non-malignant groups, the cells may be poorer in microsomes than in mitochondria.

Type of Breast Tissue	Mean	S.D.	Range
Normal	22.0	2.7	19.0-25.5
Fibroadenomata	19.1	5.9	11.5-27.9
Cystic Mastopathy	15.4	3.2	11.4-18.4
Carcinomata	27.4	14.8	11.0-49.8

TABLE 55.

PROTEIN CONTENT OF SUPERNATANT OF HUMAN BREAST TISSUE:

Results in mg. Protein per g. wet weight of Tissue

	Number	Mean	Range
Non-Malignant "Mitochondria"	5	1.24	0.42-3.20
Malignant "Mitochondria"	3	5.50	1.1-10.3
Non-Malignant "Microsomes"	2	0.30	0.27-0.33
Malignant "Microsomes"	3	1.25	0.06-1.80

TABLE 56.

PROTEIN CONTENT OF PARTICULATE FRACTIONS OF NON-MALIGNANT AND MALIGNANT BREAST TISSUE.

Results in mg. Protein per g. wet weight of Tissue

In line with these data, it has been calculated that the mean percentage of the cytoplasmic protein present in the supernatant is 95% in the non-malignant group and 74% in the malignant group, based on three samples of each group.

CERVIX

Comparison Between Normal Tissue and Carcinoma

The results obtained in 16 normals and in 25 cases of cervical carcinoma point to a higher content of nucleases and adenosine deaminase in the latter group. The individual data on the supernatant fractions are presented graphically in Figures 24-25 and the mean values with statistical treatment in Tables 57 - 61 . In six only of the normal tissues was it considered that the yield of particles was adequate to permit calculation of the distribution of enzyme activity within the cytoplasmic fractions; in one sample of the normal series, technical considerations vitiated a relationship being established between supernatant activity and wet weight of the sample, thereby reducing to 15 the number of samples upon which the mean enzyme activity per g. wet weight was based.

It will be seen from Figures 24 and 25 and Tables 57 and 58 that there is virtually a complete separation of the normal from the carcinoma group when the activities of the two ribonucleases are expressed per mg. protein or per g. wet weight. When alk. RNAase is measured per mg. protein or per g. wet weight, only one sample of the normal series exceeds in activity that of the lowest in the carcinoma series. When acid RNAase is measured per mg. protein, the same situation applies, but when measured per g. wet weight, the activity of the highest normal is 2.2 units, that of the lowest carcinoma 3.1 units. The mean alk. RNAase activity of the normal supernatants per mg. protein is almost twice that of the normal thyroid, but when measured per g. wet weight, it is only half that of the

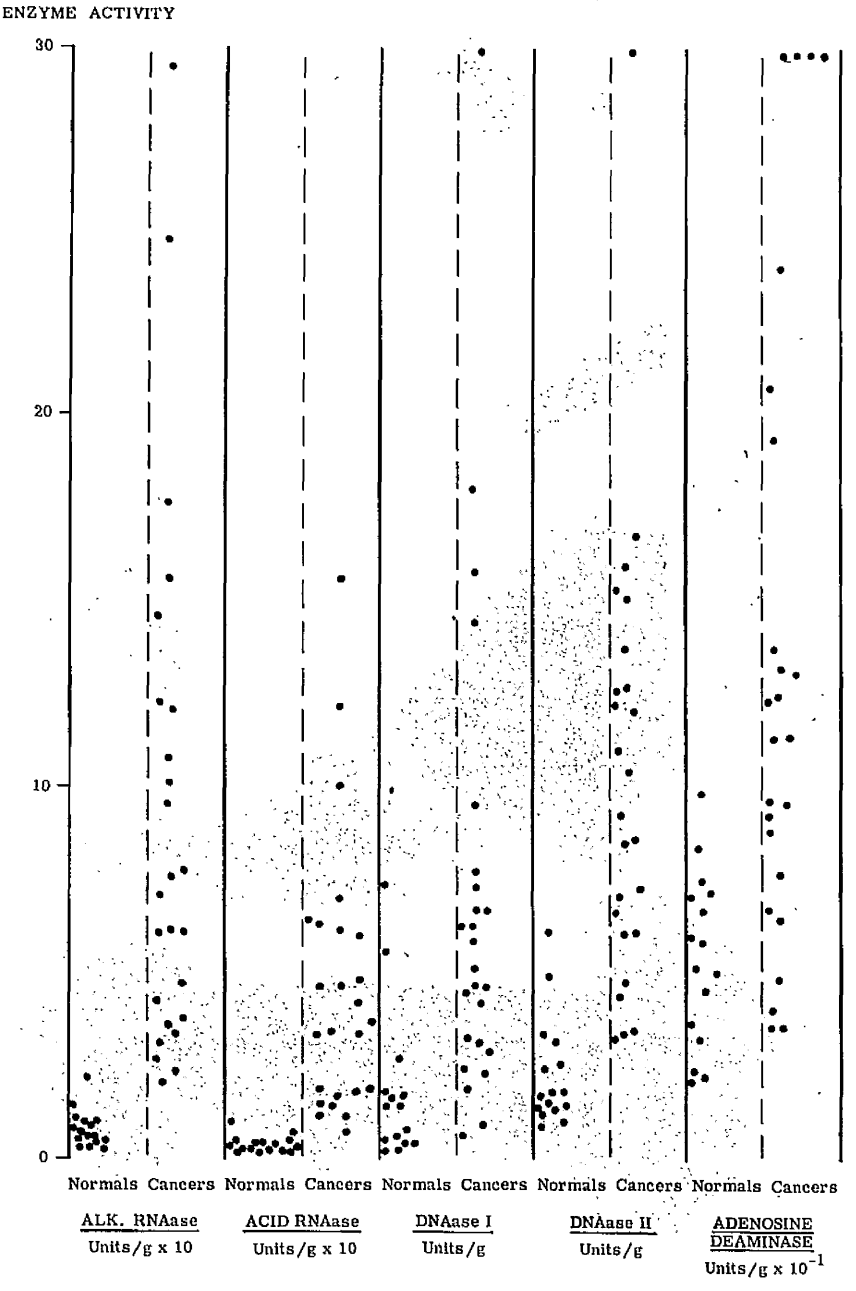


FIGURE 24

SCATTERGRAM TO SHOW DISTRIBUTION OF ENZYME ACTIVITIES PER mg. PROTEIN IN SUPERNATANT OF NORMAL AND CANCEROUS CERVIX UTERI

(An error has occurred in the above plate. For " Units/g " read " Units/ mg. Protein)

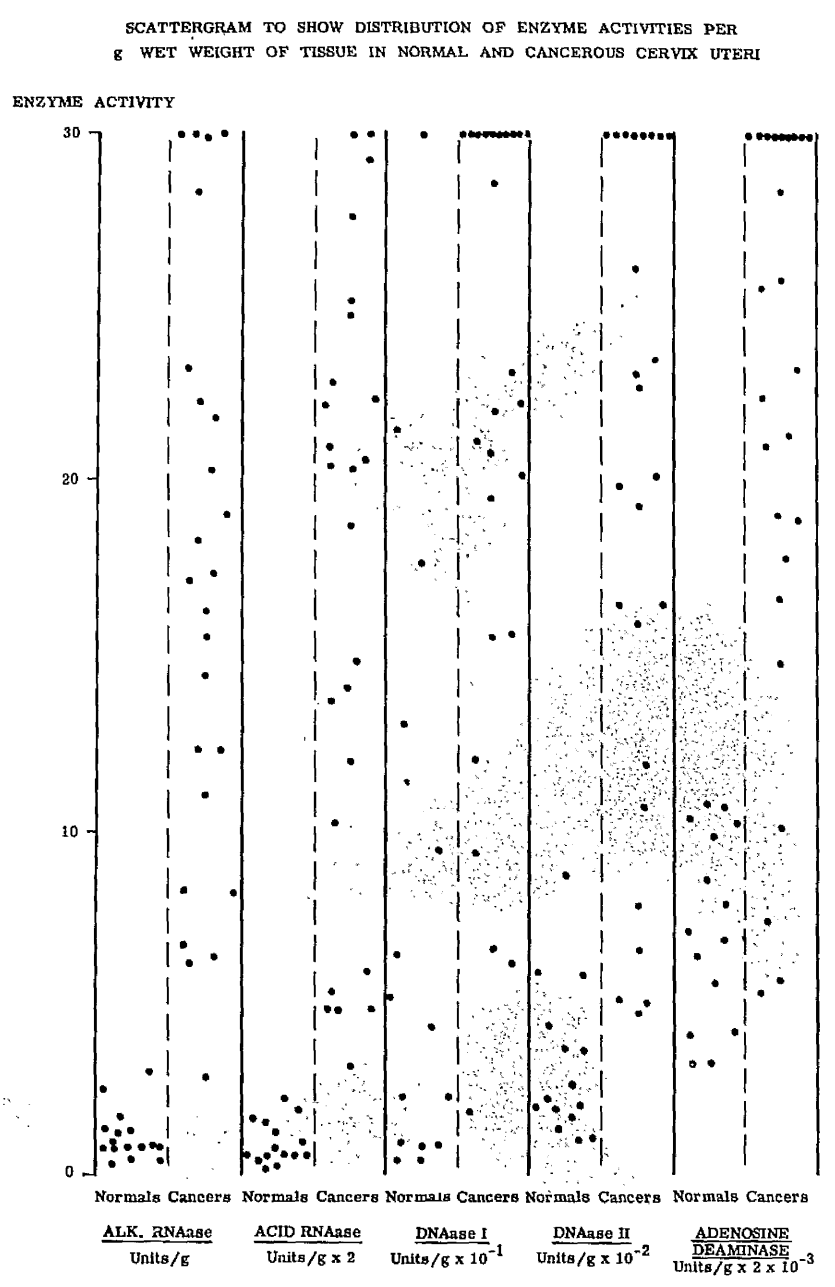


FIGURE 25

SCATTERGRAM TO SHOW DISTRIBUTION OF ENZYME ACTIVITIES PER UNIT WEIGHT OF TISSUE IN SUPERNATANT OF NORMAL AND CANCEROUS CERVIX UTERI

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	16	15	6
	Means	0.078	2.27	92.8
	S.D.	0.047	1.60	4.4
	Range	0.033-.211	0.62-5.91	86.1-97.8
CANCERS	No. of tissues	25	25	19
	Means	0.91	35.4	76.2
	S.D.	0.69	19.4	6.8
	Range	0.20-2.85	5.6-78.9	62.8-85.1
Statistical Analysis	t	4.77	6.64	5.57
	P	< 0.001	< 0.001	< 0.001

TABLE 57.

ALKALINE RIBONUCLEASE ACTIVITY IN SUPERNATANT OF HUMAN CERVIX UTERI

Comparison of Normal with Carcinoma

TABLE 58.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	16	15	6
	Means	0 .033	0.94	89.8
	S.D.	0.023	0.63	7.4
	Range	0 .007-.095	0.23-2.20	75.6-98.1
CANCERS	No. of tissues	25	25	19
	Means	0.51	17.8	72.4
	S.D.	0.28	10.5	11.8
	Range	0 .070-1.56	3.1-45.2	49.2-93.8
Statistical Analysis	t	6.86	3.86	3.39
	P	< 0 .001	< 0 .001	< 0.005->.001

TABLE 58.

ACID RIBONUCLEASE ACTIVITY IN SUPERNATANT OF HUMAN CERVIX UTERI

Comparison of Normal with Carcinoma

normal thyroid; in respect of both modes of expression the activity far exceeds that of normal breast. In those samples where such an assessment was possible, a mean of 93% of the cytoplasmic activity was found in the supernatant fraction (Table 57). The mean values for the carcinomata are 12-15 times greater than that of the normal, and only 76% of the cytoplasmic activity is found in the supernatant. All these differences between normal and carcinoma groups are highly significant ($P < 0.001$).

The mean activity of acid RNAase per mg. protein in the supernatant of normal cervix is less than half that of alk. RNAase, and therefore approximates to that of normal thyroid; but when measured per g. wet weight, the activity is less than a third of that found in the latter tissue. Once again, the activity irrespective of the mode of expression far exceeds that of normal human breast. Approximately 90% of the total cytoplasmic activity appears in the supernatant (Table 58). Since the activity of acid RNAase in the supernatant of the carcinoma group does not fall to quite the same extent, the differences between the normal and carcinoma groups are increased. Thus the mean activity per mg. protein in the latter group is more than 15 times that of the former, and the mean activity per g. wet weight in the carcinoma group is almost 20 times greater than that of the normal, both differences being significant at the 0.1% level. Only 72.4% of the total cytoplasmic activity of acid RNAase is found in the supernatant of the carcinomata ($P < 0.005$).

From Figure 24 it will be observed that considerable overlap between the normal and carcinoma groups exists in respect of their DNAase I content per mg. protein, and it is utterly impossible to draw a meaningful line of separation between them. Nevertheless it can be appreciated that there

TABLE 59.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	16	15	6
	Means	2.52	72.1	75.2
	S.D.	3.60	103.4	23.7
	Range	0.22-13.82	5.0-383.0	30.8-94.1
CANCERS	No. of tissues	25	25	16
	Means	7.23	291	65.8
	S.D.	7.42	224	16.4
	Range	0.59-35.90	65-968	38.8-92.4
Statistical Analysis	t	2.33	3.57	1.06
	P	$< 0.05 > .025$	< 0.001	

TABLE 59.

DEOXYRIBONUCLEASE I ACTIVITY

SUPERNATANT OF HUMAN

CERVIX UTERI

Comparison of Normal with Carcinoma

is a tendency for the carcinoma group to have a higher content of this enzyme than the normal group, and indeed when the activity is measured per g. wet weight of tissue, four only of the normal tissues exceed a value of 100 units while four only of the carcinoma group have activity lower than this value. The mean value of the former group per mg. protein is in fact twice that of the latter ($P < 0.05$) while the superiority of the carcinomata over the normals is four-fold when the activity is referred to the wet weight of the tissue ($P < 0.001$). The mean percentage of the total cytoplasmic activity of DNAase I found in the supernatant is 75.2% for the normals and 65.8% for the carcinomata (Table 59) but this difference is not statistically significant. It should be mentioned at this juncture that the mean DNAase I activity of the normal cervix per mg. protein is about four times that of normal thyroid, though there is little difference between the tissues when the results are measured per g. wet weight. On the other hand, the reverse comparison may be drawn with the DNAase I activity of human breast supernatant, the activity of which per mg. protein approximates to that of the cervix supernatant, while the mean wet weight activity of breast tissue is only half that of the cervix.

It is possible to make a clearer separation between the two groups of cervical tissue with respect to DNAase II activity than was the case with the previous enzyme (Figure 24). Four only of the normal tissues have activity per mg. protein which exceeds the lowest value found in the carcinoma series; while three only of the normals have activity per g. wet weight which exceeds the lowest value found in the carcinoma group. The mean activity of the normal cervix per mg. protein is intermediate between that of normal breast and normal thyroid; the mean activity per

TABLE 60.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	16	15	6
	Means	11.43	317	92.1
	S.D.	7.3	221	9.3
	Range	4.44-30.6	102-858	80.8-99.0
CANCERS	No. of tissues	25	25	16
	Means	51.4	2366	85.4
	S.D.	30.2	1584	5.9
	Range	16.4-154.7	474-7193	71.8-93.4
Statistical Analysis	t	5.16	4.99	2.02
	P	< 0.001	< 0.001	< 0.1->.05

TABLE 60.

DEOXYRIBONUCLEASE II ACTIVITY

SUPERNATANT OF HUMAN

CERVIX UTERI

Comparison of Normal with Carcinoma

g. wet weight is three times that of normal breast but only one-sixth of the mean activity of normal thyroid. The mean supernatant activity of the cervical carcinomata is almost five times that of the normals per mg. protein and almost eight times that of the normals per g. wet weight, both these differences being highly significant ($P < 0.001$). Of the total cytoplasmic activity, 92.1% was found in the supernatant fraction of the normals (Table 60) and only 85.4% in the supernatant of the carcinomata; this difference is just outside the 5% level of significance.

The difference between the two groups of cervical tissues with respect to adenosine deaminase activity is not as clear-cut as with DNAase II while being more so than was the case with DNAase I. Considering first of all the relationship between the two groups when the activity is measured per mg. protein, it will be seen (Figure 24) that two of the normals have a value in excess of 80 units while seven of the carcinomata fall below this value. Turning to the content per g. wet weight, four of the normals have more than 2000 units and three of the carcinomata less than this figure. It is interesting that the activity of the normal cervix supernatant per mg. protein is almost four times that of normal breast and thyroid supernatant; the activity per g. wet weight in the supernatant of normal cervix is a little greater than that of the normal thyroid and more than three times that of the normal breast (Table 61). The mean activity of the carcinomata is three times that of the normals per mg. protein and about five times that of the normals per g. wet weight; both these differences are very significant ($P < 0.005$).

An attempt was made to correlate the enzyme content of the carcinomata with the degree of malignancy of the samples. For this purpose, the

		Activity per mg. protein	Activity per g. wet weight
NORMALS	No. of tissues	16	15
	Means	54.2	1450
	S.D.	23.1	557
	Range	20.7-98.3	632-2146
CANCERS	No. of tissues	25	25
	Means	163.2	6417
	S.D.	144.0	6481
	Range	36.1-540.0	1044-33147
Statistical Analysis	t	2.99	3.51
	P	< 0.005->.001	< 0.005->.001

TABLE 61.

ADENOSINE DEAMINASE

ACTIVITY

SUPERNATANT OF HUMAN

CERVIX UTERI

Comparison of Normal with Carcinoma

carcinomata were divided into the following three groups:

a) Highly Malignant. All cases that presented with lesions classified clinically as Stage III were placed in this group, together with those cases classified clinically as Stage II in whom the routine histology report indicated a high degree of malignancy according to three criteria, namely, the degree of differentiation, the mitotic rate, and the evidence of invasiveness in the specimen examined. Eleven cases satisfied these criteria.

b) Moderately Malignant. Those cases classified clinically as Stage II in whom the routine histology report specified the lesion to be well-differentiated, with low invasiveness and low mitotic rate in the specimen examined were placed in this group. Six cases satisfied these criteria.

c) Intermediate Group. This comprised eight patients who could not be placed in either of the above groups. In some, the histology report was not satisfactory; in others, the lesion was well described but appeared to possess ambiguous features e.g. well-differentiated but high mitotic rate or poorly-differentiated with low mitotic rate. In three cases, the specimen examined by the histologist had some other pathological feature which prevented a clear-cut evaluation of the malignant process being reached, namely, large areas of necrotic tissue, intense stromal reaction, and chronic inflammatory infiltrate.

The mean results obtained for all three groups are presented in Table 70. The most obvious feature is the lack of correlation between the degree of malignancy implied in the classification and the enzyme content of the samples. Only with respect to DNAase II content can the groups be arranged in an order corresponding to their suspected malignancy.

There was no clear-cut difference between the Highly and the Moderately Malignant groups with respect to the ribonucleases and DNAase I; indeed, the Intermediate Group appeared to be more richly endowed in these enzymes than the Highly Malignant group. The content of DNAase II and adenosine deaminase of the Highly Malignant group is approximately double that of the Moderately Malignant group, but none of the differences encountered were statistically significant in the present material, and since the content of adenosine deaminase in the Intermediate Group exceeds that of the Highly Malignant group, it is doubtful if this enzyme has any real relationship to the malignant process.

The Particulate Fractions of Cervical Carcinomata

The separation of a "Mitochondrial" and a "Microsomal" fraction from the cytoplasm of cervical carcinomata was relatively easily accomplished, failure occuring only where the specimen was too small to permit the collection of a useful amount of material. Eighteen samples were fractionated by an identical technique to that employed with the samples of thyroid tissue using the same centrifugal fields. Unfortunately, it was not possible to subject the material thus prepared to the same scrutiny as was devoted to the preparations from thyroid tissue. The exact nature of these fractions must therefore remain a matter of some uncertainty.

The results obtained for the nuclease content of these particulate preparations are presented in Tables 66 - 69. The first point that emerges from an examination of these results is the similarity in the distribution and activity of all four nucleases studied between the two fractions, to such an extent that there is no significant difference for

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	7	5	6
	Means	0.096	0.132	7.2
	S.D.	0.089	0.083	4.4
	Range	0.021-.276	0.036-.260	2.2-13.9
CANCERS	No. of tissues	19	19	19
	Means	0.87	13.4	23.8
	S.D.	0.55	8.7	6.7
	Range	0.099-2.16	1.5-33.5	14.7-37.2
Statistical Analysis	t	3.57	3.33	5.73
	P	< 0.005->.001	< 0.005->.001	< 0.001

TABLE 62.

ALKALINE RIBONUCLEASE ACTIVITY IN CYTOPLASMIC PARTICLES OF HUMAN CERVIX UTERI

Comparison of Normal with Carcinoma

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	7	5	6
	Means	0 .050	0 .064	10.1
	S.D.	0 .045	0 .045	7.6
	Range	0 .004-.130	0 .004-.130	1.9-24.4
CANCERS	No. of tissues	19	19	19
	Means	0.49	7.9	27.6
	S.D.	0.34	5.1	10.4
	Range	0 .036-1.29	0.9-16.8	6.2-50.8
Statistical Analysis	t	3.44	3.44	3.78
	P	< 0 .005->.001	< 0 .005->.001	< 0 .001

TABLE 63.

ACID RIBONUCLEASE ACTIVITY IN CYTOPLASMIC PARTICLES OF HUMAN CERVIX UTERI

Comparison of normal with Carcinoma

TABLE 64.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
NORMALS	No. of tissues	7	5	6
	Means	6.99	9.60	24.8
	S.D.	2.66	9.1	23.1
	Range	0.85-25.7	1.4-21.4	5.9-69.2
CANCERS	No. of tissues	16	16	16
	Means	9.91	173	34.2
	S.D.	10.24	131	14.1
	Range	2.96-29.77	17-468	7.6-61.2
Statistical Analysis	t	0.64	2.75	1.24
	P	Not sig.	< .02->.01	Not sig.

TABLE 64.

DEOXYRIBONUCLEASE I ACTIVITY IN CYTOPLASMIC PARTICLES OF HUMAN CERVIX UTERI

Comparison of Normal with Carcinoma

		Activity per mg. protein	Activity per g. wet weight	Activity % total cytoplasm
NORMALS	No. of tissues	7	5	6
	Means	10.4	18.0	7.9
	S.D.	3.03	9.6	4.6
	Range	1.7-29.2	2.9-27.8	1.0-19.2
CANCERS	No. of tissues	16	16	16
	Means	27.7	488	14.6
	S.D.	15.6	249	2.4
	Range	11.9-73.5	80-1154	9.1-28.2
Statistical Analysis	t	2.69	4.13	3.81
	P	$< 0.02 \rightarrow .01$	< 0.001	$< 0.005 \rightarrow .001$

TABLE 65.

DEOXYRIBONUCLEASE II ACTIVITY IN CYTOPLASMIC PARTICLES OF HUMAN CERVIX UTERI

Comparison of Normal with Carcinoma

any of the twelve parameters recorded. With the exception of DNAase II activity, the "Mitochondrial" fraction has slightly greater activity for all nucleases when measured per mg. protein; when, however, the activity is measured per g. wet weight, the "Microsomal" is the more active fraction with respect to all four enzymes, and as a consequence, this fraction accounts for a greater percentage of the total cytoplasmic activity of all four nucleases than does the "Mitochondrial" fraction. When a comparison is made between the activity of each enzyme per mg. protein of the particulate fractions and the activity of the corresponding enzyme per mg. of supernatant protein in the carcinoma series, it will be observed that the supernatant is marginally more active with respect to both ribonucleases, and twice as active with respect to DNAase II, but the DNAase I activity of the particle fractions is almost 50% higher than that of the supernatant.

Comparison of Particulate Fractions in Normal Cervix and Cervical Carcinoma

It is regrettable that the technical difficulties which emerged during the preparation of particulate fractions from non-malignant breast tissues recurred during the preparation of these fractions from the normal cervix. Much the same considerations apply to these tissues, namely, the intrinsic poverty of sub-cellular particles in these organs together with the high content of collagen which in many cases vitiated the establishment of a quantitative relationship between the yield of particles and the weight of the sample from which they were prepared. However, since the results on the two particulate fractions prepared from cervical carcinomata were so similar, and since in two of the normal samples where it was possible to separate adequate quantities of the two fractions the same similarity

was evident, it was considered justifiable for comparative purposes to pool the material obtained by differential centrifugation of the homogenates of normal cervix so that sufficient material was available for the desired biochemical analyses. Seven such "Combined Particle" fractions from normal cervix thereby became available for study. In one, the yield was considered to be unduly low because of interference from collagen; in another, because of collapse of a tube during centrifugation it was not possible to relate the yield to the wet weight of the sample but in the remaining tube, the relationship of the particles to supernatant was undisturbed. Thus it happens that in 7 samples the activity could be related to the protein content of the fraction, in 5 to the weight of the tissue, and in 6 to the total activity of the cytoplasm.

For the purposes of this comparison, it was necessary to re-calculate the data previously obtained for the individual particulate fractions of cervical carcinomata so that they now applied to the combined particles* One more sample in which the particulate fractions were combined was added to the carcinoma group. The results of this comparison are presented in Tables 62 - 65.

The first point which emerges from a scrutiny of these results is the superiority of the particulate material from cervical carcinomata over that of the material from normal cervix in respect of their content of all four nucleases. The mean activity of alk. RNAase in the "Combined Particles" of the carcinomata is ten-fold that of the normal "Combined Particles" when the activity is expressed per mg. protein and one hundred-fold when expressed per g. wet weight (Table 62); these differences are highly significant ($P < 0.005$). Furthermore, the particulate activity accounts

* An outline of this calculation is given on Page 241.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
"MITOCHONDRIAL" Fraction	No. of tissues	18	18	18
	Means	0.87	6.56	11.9
	S.D.	0.58	4.02	3.1
	Range	0.09-2.55	1.34-17.2	7.1-19.1
"MICROSOMAL" Fraction	No. of tissues	18	18	18
	Means	0.83	7.20	12.1
	S.D.	0.49	4.3	3.9
	Range	0.10-1.77	0.97-16.31	6.6-18.2

TABLE 66.

ALKALINE RIBONUCLEASE ACTIVITY IN CYTOPLASMIC PARTICLES OF HUMAN CERVIX UTERI

Comparison of "Mitochondrial" Fraction with "Microsomal" Fraction

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
"MITOCHONDRIAL" Fraction	No. of tissues	18	18	18
	Means	0.51	3.70	13.7
	S.D.	0.35	2.54	6.0
	Range	0.05-1.48	0.58-7.30	4.0-34.6
"MICROSOMAL" Fraction	No. of tissues	18	18	18
	Means	0.49	4.36	14.1
	S.D.	0.32	2.8	4.3
	Range	0.03-1.10	0.32-8.71	2.2-23.9

TABLE 67.

ACID RIBONUCLEASE ACTIVITY IN CYTOPLASMIC PARTICLES OF HUMAN CERVIX UTERI

Comparison of "Mitochondrial" Fraction with "Microsomal" Fraction

for 23.8% of the total cytoplasmic alk. RNAase activity in the carcinomata but only 7.2% in the normals ($P < 0.001$). The acid RNAase activity of the particulate material of cervical carcinomata (Table 63) is also ten times that of the normal cervical particulate fraction when the activity is expressed per mg. protein and one hundred-fold when expressed per g. wet weight ($P < 0.005$); and though the percentage of the total cytoplasmic content of this enzyme found in the particles is increased to 10.1% in the normals, the percentage of the cytoplasmic activity found in the particulate fractions of the carcinomata is also increased compared with the previous enzyme so that the difference between normals and carcinomata in this respect is still significant at the 0.1% level of probability.

The mean DNAase I content of the "Combined Particles" from cervical carcinomata is 9.91 units per mg. protein which is higher than the corresponding value for normal cervical particles, 6.99 units; the difference is not, however, significant (Table 64). When the particulate activity is related to the wet weight of tissue, the mean value for the carcinoma series is twenty times that for the normals ($P < 0.02$). A higher percentage of the total cytoplasmic activity of DNAase I of the normal cervix resides in the particles than was the case for the two ribonucleases, and although the percentage of the total cytoplasmic activity of DNAase I found in the "Combined Particles" of the carcinomata is as high as 34.2%, the difference between normals and carcinomata in this respect is not significant.

The "Combined Particles" from normal cervix gave a mean DNAase II activity of 10.4 units per mg. protein and 18.0 units per g. wet weight of tissue (Table 65). The corresponding mean activities in the

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
"MITOCHONDRIAL" Fraction	No. of tissues	15	15	15
	Means	10.23	76.2	14.5
	S.D.	12.81	86.8	9.4
	Range	0.51-49.30	9.0-338.0	0.5-38.9
"MICROSOMAL" Fraction	No. of tissues	15	15	15
	Means	10.22	103.2	20.6
	S.D.	7.26	72.4	14.5
	Range	1.44-21.8	5.5-255 .0	2.9-55.3
Statistical Analysis	t	-	0.92	1.34
	P			

TABLE 68.

DEOXYRIBONUCLEASE I ACTIVITY IN CYTOPLASMIC PARTICLES OF HUMAN CERVIX UTERI

Comparison of "Mitochondrial" Fraction with "Microsomal" Fraction

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
"MITOCHONDRIAL" Fraction	No. of tissues	15	15	15
	Means	26.8	203	6.5
	S.D.	15.5	123	2.9
	Range	9.4-62.1	35-346	2.6-11.4
"MICROSOMAL" Fraction	No. of tissues	15	15	15
	Means	28.5	285	8.1
	S.D.	17.3	193	3.9
	Range	12.8-86.2	55-760	2.4-17.6
Statistical Analysis	t	-	1.33	1.28
	P			

TABLE 69.

DEOXYRIBONUCLEASE II ACTIVITY IN CYTOPLASMIC PARTICLES OF HUMAN CERVIX UTERI

Comparison of "Mitochondrial" Fraction with "Microsomal" Fraction

carcinoma series were 27.7 units per mg. protein ($P < 0.02$) and 488 units per g. wet weight ($P < 0.001$). The percentage of total cytoplasmic DNAase II activity measured in the "Combined Particles" was twice as great in the carcinomata compared with the normals, and this difference, too, was statistically significant ($P < 0.005$).

It remains to comment briefly on the results for the enzyme activity per mg. protein found in the particulate fraction of normal cervix compared with the activity found in the supernatant of normal cervix. The situation differs somewhat from that which prevailed when this comparison was drawn for the carcinomata, for the activity of both ribonucleases in the particles is 25-50% greater than the activity of the supernatant; the DNAase I activity of the particles is more than three-fold that of the supernatant; only with respect to DNAase II is the activity of the particles inferior to that of the supernatant, and here the difference is a relatively small one.

TABLE 70.

	Highly Malignant	Intermediate Group	Moderately Malignant
No. of Cases	11	8	6
Alk. RNAase per mg. Protein	0.88	1.03	0.85
Alk. RNAase per g. wet weight	39.4	35.9	30.3
Acid RNAase per mg. Protein	0.44	0.46	0.53
Acid RNAase per g. wet weight	18.8	16.8	18.9
DNAase I per mg. Protein	5.57	10.13	6.37
DNAase I per g. wet weight	255	367	247
DNAase II per mg. Protein	63.8	48.0	38.8
DNAase II per g. wet weight	2983	2233	1643
A.D. per mg. Protein	177	199	99
A.D. per g. wet weight	6930	7757	3760

TABLE 70.

MEAN ACTIVITIES OF FIVE SUPERNATANT ENZYMES

IN CASES OF CERVICAL CARCINOMA

GROUPED ACCORDING TO DEGREE OF MALIGNANCY

Protein Content of Normal Cervix and Cervical Carcinoma

The results of estimations of the protein content of the supernatant and particulate fractions of normal cervix are presented in Table 71 . The protein content of the supernatant is of the same order as that of normal human breast tissue, and only about a quarter of the protein content of normal thyroid tissue. The protein content of the particulate fractions measured as "Combined Particles" approximates much more closely to the situation found in breast tissue than to that found in thyroid tissue, the mean of six samples being as low as 2.6 mg. protein per g. wet weight of cervix. As much as 94.4% of the total cytoplasmic protein is located in the supernatant, a result not unlike that obtained with breast tissue, and only 5.7% in the particles.

Two principal features distinguish the carcinomata with respect to protein content. The first is the fact that in the supernatant and in the particulate fractions, the yield of protein per g. wet weight of tissue is greater than in the normal cervix (Table 72). The mean content of supernatant protein per g. wet weight is 60% higher than that of the normals ($P<0.05$). It will be observed that this mean is associated with a high standard deviation; this arises largely through the contribution to the variance exercised by one specimen, the protein content of which was more than two-and-a-half times that of the next highest in the series; it is probable that failure to eradicate blood-clot from the sample contributed to this very high result. The mean protein content of the "Mitochondrial" fraction of cervical carcinomata is 8.5 mg. per g. wet weight of tissue, while the "Microsomal" fraction has a mean protein content of 9.5 mg. per g. wet weight. When the mean protein content of the

		Protein mg. per g. wet weight	Percentage of Total Cytoplasmic Protein
SUPERNATANT	No. of tissues	15	6
	Mean	28.0	94.4
	S.D.	7.5	1.1
	Range	16.4-35.7	92.5-95.5
	t	2.03	6.45
	P	< 0.05	< 0.001
"COMBINED PARTICLES"	No. of tissues	6	6
	Mean	2.6	5.7
	S.D.	2.1	1.1
	Range	0.9-6.5	4.5-7.5
	t	4.95	6.06
	P	< 0.001	< 0.001

TABLE 71.

PROTEIN CONTENT OF CYTOPLASMIC FRACTIONS

OF NORMAL HUMAN CERVIX UTERI

Statistical Results refer to Comparison with

Protein Content of Cervical Carcinomata

		Protein mg. per g. wet weight	Percentage of Total Cytoplasmic Protein
SUPERNATANT	No. of tissues	25	20
	Mean	46.3	75.2
	S.D.	34.2	7.7
	Range	13.9-184.1	56.6-88.7
"MITOCHONDRIAL" FRACTION	No. of tissues	18	18
	Mean	8.5	11.9
	S.D.	3.7	4.3
	Range	3.3-16.8	6.6-23.4
"MICROSOMAL" FRACTION	No. of tissues	18	18
	Mean	9.5	13.6
	S.D.	3.7	4.5
	Range	3.9-18.2	4.9-23.2
"COMBINED PARTICLES"	No. of tissues	19	19
	Mean	17.3	24.8
	S.D.	7.1	7.6
	Range	3.6-34.1	11.3-43.4

TABLE 72.

PROTEIN CONTENT OF CYTOPLASMIC FRACTIONS

OF CERVICAL CARCINOMATA

"Combined Particles" was calculated for comparison with the corresponding fraction of the normal cervix, the value was found to be 17.3 mg. per g. wet weight of tissue. Applying the 't' test to the data showed the particulate content of protein to be significantly higher than that of the normal cervix at the 0.1% level of probability.

The second feature which distinguishes the cervical carcinomata from the normal cervix with respect to their protein content is the relative distribution of the cytoplasmic protein between supernatant and particles; for the percentage of total cytoplasmic protein found in the supernatant is only 75.2% whereas that of the "Combined Particle" fraction is 24.8%. These differences from the mean percentages of protein incorporated into the corresponding fractions of the normal cervix are both significant at the 0.1% level. In passing, mention must be made of two aspects of the protein results for the "Combined Particle" fraction of cervical carcinomata that may at first sight be considered anomalous. The first is the low value of 3.6 quoted for the lower limit of the observed range of protein per g. wet weight of tissue, this being lower than the sum of the two lowest values for the "Mitochondrial" and "Microsomal" fractions. In fact this result was given by the sample in which, because of the low yield of particles, the two fractions were pooled prior to analysis as was the case with most of the normal samples. The other seemingly anomalous feature is the apparent discrepancy between the protein content of the two fractions per g. wet weight, and the percentage distribution of cytoplasmic protein between the two. The two sets of data may be reconciled by the knowledge that the four samples of carcinomata from which particulate fractions could not be obtained all had a content of supernatant protein

substantially below the mean for the group; when they are excluded the mean rises to 50.7 mg. protein per g. wet weight so that the ratio of supernatant protein to particulate protein 50.7:17.3 is in accord with the ratio 75.2:24.8 which reflects the relative percentage of cytoplasmic protein in the two fractions.

Effect of Radiation Upon Enzyme Content of Cervical Carcinomata

It will be recalled that a total of sixteen specimens were obtained from patients with cervical carcinomata subjected to radiation; in 13 cases, the post-radiation specimen was obtained one week after radium implantation in which the dose administered was 6,250 rads to the surface of the cervix over a 48-50 hour period. In three cases, the post-radiation specimen was taken after radium implantation followed by supervoltage therapy, the total dose in these subjects being of the order of 8,000 rads to the surface of the cervix over a four week period. The inclusion of these last three specimens in no way biases the results, since their enzyme content did not alter any more consistently in one direction than that of the samples taken from patients who had not been treated with supervoltage therapy. The advantage of including them lay in the resultant increase in numbers which rendered statistical analysis more productive of significant findings.

One feature which may appear baffling on preliminary examination of the results is the variation in the numbers upon which the various mean values are based. In explanation, it can be stated that a supernatant fraction was prepared from all 16 specimens, but in one, an accident occurring during centrifugation prevented a relationship being established

between the protein and enzyme content of the supernatant and the weight of the sample. The post-radiation samples were in general much smaller than the pre-radiation samples, so that from several it was not feasible to collect sufficient particulate material for analysis; even where a satisfactory particulate fraction was prepared, this was not always adequate for all estimations and priority was given to the assay of ribonucleases since less material was required than for the deoxyribonucleases.

From 11 samples, well-defined "Mitochondrial" and "Microsomal" fractions were derived. Three further samples gave rise to particulate fractions in which the amount of material in the separate fractions was not adequate for analysis, but when pooled, sufficient material became available. On naked eye examination of these fractions prior to pooling of the material, the "Mitochondrial" fraction was the predominant one. Consequently the data derived from the analyses carried out on the pooled material were compared with data derived by combining the two particulate fractions of the pre-radiation specimen from the same patient, and added to the results of the "Mitochondrial" fraction; thus the number of observations on the effect of radiation on the enzyme content of this fraction is 14 for the two ribonucleases. However, in calculating the percentage of the cytoplasmic activity present in the various fractions, the results in these three samples were not included with the "Mitochondrial" fraction which has for all four nucleases a number of observations on this parameter which are three fewer than the observations of activity per mg. protein. Nevertheless, as the total cytoplasmic activity was now known, it was possible to calculate the percentage distribution of protein and enzymes in the supernatant fraction of these three tissues,

a step which would not have been possible if this procedure had not been adopted.

As a general rule, it can be stated that cases of cervical carcinoma which were early or moderately advanced had a second radium insertion and therefore became available for the removal of a further biopsy specimen. Advanced cases were treated with only one insertion of radium, the balance being made up with supervoltage therapy; in addition, two cases who would normally have had two radium implants were considered unfit for two general anaesthetics within such a short period, and one implant only was administered. On the other hand not all cases coming to theatre for a second insertion were suitable for removal of a second biopsy. In some, the lesion took the form of an ulcer from which material could not easily be obtained; in others, the effect of the radium was so dramatic that there was little tumour material available; in yet another group, the vascularity of the cervix was such as to occasion brisk haemorrhage when the first biopsy was carried out, so that on ethical grounds an attempt to secure a second sample was abandoned. It follows from all this that no consistent difference in malignancy separated the group from which second samples were obtained and that from which such samples could not be obtained.

It is only to be expected that some difference in the mean values for the pre-radiation series from those given by all 25 samples of cervical carcinomata would arise. It is gratifying that these differences are quite small, many of the values actually being identical. For the purpose of evaluating the changes effected by irradiation of the tumour, the paired results on each case were treated according to the Null

TABLE 73.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
ALKALINE RIBONUCLEASE	Mean pre	0.33	38.3	74.9
	Mean post	1.28	37.4	70.9
	No. Increased	13	6	4
	No. Decreased	3	9	10
	No. Static	0	0	0
	to	2.61		2.30
	P	< 0.02->0.01	Not sig.	< 0.05->0.025
ACID RIBONUCLEASE	Mean pre	0.51	19.6	70.9
	Mean post	0.67	19.7	67.2
	No. Increased	10	6	6
	No. Decreased	5	9	7
	No. Static	1	0	1
	to	2.0		
	P	< 0.1->0.05	Not sig.	Not sig.

TABLE 73.

EFFECT OF RADIATION UPON ACTIVITY OF RIBONUCLEASES IN SUPERNATANT OF HUMAN CERVIX UTERI

Hypothesis and the value of 't' calculated. This step was omitted only where the difference between the means was so slight, or the number of samples showing a change in one direction so few as to vitiate all hope of demonstrating a significant finding.

The changes in activity of both ribonucleases in the supernatant which followed radiation of the cervical carcinomata are presented in Table 73. The mean activity of alk. RNAase per mg. protein rose by 50% in the post-radiation specimens, and in 13 out of the 16 individual specimens ($P < 0.02$). Relative to the wet weight of the samples there was however a slight fall in the mean activity of this enzyme. Although the mean percentage of the total cytoplasmic activity found in the supernatant fell by only 4%, this occurred in a sufficiently large number of the cases to render the fall statistically significant ($P < 0.05$). The mean activity of acid RNAase per mg. protein rose by one-third in the supernatant of the post-radiation group, but this rise was restricted to 10 samples and fell just short of statistical significance. Little change in the activity per g. wet weight was noted, and although the mean percentage of the cytoplasmic acid RNAase located in the supernatant fell to the same extent as did that of alk. RNAase, the number of individual cases in which a rise in the percentage was detected almost equalled the number displaying a decrease, so that no significance attaches to this result.

None of the changes recorded in the mean values for DNAase I activity after radiation (Table 74) are of any significance, since scrutiny of the behaviour of individual samples shows that the changes in each direction are nearly equal in numerical terms. With DNAase II, the

TABLE 74.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
DEOXYRIBONUCLEASE I	Mean pre	6.58	333	64.0
	Mean post	7.56	231	61.2
	No. Increased	8	6	5
	No. Decreased	7	8	6
	No. Static	1	1	1
	to		1.32	
	P	Not sig.	Not sig.	Not sig.
DEOXYRIBONUCLEASE II	Mean pre	54.2	2747	84.3
	Mean post	44.9	1541	83.1
	No. Increased	5	2	8
	No. Decreased	11	12	3
	No. Static	0	1	1
	to	0.61	2.89	
	P	Not sig.	< 0.02->.01	Not sig.

TABLE 74.

EFFECT OF RADIATION UPON ACTIVITY OF DEOXYRIBONUCLEASES IN SUPERNATANT OF HUMAN CERVIX UTERI

	Activity per mg. protein	Activity per g. wet weight
Mean pre	148.9	7659
Mean post	108.5	3729
No. Increased	6	3
No. Decreased	9	11
No. Static	1	1
to	1.36	2.73
P	Not sig.	$< 0.02 - > .01$

TABLE 75.

EFFECT OF RADIATION UPON ACTIVITY OF ADENOSINE DEAMINASE IN SUPERNATANT OF HUMAN CERVIX UTERI

activity after radiation presented several bizarre features. Although 11 of the 16 samples showed a fall in the activity per mg. protein, 5 of the samples demonstrated a not inconsiderable rise so that the value of 't_o' is quite small. When the activity was related to the weight of sample, 12 of the 16 showed a fall, and the mean post-radiation activity per g. wet weight is little more than half the mean of the pre-radiation samples ($P < 0.02$). Although the percentage of the total cytoplasmic activity of DNAase II rose in 8 of 12 samples after radiation, the mean value actually fell slightly.

The mean activity of adenosine deaminase in the supernatant per mg. protein fell by 30% in the post-radiation group as a whole (Table 75), and in 9 out of 16 samples, but this fall was not significant. On the other hand, the activity per g. wet weight is halved, the fall occurring in 11 out of 15 cases ($P < 0.02$).

All fourteen "Mitochondrial" fractions showed an increase in alk. RNAase activity per mg. protein (Table 76), the mean value for the post-radiation series being about 70% higher than that of the pre-radiation series ($P < 0.001$). The mean activity of this fraction per g. wet weight of tissue is 50% higher in the post-radiation series, ten of the 13 samples showing an increase compared with the activity of the corresponding pre-radiation specimen ($P < 0.01$). The percentage of the total cytoplasmic content of this enzyme located in the "Mitochondrial" fraction rose in 9 out of 11 cases following radiation, the mean value increasing from 12.3% to 15.3% ($P < 0.005$).

The effect of radiation upon the acid RNAase activity of the "Mitochondrial" fraction is similar to the effect reported above for alk. RNAase. Thus, there is a 75% increase in the mean activity of the

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
ALKALINE RIBONUCLEASE	Mean pre	0.87	6.56	12.3
	Mean post	1.45	9.68	15.3
	No. Increased	14	10	9
	No. Decreased	0	3	1
	No. Static	0	0	1
	to	4.16	3.07	4.06
	P	< 0.001	< 0.01->.005	< 0.005->.001
ACID RIBONUCLEASE	Mean pre	0.51	3.7	14.3
	Mean post	0..89	6.37	17.8
	No. Increased	12	9	7
	No. Decreased	2	4	3
	No. Static	0	0	1
	to	3.00	2.34	1.60
	P	< 0 .01->.005	< 0 .05->.025	< 0 .1->.05

TABLE 76.

EFFECT OF RADIATION UPON ACTIVITY OF RIBONUCLEASES IN "MITOCHONDRIAL" FRACTION OF HUMAN CERVIX UTERI

TABLE 77.

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
DEOXYRIBONUCLEASE I	Mean pre	10.2	76.2	16.0
	Mean post	10.1	77.5	24.9
	No. Increased	3	6	8
	No. Decreased	6	3	1
	No. Static	3	2	0
	to			3.04
	P	Not sig.	Not sig.	< 0.02->.01
DEOXYRIBONUCLEASE II	Mean pre	26.8	203	6.7
	Mean post	19.0	167	9.3
	No. Increased	4	1	3
	No. Decreased	8	9	4
	No. Static	0	1	2
	to	1.03	1.64	
	P	Not sig.	< 0.1->.05	Not sig.

TABLE 77.

EFFECT OF RADIATION UPON ACTIVITY

OF DEOXYRIBONUCLEASES IN "MITOCHONDRIAL" FRACTION

OF HUMAN CERVIX UTERI

enzyme in this fraction after radiation, 12 out of 14 cases showing an elevation ($P < 0.01$). The activity per g. wet weight found in this fraction also rises by about 75% following radiation, though the elevation is restricted to 9 cases out of 13 ($P < 0.05$). Although the mean percentage of total cytoplasmic acid RNAase in the "Mitochondrial" fraction rises after radiation by much the same increment as was found in this parameter for alk. RNAase, the elevation is restricted to 7 out of 11 cases and is not significant in the present series.

The content of DNAase I in the "Mitochondrial" fraction does not show much change from that found in the pre-radiation series (Table 77). Indeed the mean activities before and after radiation, whether expressed per mg. protein or per g. wet weight are remarkably close to each other. On the other hand, the share of the total cytoplasmic content of this enzyme contributed by this fraction rises by 9%, 8 out of 9 individual samples showing an elevation. The content of DNAase II in this fraction, on the other hand, shows a fall in the present series. Although the mean activity of this enzyme per mg. protein in the "Mitochondrial" fraction fell by one-third of the pre-radiation value, the effect was not significant since 4 of the 12 samples showed a not inconsiderable rise. Likewise 9 out of 11 samples showed a diminished enzyme content in this fraction per g. wet weight of tissue after radiation, but the increase in activity which occurred in one case was so great as to render the changes in the group as a whole insignificant. This tendency for a general trend to be counterbalanced by an opposite change in a minority of specimens is even more obvious when the data on the percentage distribution of the total cytoplasmic activity of DNAase II in this fraction are examined;

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
ALKALINE RIBONUCLEASE	Mean pre	0.83	7.20	12.8
	Mean post	1.37	8.87	13.8
	No. Increased	10	7	8
	No. Decreased	1	4	3
	No. Static	0	0	0
	to	3.85	0.96	0.78
	P	< 0.005->.001	Not sig.	Not sig.
ACID RIBONUCLEASE	Mean pre	0.49	4.36	14.8
	Mean post	0.87	5.78	15.0
	No. Increased	8	7	6
	No. Decreased	2	4	5
	No. Static	1	0	0
	to	2.90	1.08	
	P	< 0 .02->.01	Not sig.	Not sig.

TABLE 78.

EFFECT OF RADIATION UPON ACTIVITY

OF RIBONUCLEASES IN "MICROSOMAL" FRACTION

OF HUMAN CERVIX UTERI

		Activity per mg. protein	Activity per g. wet weight	Activity % total Cytoplasm
DEOXYRIBONUCLEASE I	Mean pre	10.2	103.0	20.0
	Mean post	7.2	38.2	13.9
	No. Increased	3	1	2
	No. Decreased	6	8	7
	No. Static	0	0	0
	to	1.30	4.31	2.84
	P	Not sig.	< 0.005->.001	< 0.025->.020
DEOXYRIBONUCLEASE II	Mean pre	28.5	285	9.0
	Mean post	23.1	155	7.6
	No. Increased	2	1	1
	No. Decreased	7	8	6
	No. Static	0	0	2
	to	1.11	3.01	0.73
	P	Not sig.	<0.02->.01	Not sig.

TABLE 79.

EFFECT OF RADIATION UPON ACTIVITY OF DEOXYRIBONUCLEASES IN "MICROSOMAL" FRACTION OF HUMAN CERVIX UTERI

although only 3 out of 9 samples show an increase in this parameter, they effectively raise the mean to a value which is 2.6% above that of the pre-radiation group.

The behaviour of the "Microsomal" fraction after radiation was in most respects qualitatively similar to that reported above for the "Mitochondrial" fraction. Thus, the mean alk. RNAase of the fraction per mg. protein (Table 78) rose by 65%, an elevation being recorded in 10 cases out of 11 ($P < 0.005$), and the acid RNAase rose by 77% of the mean activity per mg. protein of the pre-radiation series, an elevation being recorded in 8 cases out of 11 ($P < 0.02$). Although 7 out of 11 cases displayed a rise in the activity of these enzymes in the "Microsomal" fractions per g. wet weight of tissue, the mean elevations being by 23% of the pre-radiation value for alk. RNAase and by 33% of the pre-radiation value for acid RNAase, with neither enzyme was the rise statistically significant. A slight increase in the percentage of the total cytoplasmic content of both ribonucleases found in this fraction occurred after radiation, but the changes were quite variable in individual samples and fell far short of statistical significance.

The content of DNAase I in the "Microsomal" fraction, on the other hand, showed a fall after radiation, the mean activity per mg. protein falling from 10.2 to 7.2 units and in 6 out of 9 cases (Table 79), and the mean activity per g. wet weight of tissue decreased from 103.0 to 38.2 units and in 8 out of 9 cases; the latter decrease only was significant ($P < 0.005$). Likewise the percentage of total cytoplasmic DNAase I activity found in the "Microsomal" fraction dropped from 20.0% to 13.9%, this fall occurring in 7 out of 9 individual cases ($P < 0.025$). The content of DNAase II in this fraction also decreased, though not to

quite the same extent as the previous enzyme. The mean activity per mg. protein diminished by almost 20% and in 7 out of 9 individual cases, but this decrease was not significant in the present series. The mean activity of the fraction per g. wet weight of tissue diminished by 45% after radiation and in 8 out of 9 individual cases ($P < 0.02$). Although the percentage of the total cytoplasmic activity found in the "Microsomal" fraction fell in 6 out of 9 cases, the overall effect was to reduce the mean value by only 1.4%.

The effect of radiation upon the protein content of the various fractions of cervical carcinomata is shown in Tables 80 and 81. The first feature of note is the remarkable fall which took place in the protein content of the supernatant. The mean content per g. wet weight in this fraction was almost halved, a fall, usually dramatic, occurring in 13 out of 15 cases ($P < 0.02$). In 10 out of 14 cases, this resulted in a drop in the share of the total cytoplasmic protein found in the supernatant, so that the mean percentage fell by 10% ($P < 0.05$). The mean protein content of the "Mitochondrial" fraction on the other hand, rose by about 1 mg. per g. wet weight of tissue, and the percentage of the total cytoplasmic protein found in this fraction went up from a mean value of 12.4% before radiation to a mean of 18.2% after radiation, this latter result being significant at the 1% level. Little change took place in the protein content of the "Microsomal" fraction as a result of radiation. The mean protein content per g. wet weight of tissue fell from 8.9 mg. to 8.3 mg. and although the percentage of total cytoplasmic protein found in this fraction increased in 8 of 11 cases, the overall effect was to raise the mean percentage from 11.4 before radiation to

		Protein mg. per g. wet weight	Percentage of Total Cytoplasmic Protein
SUPERNATANT	Mean pre	59.9	77.2
	Mean post	32.9	67.1
	No. Increased	2	4
	No. Decreased	13	10
	No. Static	0	0
	to	2.97	2.46
	P	< 0.02	< 0.05
"MITOCHONDRIAL" FRACTION	Mean pre	8.7	12.4
	Mean post	9.6	18.2
	No. Increased	7	8
	No. Decreased	4	1
	No. Static	2	2
	to	0.62	3.90
	P	-	< 0.01

TABLE 80.

EFFECT OF RADIATION UPON PROTEIN CONTENT OF SUPERNATANT AND "MITOCHONDRIAL" FRACTION OF CERVICAL CARCINOMATA

	Protein mg. per g. wet weight	Percentage of Total Cytoplasmic Protein
Mean pre	8.9	11.4
Mean post	8.3	14.5
No. Increased	3	8
No. Decreased	5	2
No. Static	3	1
to	0.46	1.29

TABLE 81.

EFFECT OF RADIATION UPON PROTEIN CONTENT OF "MICROSOMAL" FRACTION OF CERVICAL CARCINOMATA

14.5 after radiation, and this difference was not significant.

A careful scrutiny of the data of individual cases did not reveal any consistent factors which could be correlated with the observed changes, other then the general rule that the higher the enzyme content in the pre-radiation sample, the more likely was it to demonstrate a fall after radiation, and vice-versa. There were however many exceptions to this. Neither the age of the patient, the degree of malignancy, the histological or clinical state of the disease, appeared to determine the response to radiation; nor could changes in one enzyme be correlated with changes in another enzyme except in so far as the fall in protein content of the tissue sample was likely to lower the enzyme content of the sample generally when the activity was related to the wet weight. Indeed the pattern of enzyme response was so varied, that when the data for the specific activity of the four nucleases and adenosine deaminase per mg. protein in the supernatant were arranged for each patient and marked according as to whether it rose or fell after radiation, 11 different patterns were given by the 16 patients out of a possible 32 patterns, only one of which appeared as often as thrice in the entire series.

Studies on Effect of Radiation Upon Serum and Urine Nucleases and Urine Deoxynucleotides

Samples of blood and urine were taken from 10 patients before radiation, and at various time intervals after radiation as described in Materials and Methods. The fluctuations of these components of serum and urine were quite large in the course of this work on individual patients. In general, the ribonucleases of serum and urine, and the DNAase I content of urine gave reasonably good reproducibility for the various test samples taken prior to radiation. Quite frequently, the results of these tests on three separate occasions in the same patient agreed to within 10%, and it was rare to find consecutive tests before radiation differing by more than 25%. One value for the 24-hour alk. RNAase activity of the urine of a particular patient was 75% higher than that of its companion urine collection the previous day. No greater divergence than this occurred in the pre-radiation assays for these enzymes carried out on the same patient. On the other hand, the difference in the values between particular patients prior to radiation could be three-fold. After radiation, much more scatter was evident in the results obtained over a weekly period from an individual patient. Thus, for example, it was not uncommon for two out of three specimens in a given week to yield results twice as high as those of the third specimen for one or more enzyme activities, and the lowest result given by one patient in a particular week could be as low as one-sixth of the highest value given for that enzyme in another patient for the corresponding week of therapy.

With the deoxyribonucleases of serum and the DNAase II activity of

urine, the variance within patients and between patients was greater still, primarily due to the very low values for these enzymes detected in the test material. It was far from rare for no activity whatever to be detected; yet on other occasions, sporadic elevation of activity took place. This variance was no greater after radiation than before radiation for these enzymes. The estimation of urinary deoxynucleotides was more satisfactory from the point of view of day-to-day variation shown by an individual patient, but the differences between patients during the preliminary investigations and at the same point of treatment could be eight-fold.

After careful scrutiny of the results, it was decided to present the data in the form of tables, averaging all the pre-radiation results on an individual patient to produce a single base-line value for that patient. The data on the specimens taken during and immediately after the two insertions of radium were treated as four separate results for each patient, though, of course, all four results were only available from the six patients out of the ten who underwent both radium insertions. Thereafter, all the results on a single patient were averaged for each week of the four during which they remained in hospital, so that for each parameter of enzyme activity and deoxynucleotide content there are four figures available from each patient to cover the period of supervoltage therapy, each figure in turn being the mean of two or three separate observations on that patient. This device was regarded as being entirely justifiable because of the impossibility of taking a specimen at a set time interval after radiation throughout the whole four-week period, since the dose was frequently varied from one treatment to another depending upon the therapist's estimate of progress; radiation was given on some days of the

week and not on others; the time of day on which radiation was given varied from day to day according to the organisation of the clinic; and the use of medication was required to alleviate undesirable side-effects in some patients. All these factors summated to a formidable accumulation of variables which could not possibly be controlled in the design of the experiment, for which reason a value for a particular week in a given patient based upon more than one observation in that week was a step towards obtaining a valid estimate of the patient's response at that stage in the treatment.

To summarise, there were in all ten patients, seven sets of data for the four nucleases of serum and urine, and for the deoxynucleotide content of urine, arranged thus: Pre-Radiation (Mean of two or three); First Insertion (Single Observation); First Withdrawal (Single Observation); First Week (Mean of two or three); Second Week (Mean); Third Week (Mean); Fourth Week (Mean). In six of these ten patients there were further specimens thus: Second Insertion (Single Observation); Second Withdrawal (Single Observation). The seven results thus obtained in all ten patients were subjected to a two-way analysis of variance.

The mean values for each interval of therapy are presented in the form of a table at the foot of which the results of the analysis of variance are also given. The mean values obtained for the Second Insertion and Second Withdrawal of Radium in six patients are also presented in these tables although it must be understood that they were not included in the analysis of variance. The results of all estimations carried out on urine were expressed as a concentration per ml. of urine, as a 24-hour output, and as a concentration per mg. of creatinine.

Although the purpose of the experiment was to compare the results of investigations carried out during and after the exposure of the patient to therapeutic doses of radiation with those obtained before the commencement of therapy in the same patient, a set of data was also obtained from ten healthy ambulant subjects. Six of these were females and four were males, one serum specimen and one 24-hour urine specimen being obtained under normal working conditions. This data appears in Tables 82 and 84 . The average age of the group was less than half that of the patients with cervical carcinoma. As indicated, the sex compositon was different. The dietary conditions certainly varied in the two groups; and while one group was confined to bed, the other was engaged in a normal working day. It is therefore irrelevant to compare the data in the two groups, and the results on the normal subjects are presented merely to serve as an indication of the level of activity and the degree of variance one might expect to find in a healthy mixed population.

Effect of Radiation Upon Serum Nuclease Activities

The results obtained in the ten subjects for all four nucleases are presented in Table 83 . The mean values of the two ribonucleases in the pre-therapy period are close to those of the normal group. During therapy, there were slight elevations of alk. RNAase activity, the mean value for the first week of therapy being the only one which fell below that of the control period; and all the mean values of serum acid RNAase activity during therapy exceeded that of the control period; but such differences as occurred could not be statistically related to the treatment. The very

	Mean	S.D.	Range
Alk. RNAase	1.26	0.25	0.80-1.65
Acid RNAase	0.64	0.21	0.32-1.02
DNAase I	7.2	3.3	0-11.6
DNAase II	2.3	3.1	0-7.2

TABLE 82.

RESULTS OF SERUM NUCLEASE ASSAYS

ON TEN HEALTHY SUBJECTS

All the data refer to activities
per ml. of serum

TABLE 83.

		SERUM RIBONUCLEASES ACTIVITY		SERUM DEOXYRIBONUCLEASES ACTIVITY	
		Alkaline	Acid	I	II
Deep X-Ray Therapy	Pre Therapy	1.18	0.59	3.8	1.1
	First Insertion	1.35	0.62	5.0	2.5
	First Withdrawal	1.30	0.62	2.4	0.9
	Second Insertion	1.22	0.70	4.4	2.0
	Second Withdrawal	1.18	0.62	3.0	2.0
	First Week	1.16	0.62	3.8	3.0
	Second Week	1.20	0.62	3.1	2.1
	Third Week	1.49	0.74	2.3	1.3
	Fourth Week	1.38	0.67	2.9	0.8
Variance Ratio	Between Subjects	28.89	25.03	2.8	3.3
	Between Treatments	1.63	0.91	1.8	1.53

TABLE 83.

EFFECT OF RADIUM IMPLANTATION AND DEEP X-RAY THERAPY UPON SERUM NUCLEASE ACTIVITIES

Mean Results on Ten Subjects:
For Explanation of Time Intervals and Definition of Units, See Text

All activities as units per ml.

high variance between subjects was a feature of these results.

The mean value for DNAase I in the control period was half that recorded in the group of normal subjects. A slight increase in the mean serum activity of this enzyme occurred during the insertion of radium, but none of the other mean values during therapy are higher than that of the control period. The mean value for serum DNAase II activity in the control period was also half that of the normal group. Most of the mean values for this enzyme during therapy exceeded that of the control period. The statistical analysis of the data did not reveal any significant effect of therapy upon serum deoxyribonuclease activities, although the variance between subjects was less than that encountered in the data on serum ribonucleases.

Effect of Radiation upon Urine Nucleases

The results for the excretion of nucleases in the urine of patients before and after radiation are presented as a concentration of enzyme activity per ml. of urine in Table 85, as the total enzyme output per 24-hours in Table 86, and as a concentration of enzyme activity per mg. creatinine in Table 87.

The mean concentration of alk. DNAase activity per ml. of urine is lower than that of the normal group in the pre-radiation period. With each insertion of radium, the concentration rises, and after each withdrawal of radium falls again. During the first two weeks of supervoltage therapy the mean value is close to that of the control period, but over the last two weeks an elevation occurs such that the mean for the fourth week is more than 30% higher than the basal value. This elevation is

TABLE 84.

RESULTS OF ASSAY OF NUCLEASES

AND TOTAL DEOXYNUCLEOTIDES IN URINE

OF TEN HEALTHY SUBJECTS

Units for Enzymes are those defined in text - see "Materials and Methods". Units for Deoxynucleotides equivalent to milligrammes DNA detected by Stumpf Reaction. The units have been adjusted, in some cases multiplying by 10^x, in order to facilitate comparison with the results as presented for the Radiation Series.

TABLE 84.

		Units per ml. Urine	Units per 24 hours	Units per mg. Creatinine
Alk. RNAase	Mean	3.92	451×10^{-1}	4.40
	S.D.	1.33	192×10^{-1}	1.83
	Range	1.80-5.90	$251-814 \times 10^{-1}$	2.44-8.16
Acid RNAase	Mean	2.43	267×10^{-1}	2.58
	S.D.	1.10	156×10^{-1}	1.54
	Range	0.80-4.38	$122-671 \times 10^{-1}$	1.20-6.48
DNAase I	Mean	278	338×10^{3}	342
	S.D.	20	31×10^{3}	33
	Range	244-306	$274-402 \times 10^{3}$	248-401
DNAase II	Mean	5.2	7.5×10^{3}	7.9
	S.D.	4.1	6.8×10^{3}	6.7
	Range	0-11	$0-21 \times 10^{3}$	0-23
Total Deoxynucleotides	Mean	43.5×10^{-2}	513	509×10^{-3}
	S.D.	22.3×10^{-2}	334	335×10^{-3}
	Range	$9.5-78.8 \times 10^{-2}$	145-1182	$142-1196 \times 10^{-3}$

statistically significant at the 2.5% level, and the elevation during the first radium insertion is just less than that required to reach significance at the 5% level. The mean pre-radiation value for the 24-hour output of alk. RNAase in the carcinoma group is a little over 10% lower than that of the control subjects. The output falls somewhat during the first insertion of radium and rises quite substantially after withdrawal. The mean output during the first two weeks of therapy is below that of the basal value and the output during the last two above the basal value but none of these differences are statistically significant. The mean concentration of alk. RNAase before radiation per mg. creatinine is likewise about 10% lower than that of the normal subjects. The mean value shows no change during the first radium insertion but increases by about 25% during the first withdrawal, though this increase is outside the 5% significance limit. During the period of supervoltage therapy, a steady rise in the mean takes place, the value for the third week being significantly higher than that of the basal period at the 2.5% level, and the mean for the fourth week significantly higher at the 5% level. The variance between subjects was very great when alk. RNAase activity was expressed as a concentration per ml. and very much less when measured as a 24-hour output; the variance between subjects when the results were expressed in relation to urinary creatinine was intermediate between these two.

The behaviour of urinary acid RNAase activity after radiation shared many features in common with that of alk. RNAase. To begin with, the mean pre-radiation concentration per ml. during the pre-radiation period, which was only 76% of the mean value found in the normal subjects,

		URINE RIBONUCLEASES ACTIVITY		URINE DEOXYRIBONUCLEASES ACTIVITY	
		Alkaline	Acid	I	II
Deep X-Ray Therapy	Pre Therapy	2.95	1.86	251	22.4
	First Insertion	3.79	2.68*	209	8.7
	First Withdrawal	3.08	2.06	185	8.1
	Second Insertion	3.63	2.54	228	8.0
	Second Withdrawal	2.52	1.83	193	8.2
	First Week	2.98	1.95	196	5.8
	Second Week	2.95	1.99	191	6.0
	Third Week	3.61	2.43	229	16.7
	Fourth Week	4.23*	3.00*	241	9.1
Variance Ratio	Between Subjects	10.92	9.01	3.32	1.28
	Between Treatments	2.68	2.92	1.12	0.78
	Significance	P<0.025	P<0.01	Not Significant	
	Fiducial Interval	t	t		
		0.05 0.88	0.05 0.70		
		0.025 1.02	0.01 0.93		

TABLE 85.

EFFECT OF RADIUM IMPLANTATION

AND DEEP X-RAY THERAPY UPON

URINE NUCLEASE CONCENTRATION

Mean Results on Ten Subjects: units per ml.

For Explanation of Time Intervals and

Definition of Units, See Text

* Denotes Significant Result

	URINE RIBONUCLEASES ACTIVITY		URINE DEOXYRIBONUCLEASES ACTIVITY	
	Total 24-Hour Output x 10^{-1}		Total 24-Hour Output x 10^{-3}	
	Alkaline	Acid	I	II
Pre Therapy	393	246	336	15.8
First Insertion	341	227	209*	9.5
First Withdrawal	472	302	279	13.5
Second Insertion	316	194	227	9.6
Second Withdrawal	322	204	307	8.6
First Week	357	218	277	8.1
Second Week	377	229	276	9.7
Third Week	441	258	305	11.3
Fourth Week	439	280	296	12.3
Variance Ratio: Between Subjects	4.63	4.14	17.53	3.23
Variance Ratio: Between Treatments	1.46	1.16	2.77	0.50
Significance	Not significant		P<0.025	Not sig.
Fiducial Interval			t0.025 76	

Deep X-Ray Therapy

TABLE 86.

EFFECT OF RADIUM IMPLANTATION AND DEEP X-RAY THERAPY UPON TOTAL 24-HOUR URINE NUCLEASE ACTIVITIES

Mean Results on Ten Subjects:
For Explanation of Time Intervals and Definition of Units, See Text

* Denotes Significant Result

TABLE 87.

	URINE RIBONUCLEASES ACTIVITY		URINE DEOXYRIBONUCLEASES ACTIVITY	
	Alkaline	Acid	I	II
Pre Therapy	3.87	2.40	322	14.2
First Insertion	3.80	2.48	193 *	8.6
First Withdrawal	4.86	3.07	282	12.8
Second Insertion	3.46	2.28	242	10.8
Second Withdrawal	3.38	2.24	242	8.6
Deep X-Ray Therapy: First Week	3.72	2.23	292	7.7
Second Week	4.43	2.65	270	9.3
Third Week	5.46 *	3.33 *	338	19.1
Fourth Week	5.14 *	3.34 *	314	12.0
Variance Ratio: Between Subjects	7.20	9.63	9.72	3.78
Between Treatments	2.75	2.35	5.04	0.89
Significance	P<0.025	P<0.05	P<0.001	Not sig.
Fiducial Interval	t0.05 1.196 t0.025 1.379	t0.05 9.842	t0.001 105	

TABLE 87.

EFFECT OF RADIUM IMPLANTATION

AND DEEP X-RAY THERAPY UPON

URINE NUCLEASE ACTIVITIES

Mean Results on Ten Subjects

Expressed as Units per mg. Creatinine

* Denotes Significant Result

rose by nearly 50% during the first insertion of radium and fell to the basal value after withdrawal; this pattern was repeated in the six subjects who had a second radium insertion. During supervoltage therapy, a steady elevation of activity occurred such that the mean value during the fourth week was 60% higher than the basal value. The elevation during the first insertion was significant at the 5% level, and the rise during the fourth week was significant at the 1% level. The mean output of the enzyme per 24-hours in the period prior to radiation, which was a little lower than that of the normal subjects, showed a fall during the first radium insertion and a rise upon withdrawal of the radium; the mean values during the first two weeks of supervoltage therapy were somewhat below the basal value, and during the last two weeks they were somewhat above the basal level, but none of these changes from the pre-radiation period were of significance. When the urine acid RNAase output was related to urinary creatinine, the mean for the pre-radiation period was a little lower than that of the normal subjects. There was a slight increase in this value during the first insertion of radium followed by a rise of about 25% after withdrawal. A steady elevation in the value occurred during the period of supervoltage therapy, that for the first week of therapy being somewhat below the basal value, and that for the fourth week being 140% of the basal value. The mean values for the last two weeks of therapy are indeed significantly above that of the pre-radiation period ($P < 0.05$ for both). In contrast to the previous enzyme, the variance between subjects when the activity of acid RNAase was related to urinary creatinine is somewhat higher than that found when the results were expressed merely as a concentration per ml., while the variance between subjects of the 24-hour

output of the enzyme is very much lower than both.

The mean DNAase I concentration of the urine in the carcinoma patients prior to radiation was 251 units per ml. compared with a mean of 276 units per ml. in the normal group. This was higher than any mean value during the period of treatment. The lowest mean value was recorded after the first withdrawal of radium and gradually rose during supervoltage therapy although the basal level was not quite reached. This fall during therapy was not however significant when subjected to analysis of variance. When the output was calculated per 24-hours, the same general fall was observed. More particularly, the basal value, which was close to the mean for the normal group, dropped by 40% during the first insertion and rose to 80% upon withdrawal of the radium. A similar fall followed by a rise resulted in those patients subjected to a second insertion of radium. During the four weeks of supervoltage therapy, the mean values fluctuated over a narrow range, the highest of the four means being 10% lower than the mean for the pre-radiation period. The fall during the first insertion was significant at the 2.5% level; none of the other mean values differed significantly from the basal mean. Much the same pattern was observed when the output of DNAase I was related to urinary creatinine. The mean for the pre-radiation period was about 7% lower than that of the normal group and a fall to about 60% of this value, significant at the 0.1% level, occurred during the first radium insertion, followed by a rise to almost 90% of the initial mean. In those patients who had a second insertion, the mean decreased, though less drastically, and showed no elevation upon withdrawal. During the four weeks of supervoltage therapy, the mean fluctuated to values a little above or a little below the basal mean.

The variance between subjects was very great when the enzyme activity was measured per ml. of urine, somewhat less when measured per mg. of creatinine, and least of all when measured as a 24-hour output.

The mean activity of DNAase II per ml. of urine in the pre-radiation period was four times that of the normal subjects, and very much higher than that of any subsequent period during therapy. However, it is necessary to indicate that this high mean is unrepresentative of the group as a whole, since one subject who averaged 170 units per ml. prior to radiation contributed 75% of the total activity from which the mean value is derived. From the time of the first insertion of radium onwards, the mean value for the group remained close to 8 units per ml. of urine, with the exception of the third week of supervoltage therapy, when a rise to a mean value of 16.7 units per ml. took place. When the DNAase II output was measured per 24-hours, the mean for the pre-radiation period was only twice the mean for the normal group, and though in no subsequent period was the mean excretion of DNAase II for the carcinoma patients as high as that of the basal period, the fall which took place during the first insertion of radium was not as striking as that occurring when the results are expressed simply as a concentration. Furthermore, following withdrawal of radium, and during the last two weeks of supervoltage therapy, the mean 24-hour output of DNAase II approached the value found in the pre-radiation period. A similar picture emerges when the results are expressed per mg. of creatinine. The mean activity for the period prior to radiation was about twice that of the normal subjects and was followed by a fall during the first insertion of radium. During withdrawal of the radium, and again during the last two weeks of supervoltage therapy the

mean value rose to a level close to that of the control period: during the third week the value for the control period was actually exceeded. The variance between subjects is less for this enzyme than for the other four urinary nucleases, but no significant findings emerged upon analysis of the results.

Effect of Radiation upon Urinary Deoxynucleotide Excretion

As described in the section dealing with Materials and Methods, the total urinary deoxynucleotides were measured by the method of Stumpf (1947) using an acid blank to correct for non-specific colour formation in the urine samples. The mean concentration, measured as mg. DNA per 100 ml. urine was 27.7 for the carcinoma patients prior to commencement of radiotherapy compared with the mean of 43.5 for the normal group, but this difference was not significant because of the high variance associated with both groups*. Minor fluctuations in the mean concentration took place during the early therapeutic proceedures, but from the second week of supervoltage therapy onwards, this value was above 40 mg. DNA per 100ml. The elevation was not statistically significant. The mean 24-hour output of deoxynucleotides in the carcinoma patients prior to radiation was only 75% of the mean for the normal subjects. A fall in the mean during insertion and a rise following withdrawal characterised both radium treatments. During the four weeks of deep X-ray therapy the output was higher than that which occurred in the pre-radiation period, but once again, the elevation was not statistically significant. The output of deoxynucleotides in the carcinoma patients prior to radiation relative to that of creatinine was about 75% of that occurring in the normal group. A fall

* See Tables 84 and 88.

TABLE 88.

		mg. DNA per 100 ml.	mg. DNA per 24 hours	mg. DNA per g. Creatinine
	Pre Therapy	27.7	381	388
	First Insertion	24.8	222	229 *
	First Withdrawal	30.4	429	605
	Second Insertion	25.6	208	242
	Second Withdrawal	43.0	360	370
Deep X-Ray Therapy	First Week	27.9	438	431
	Second Week	45.8	532	596
	Third Week	44.3	465	567
	Fourth Week	41.8	508	499
Variance Ratio	Between Subjects	23.0	15.6	25.0
	Between Treatments	1.50	1.67	2.31
	Significance	Not sig.	Not sig.	P<0.05
	Fiducial Interval			t0.05 253

TABLE 88.

EFFECT OF RADIUM IMPLANTATION

AND DEEP X-RAY THERAPY

UPON URINARY EXCRETION OF DEOXYNUCLEOTIDES

AS MEASURED BY THE STUMPF REACTION

Mean Results on Ten Subjects

* Denotes Significant Result

took place during the first insertion of radium followed by a dramatic rise in the mean value, and the same pattern was repeated in the six subjects who received a second radium treatment. The mean values recorded during the four weeks of deep X-ray therapy were consistently higher than the mean for the control period. However, when the results are subjected to analysis of variance, the only finding of significance is that the output per mg. creatinine during the insertion of radium is low compared with that which followed withdrawal of the radium and the last three weeks of supervoltage therapy ($P < 0.05$); but it is not significantly lower than the output of deoxynucleotides relative to creatinine which took place during the control period, which value in turn is not significantly lower than that of any of the values occurring during therapy. The variance between subjects is very high, but less so when the deoxynucleotides are measured as a 24-hour output per patient.

So far as the results on nuclease and deoxynucleotide output following radiation are concerned, it remains to add that a careful attempt was made to relate a biochemical response - either an increase or a decrease - to a clinical response. In the time that has elapsed since treatment, the radiotherapist's assessment of the general well-being of the patient and the change in size of the tumour form the only basis for such a relationship. All the patients are still alive, the longest survival being for 28 months, so that it is not possible to divide the patients into groups based upon survival. At the present time, three of the ten patients appear to have done less well than the group as a whole, as gauged by subjective and objective clinical criteria such as persistance of discharge, bleeding, pelvic discomfort and irritation, weight change, and tumour

		Group A	Entire Group
Alk. RNAase per mg. Creatinine	Third Week	107	141
	Fourth Week	114	133
Acid RNAase per mg. Creatinine	Third Week	110	139
	Fourth Week	117	139
Total Deoxynucleotides per mg. Creatinine	Third Week	110	146
	Fourth Week	83	127

TABLE 89.

The response shown by three patients (Group A) is compared with that of all ten patients undergoing radiotherapy (Entire Group). The mean value for excretion of ribonucleases and total deoxynucleotides per mg. creatinine during the third and fourth weeks of supervoltage therapy is expressed as a percentage of the mean value for that group during the basal period.

regression. Examination of the data for these subjects induced an impression that, relative to the rest of the group, there was little response in the excretion of urinary ribonucleases and deoxynucleotides. In support of this impression, the output of ribonucleases and of deoxynucleotides with respect to the output of creatinine during the last two weeks of deep X-ray therapy have been expressed as a percentage of the mean for the pre-radiation period, the relative data for these three subjects and for the entire group of 10 subjects being presented in Table 89 . It will readily be apparent that the three subjects in question produced a poor response to radiation which fell considerably below that of the group as a whole. Two of the three subjects were placed in the Highly Malignant group and one in the Moderately Malignant group according to the criteria outlined earlier in this section. The other seven subjects were classified as follows: Highly Malignant 2; Intermediate Group 2; Moderately Malignant 3.

In view of the remarkable variance between subjects in this small series, an attempt was made to discover whether such factors as age, degree of malignancy, and body weight, could explain the difference in output of urinary nucleases and deoxynucleotides with special reference to the control period. No explanation was forthcoming from these sources. Renal tract involvement and pyuria were not present in these patients, all of whom had had cystoscopic examination at the time of the first radium insertion. Among the factors that were carefully observed during the period of radiotherapy, the development of gastro-intestinal symptoms received special attention. Three patients reacted to radiation with the development of severe diarrhoea. While the concentration of nucleases and deoxynucleotides

per ml. of urine in these patients was higher than that of the remainder, the total output per 24 hours tended to fall below average because of reduced urine volume, but the output related to urinary creatinine was not consistently different from that of the group as a whole taking into consideration the stage of therapy that had been reached at the time diarrhoea developed.

DISCUSSION

DISCUSSION

Preliminary Outline

In view of the large body of data to be considered and the variety of materials to which they apply, it is desirable to outline the scheme that will be followed in discussing the results of the present work. Each of the three organs studied will be considered separately. The significant findings will be summarised, and to this end summary tables have been prepared. The nature of the enzyme activities will be discussed in relation to their cytoplasmic location. Differences between normal and pathological tissues will be examined for any relevance they might have to the disease process.

The data relating to the effects of radiation upon cervical tumours and upon nuclease levels of blood and urine will be discussed, and compared with the results of other investigators who have examined these effects in animal experiments.

In the final sections, the general conclusions to be drawn from the present work with regard to the biological role and intracellular location of the enzymes, and the level of their activities in malignant neoplasia, will be set alongside the present concensus on these points which emerges from a review of the relevant literature.

SUMMARY TABLES A - O : GENERAL LEGEND.

S	:	Supernatant Fraction
Mt	:	Mitochondrial Fraction
Mc	:	Microsomal Fraction
DNA - P	:	Deoxyribose Nucleic Acid Phosphorus
P	:	Activity per mg. Protein
WW	:	Activity per g. wet weight of tissue
a	:	Result significant at the 5% level
b	:	Result significant at the 2% level
c	:	Result significant at the 1% level
d	:	Result significant at the 0.5% level

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SUMMARY TABLE A

RESULTS FOR ALK. RNAase ACTIVITY IN THYROID TISSUES

	Activity/mg.protein			Activity/g wet wt.			Activity % Total		
	S	Mt.	Mc.	S	Mt.	Mc.	S	Mt	Mc.
Normal	0.041	0.053	0.053	4.32	0.22	0.33	88	5.0	7.1
Thyrotoxicosis	0.045	0.095	0.095	4.43	0.34	0.31	87	6.8	6.1
Carcinomas	0.070[b]	0.079	0.060	3.94	0.46[a]	0.47	82	8.8	9.0
Hashimotos	0.338[d]	0.296[d]	0.263[c]	12.75[d]	1.38[d]	1.24[c]	82	9.3	8.2
Adenomas	0.046	0.064	0.68	3.33	0.265	0.26	85	8.4	6.8

SUMMARY TABLE B

RESULTS FOR ACID RNAase ACTIVITY IN THYROID TISSUES

	Activity/mg.protein			Activity/g wet wt.			Activity % Total		
	S	Mt.	Mc.	S	Mt.	Mc.	S	Mt.	Mc.
Normal	0.031	0.043	0.037	3.51	0.18	0.19	91	4.5	4.2
Thyrotoxicosis	0.046[a]	0.066	0.072[a]	4.58[a]	0.24	0.25	90	4.7	4.8
Carcinomas	0.047	0.043	0.034	2.62	0.27	0.31	84[a]	7.3	8.9[a]
Hashimotos	0.150[b]	0.185[a]	0.172[a]	5.80	0.82[c]	0.77[c]	75[c]	13.1[c]	12.4[c]
Adenomas	0.044	0.044	0.046	3.48	0.22	0.19	90	5.6	4.7

SUMMARY TABLE C.

RESULTS FOR DNAase I ACTIVITY IN THYROID TISSUES

	Activity/mg.protein			Activity/g wet wt.			Activity % Total		
	S	Mt.	Mc.	S	Mt.	Mc.	S	Mt.	Mc.
Normal	0.66	4.14	5.90	65	16.0	23.4	62	14.8	22.9
Thyrotoxicosis	0.76	5.83[a]	6.45	76	22.0	21.2	60	19.0	19.9
Carcinomas	1.81[b]	6.81	8.44	109	43.4[a]	71.5[c]	50	18.5	31.8
Hashimotos	2.98[d]	4.73	7.90	125	29.1	45.3	61	16.9	24.3
Adenomas	0.98	5.95	7.46	84	30.8	30.5	57	20.6	22.0

SUMMARY TABLE D

RESULTS FOR DNAase II ACTIVITY IN THYROID TISSUES

	Activity/mg.protein			Activity/g wet wt.			Activity % Total		
	S	Mt.	Mc.	S	Mt.	Mc.	S	Mt.	Mc.
Normal	17.5	14.3	17.1	1758	53	60	94	3.1	3.3
Thyrotoxicosis	26.3[a]	39.2[a]	55.5[c]	2366[a]	146	193[a]	88	5.0	6.6[a]
Carcinomas	32.8[c]	25.1[a]	25.9	1885	156[d]	211[d]	83[b]	7.3[a]	9.3[d]
Hashimotos	46.7[d]	26.4	31.0	1886	172	261	86[a]	6.5[a]	8.0[a]
Adenomas	25.6	21.7	36.2	1970	111	155	88	4.9	6.8

SUMMARY TABLE E

RESULTS FOR ADENOSINE DEAMINASE ACTIVITY IN SUPERNATANT OF THYROID TISSUES

	Activity per mg. protein	Activity per g. wet wt.
Normal	14.2	1292
Thyrotoxicosis	23.0[a]	1976[a]
Carcinoma	86.8[a]	5580
Hashimotos	187.0[d]	7533[d]
Adenoma	20.8	1524

SUMMARY TABLE I

RESULTS FOR PROTEIN CONTENT OF THYROID TISSUES

	Activity per g. wet wt.			Activity % Total Cytoplasm		
	S	Mt.	Mc.	S	Mt.	Mc.
Normal	122	4.3	5.8	91	3.9	4.9
Thyrotoxicosis	106	3.6	3.3[a]	93	3.4	3.2
Carcinoma	58[c]	6.4	8.4	80[c]	8.6[a]	11.1[b]
Hashimoto	40[c]	4.6	4.6	83	8.5[a]	8.4[b]
Adenoma	85	5.2	3.8	89	6.4	4.6

SUMMARY TABLE G

RESULTS OF ENZYME ACTIVITIES (per mg. protein etc.)

IN SUPERNATANT OF THYROID TISSUES - SERIES II

Activity per mg. protein

	Normal	Thyrotoxicosis	Adenoma
Alk. RNAse	0.054	0.061	0.076
Acid RNAse	0.042	0.056	0.051
DNAse I	0.94	1.21 [c]	0.82
DNAse II	22.4	44.3 [c]	21.5
Adenosine deaminase	15.6	25.1 [a]	14.2

Activity per g. wet wt.

	Normal	Thyrotoxicosis	Adenoma
Alk. RNAse	6.13	6.66	7.49
Acid RNAse	4.77	6.23	5.27
DNAse I	106.	128. [c]	126.
DNAse II	2520.	4702. [c]	2152.
Adenosine deaminase	1736.	2617 [a]	1366.

Activity per mg. DNAP

	Normal	Thyrotoxicosis	Adenoma
Alk. RNAse	27.4	22.4	45.2 [a]
Acid RNAse	21.6	21.0	31.6
DNAse I	494	423	542
DNAse II	11865	15654	12311
Adenosine deaminase	8233	8662	7928

SUMMARY TABLE H

RESULTS OF ENZYME ACTIVITIES IN SUPERNATANT OF BREAST TISSUES

	Alk. RNAse		Acid RNAse		DNAse I		DNAse II		Deaminase	
	P	WW	P	WW	P	WW	P	WW	P	WW
Normal	0.028	0.59	0.018	0.38	2.20	47.8	8.2	176	17.9	406
Carcinoma.	0.096[b]	2.27[c]	0.064[c]	1.44[c]	2.30	56.8	39.3[b]	1075[c]	74.3[d]	2011[c]
Fibroadenoma	0.037	0.74	0.021	0.44	1.47	26.2	9.4	187	25.5	505
Cystic Mastopathy	0.041	0.63	0.013	0.37	3.98	58.0	23.6[c]	372	39.4[a]	642

SUMMARY TABLE I

COMPARISON OF ENZYME ACTIVITIES IN SUPERNATANT OF NORMAL AND CANCEROUS CERVIX UTERI

	per mg. protein		per g. wet wt.		% total	
	normal	cancer	normal	cancer	normal	cancer
Alk. RNAse	0.078	0.91[d]	2.27	35.4[d]	93	76[d]
Acid RNAse	0.033	0.51[d]	0.94	17.8[d]	90	72[c]
DNAse I	2.52	7.23[a]	72.1	291 [d]	75	66
DNAse II	11.43	51.4[d]	317	2366[d]	92	85
Deaminase	54.2	163.2[c]	1450	6417[c]	-	-

SUMMARY TABLE J

COMPARISON OF ENZYME ACTIVITIES IN COMBINED PARTICLES OF NORMAL AND CANCEROUS CERVIX UTERI

	per mg. protein		per g. wet wt.		% total	
	normal	cancer	normal	cancer	normal	cancer
Alk. RNAse	0.096	0.87[c]	0.132	13.4[c]	7.2	23.8[d]
Acid RNAse	0.050	0.49[c]	0.064	7.9[c]	10.1	27.6[d]
DNAse I	6.99	9.91	9.60	173[b]	24.8	34.2
DNAse II	10.4	27.7[b]	18.0	488[d]	7.9	14.6[c]

SUMMARY TABLE K

PROTEIN CONTENT OF CYTOPLASMIC FRACTIONS OF NORMAL AND CANCEROUS CERVIX UTERI

	mg/g. wet weight		Percentage total cytoplasm	
	Normal	Carcinoma	Normal	Carcinoma
Supernatant	28.0	46.3[a]	94.4	75.2[d]
Mitochondria	-	8.5	-	11.9
Microsomes	-	9.5	-	13.6
Combined	2.6	17.3[d]	5.7	24.8[d]

SUMMARY TABLE L

EFFECT OF RADIATION UPON ENZYME ACTIVITIES OF SUPERNATANT OF CERVICAL CARCINOMATA

		Activity per mg. protein	Activity per g. wet wt.	Activity % total cytoplasm
Alk. RNAse	before	0.83	38.3	74.9
	after	1.28[b]	37.4	70.9[a]
Acid RNAse	before	0.51	19.6	70.9
	after	0.67	19.7	67.2
DNAse I	before	6.58	333	64.0
	after	7.56	231	61.2
DNAse II	before	54.2	2747	84.3
	after	44.9	1541[b]	83.1
Adenosine deaminase	before	148.9	7659	-
	after	108.5	3729[b]	-

SUMMARY TABLE M

EFFECT OF RADIATION UPON ENZYME ACTIVITIES OF "MITOCHONDRIAL" FRACTION OF CERVICAL CARCINOMATA

		Activity per mg. protein	Activity per g. wet wt.	Activity % total cytoplasm
Alk. RNAse	before	0.87	6.56	12.3
	after	1.45[d]	9.88[c]	15.3[c]
Acid RNAse	before	0.51	3.7	14.3
	after	0.89[c]	6.37[a]	17.8
DNAse I	before	10.2	76.2	16.0
	after	10.1	76.5	24.9[b]
DNAse II	before	26.8	203	6.7
	after	19.0	167	9.3

SUMMARY TABLE N

EFFECT OF RADIATION UPON ENZYME ACTIVITIES OF "MICROSOMAL" FRACTION OF CERVICAL CARCINOMATA

		Activity per mg. protein	Activity per g. wet wt.	Activity % total cytoplasm
Alk. RNAse	before	0.83	7.20	12.8
	after	1.37[c]	8.87	13.8
Acid RNAse	before	0.49	4.36	14.8
	after	0.87[b]	5.78	15.0
DNAse I	before	10.2	103	20.0
	after	7.2	38.2[c]	13.9[a]
DNAse II	before	28.5	285	9.0
	after	23.1	155[b]	7.6

SUMMARY TABLE 0

EFFECT OF RADIATION UPON PROTEIN CONTENT OF CYTOPLASMIC FRACTIONS OF CERVICAL CARCINOMATA

		Protein mg. per g. wet wt.	Protein Percentage of total cytoplasm
Supernatant	before	59.9	77.2
	after	32.9[b]	67.1[a]
Mitochondria	before	8.7	12.4
	after	9.6	18.2[c]
Microsomes	before	8.9	11.4
	after	8.3	14.5

THYROID

Summary of the Significant Findings (Summary Tables A - G)

The alk. RNAase content of all three fractions of the gland in Hashimoto's thyroiditis is increased relative to the protein content of the fraction and to the wet weight of the tissue. Carcinoma of the gland is associated with an increased activity in the supernatant only, and even then, this increase is relative to the protein content of the supernatant and is not found when normal and carcinoma are compared on a wet weight basis. The alk. RNAase content of the organ in thyrotoxicosis and in benign neoplasia shows no significant difference from that of the normal gland by any of the criteria studied. The distribution of the enzyme is similar in all examples of thyroid tissue, and so far as one can judge from the techniques of the present study, the enzyme is predominantly present in the supernatant although it is not concentrated relative to protein in any one fraction of the cytoplasm.

Acid RNAase is increased in all three fractions of the cytoplasm in Hashimoto's thyroiditis relative to protein content. When, however, the weight of the organ is taken into consideration, the activity of the supernatant is not significantly increased over that of the normal gland whereas the particulate fractions are very much more active in this respect than those of the normal gland. It appears that a shift in activity has taken place to the extent that the particles comprise a much higher percentage of the total cytoplasmic activity of the Hashimoto tissue and the supernatant a correspondingly lesser percentage than that of the normal thyroid supernatant. Neither benign, nor malignant neoplasia alter

acid RNAase activity or distribution in the thyroid except in so far as the percentage of the cytoplasmic activity located in the "Microsomal" fraction of carcinomata is increased and that of the supernatant decreased; but thyrotoxicosis is associated with a higher content of supernatant/acid RNAase per mg. protein and per g. wet weight; the "Microsomal" fraction of the thyrotoxic cell contains a higher specific activity of this enzyme than its counterpart in the normal thyroid. The distribution of acid RNAase in the cytoplasm favours the supernatant even more than was the case with the previous enzyme, except for thyroiditis as has already been indicated.

DNAase I is very significantly increased relative to protein in the supernatant of Hashimoto tissue, but no other change which is significantly different from the normal is apparent in this condition. The activity of this enzyme is also elevated in the supernatant of thyroid carcinoma, but only relative to its protein concentration; by contrast, the activity of both particulate fractions is elevated but only relative to the weight of the tissue. The specific activity of the "Mitochondrial" fraction is elevated in the thyrotoxic gland, but this is the only change apparent in this condition. Benign neoplasms are not associated with changes in DNAase I activity. A higher percentage of the total cytoplasmic activity of this enzyme is present in the particulate fractions of thyroid in all the conditions examined than was the case with the previous two enzymes; in every instance the "Microsomal" fraction was more active relative to protein content and contained a greater share of the total cytoplasmic activity than the "Mitochondrial" fraction. The supernatant, relative to its protein content, was much less active than the particles, and yet

contained 50-60% of the total cytoplasmic activity. Only minor and insignificant changes in the distribution of this enzyme were encountered in pathological conditions of the thyroid.

In many respects, the results for DNAase II activity are the most interesting obtained in this series. In the first place, there is a pattern of increasing activity relative to protein content evident in the supernatant if one arranges the tissues in the following order: normal, benign neoplasm, hyperplasia, cancer, and thyroiditis. Significant differences evident when the activities of the fractions are measured relative to the weight of the tissue comprise elevations in toxic supernatant and both particulate fractions of carcinomata. Although the particulate fractions of all the abnormal tissues studied have a specific activity greater than that of the corresponding fraction of the normal gland, these differences are statistically significant only for those of the toxic gland and for the "Mitochondrial" fraction of carcinomata. In the normal gland and, for the most part, in all other conditions except thyrotoxicosis, the specific activity of the supernatant is higher than that of either particulate fraction. Consequently, more than 80% of the activity of the cytoplasm is present in the supernatant in all tissues; but within this general context, the relative proportions show considerable variation. In thyrotoxicosis the "Microsomal" fraction contains a higher percentage of the cytoplasmic activity than those of the normal gland, while in cancer and thyroiditis, both particulate fractions contain more than double the percentage of the cytoplasmic activity present in the comparable fractions of the normal gland, and the supernatant in both conditions contains correspondingly less.

The activity of adenosine deaminase in the supernatant of human thyroid displays a pattern of change in the pathological states examined in the present work which is remarkably similar to that found with the previous enzyme. Thus, with respect to protein content, the tissues can be arranged in the following order: normal, adenoma thyrotoxicosis, cancer, and thyroiditis. Furthermore, this same order is followed when the activity is measured relative to the weight of the tissue.

The interpretation of these findings is assisted by knowledge of the protein content of the fractions in the conditions studied. A progressive decrease in supernatant protein per g. of tissue is found in the following order: normal, toxic, adenoma, carcinoma, and thyroiditis. The only difference from normality found in the protein content of the particulate fractions arose in the "Microsomal" fraction of toxic thyroid where a significant decrease occurred. It should perhaps be mentioned that the "Mitochondrial" and "Microsomal" fractions of the carcinomata had a higher protein content per g. of tissue than the normal series, although these differences were not statistically significant. In the normal gland, more than 90% of the protein content of the cytoplasm was located in the supernatant. In carcinoma, this percentage was considerably less and was associated with a marked increase in the relative percentages of cytoplasmic protein located in the particulate fractions. In thyroiditis, the protein content of the supernatant relative to the whole cytoplasm was decreased, though this decrease was not significant in the present series; on the other hand, the increase in the percentages located in the "Mitochondrial" and "Microsomal" fractions were significantly increased.

When a smaller number of tissues were examined under the rather

different conditions employed in Series II, the only significant differences detected in the supernatant were increased DNAase II and adenosine deaminase per mg. protein and per g. wet weight, in the toxic tissues compared with the normal gland. No differences in enzyme content of the toxic tissue relative to the normal were evident when activities were expressed relative to the DNA content of the tissues. However, when this parameter of enzyme activity was employed, the alk. RNAase activity of the adenomata was significantly higher than that of both normal and toxic glands. It must be mentioned that higher values for all the enzymes were obtained in this series, and an attempt will be made to clarify these differences below.

Enzyme Inter-Relationships in Human Thyroid

a) Alk. and Acid RNAase

The nature of the enzymic degradation of RNA by the extracts used in this study at alkaline and acid pH has not been characterised. In particular, data are lacking which would permit a decision on the question whether the activities measured at the two pH values are attributable to a single enzyme. However, if the ratios of the activities in the supernatant of normal and toxic thyroid tissue are considered, it will be apparent that in this fraction, thyrotoxicosis must bring about a preferential increase in the activity of an acid-optimal RNAase while leaving an alkaline-optimal RNAase unaffected; or alternatively, the metabolic disturbance results in a shift in pH optimum of a single enzyme towards the acid side. The second explanation is less likely since such an effect has never previously been demonstrated for this enzyme; thus,

while X-irradiation causes a shift in pH-optimum of DNAase II towards the acid (Goutier-Pirotte and Thonnard, 1956; Goutier and Goutier-Pirotte, 1957) an attempt to demonstrate this possibility for RNAase yielded negative results (Eichel and Roth, 1962). The suggestion that increase in the activity of acid RNAase might be directly associated with thyrotoxicosis gains support from the finding that administration of thyroxine to the rat causes an elevation of this enzyme in liver (Reid, 1960). Alternatively, it is possible that the increase in acid RNAase in thyrotoxicosis may arise as a consequence of changes in the cell population of the organ, but this seems unlikely since similar changes in cellularity are encountered in adenomata and in thyroiditis; in neither of these conditions was any suggestion obtained that acid RNAase activity might be increased preferentially to that of alk. RNAase. Clearly, it would be of interest to ascertain whether this increase in acid RNAase activity occurs in other organs of the thyrotoxic subject, and whether it is active thyroid hormone or one of the extra-thyroid factors held responsible for the initiation of thyrotoxicosis (Adams, 1965) which stimulates activity.

The data on the thyrotoxic tissues throw further light on these relationships by revealing that the specific activity of alk. RNAase in the "Mitochondrial" fraction is more than double that of the supernatant, while the specific activity of acid RNAase in this fraction is less than 50% above that of the supernatant. Two inferences may be drawn from this observation. The first is that the RNAase activity of supernatant and of mitochondria is due to separate enzymic species at these sites, although it is probable that during the preparative procedure some cross-contamination takes place. It is unlikely that the mitochondria

would adsorb preferentially alk. RNAase to such an extent that they had a higher specific activity than the supernatant; and this could only take place if thyroid supernatant alk. and acid RNAase were distinct enzymes with quite different capacity for adsorption to mitochondria. The converse possibility - that the supernatant enzyme is derived from ruptured mitochondria - would require the disruption of more than 90% of the mitochondria during the preparative procedure. This is unlikely on three grounds: firstly, the variation between alk. and acid RNAase activity ratios in the three fractions of the different tissues; secondly, the ability of mitochondrial preparations to liberate active enzyme when subjected to ultrasonic disintegration - especially DNAase I - a finding which indicates that in the preparations obtained, preservation of membrane integrity had occurred (see Page122); thirdly, in thyroiditis, the specific activity of supernatant alk. RNAase exceeded that of the mitochondrial enzyme, and in adenomata and carcinomata the specific activity of supernatant acid RNAase equalled or exceeded that of the mitochondrial enzyme, although the presence of non-thyroid cells in these conditions must also be kept in mind.

It remains a possibility that the activities measured can be attributed to an acid RNAase confined to the supernatant, but still with considerable activity at pH 7.4 which is increased by variable contributions from release of an alk. RNAase normally confined to the mitochondria. In turn, the acid RNAase activity of these organelles might be due to activity of alk. RNAase at pH 5.6 together with adsorption of some acid RNAase from the supernatant. This interpretation would be in accord with the situation prevailing in rat liver, where the supernatant contains an

acid RNAase (Reid and Nodes, 1963) and the mitochondria an alk. RNAase (Roth, 1954 ; 1956a; 1957 ; Roth et al., 1957 ; de Lamirande et al., 1954 ; de Lamirande and Allard, 1959a). However, both RNAases have been reported in all fractions of rat liver by various groups of investigators, few of whom attempted to separate the two activities in their material, and scant attention has been given in the past to consideration of cross-contamination. A return will be made to this point when attempting to integrate the data on all tissues obtained during the present work at the close of this discussion.

Turning now to the status of RNAase activity in the "Microsomal" fraction of human thyroid, this is hard to define on the basis of the present work, as indeed is the relation between this activity and that of the supernatant. Only in the thyrotoxic gland was the specific activity of acid RNAase greater than that of alk. RNAase in this fraction, but in itself this observation requires the presence of at least two distinct RNAases in the "Microsomal" fraction. It could be that thyroid microsomes themselves have both alk. and acid RNAase as an integral component of their enzymic apparatus with the former predominating except in thyrotoxicosis where the factors responsible for raising the activity of acid RNAase in the supernatant also conspire to elevate acid RNAase of microsomes. There is abundant evidence for the existence of both enzymes in microsomal preparations of animal tissues such as rat liver (Roth, 1960 ; 1962 ; Martin et al., 1963 ; Morais and de Lamirande, 1965), mouse pancrease (Dickman and Trupin, 1958 ; Dickman and Morrill, 1959 ; Morris and Dickman, 1960), guinea-pig liver (Leslie, 1961 ; Martin et al., 1963), guinea-pig pancreas (Siekevitz and Palade, 1960), goat brain (Datta et al., 1964), HeLa cells (Leslie, 1961), as well as in microsomes

from plant tissues (Kessler and Engelberg, 1962) and yeasts (Danner and Morgan, 1963). Another possibility is that the alk. RNAase activity of the "Microsomal" fraction is due to the mitochondria it contains, while the acid RNAase activity is due to lysosomes in the preparation. With regard to the first possibility, electron microscopy revealed that some mitochondria are undoubtedly present, but their number would be quite inadequate to account for the fact that both fractions have approximately the same specific activity; thyroid microsomes must have considerable intrinsic activity. The acid RNAase activity of the "Microsomal" fraction, if attributable to its lysosome content, would require very low numbers of these organelles in all the tissues studied except toxic thyroid, since the activity at pH 5.6 falls little short in most samples of that which might be due to function of an alkaline-optimal enzyme at this pH.

It seems more likely, in view of a similar finding with the supernatant, that the increased specific activity of acid RNAase in the "Microsomal" fraction of toxic thyroid is due to the stimulation of an acid RNAase inherently present in these particles but normally present in very low concentration or in a masked state. The latency exhibited by many microsomal RNAase preparations has been conclusively demonstrated, and is indeed a biological necessity in view of the high RNA content of these particles (Dickman and Trupin, 1958 ; Dickman and Morrill, 1959 ; Roth, 1960 ; 1962).

Because of doubts whether the centrifugal field usually employed in these studies would suffice to sediment all the microsomes, and because storage of the tissues at -20° might occasion the rupture of lysosomes, the possibility must be considered whether the supernatant activity might

be associated with microsomal material which remained suspended after centrifugation for 1 hour at 35,000g, and whether this activity could arise from ruptured lysosomes. On three occasions, the supernatant after the standard differential centrifugation was subjected to 105,000g in the Spinco preparative ultracentrifuge Model E for one hour. The specific activity was unaltered by this procedure, and the translucent pellet which was obtained, contained no more than 5% of the total activity of the supernatant. The speed of centrifugation does not therefore appear to be critical. To check the effects of storage, three glands were obtained within minutes of surgical removal and were processed without delay so that the homogenate was prepared no more than half-an-hour from the time of removal. This modification did not affect the specific activity or the distribution of the enzyme in any detectable fashion.

This discussion has taken no account of the possible existence of RNAase inhibitors in the various fractions since no data bearing upon this topic were obtained. Changes in such factors should however not be entirely ignored in attempts to interpret the changes in enzyme activity which were found in this investigation, and it is probable that information concerning the existence and concentration of such inhibitors would assist in clarifying the situation.

b) DNAase I and DNAase II

It seems likely that in the cytoplasm of human thyroid two distinct enzymes are present which participate in the depolymerisation of DNA. One, which is active under conditions optimal for DNAase I activity is present in high concentration in the particles although they account for

less than 50% of the total cytoplasmic activity measured under these conditions. The other, which is active under conditions optimal for DNAase II is distributed more evenly throughout the three fractions in so far as its specific activity is concerned, with the supernatant accounting for more than 80% of the total cytoplasmic activity. The DNAase I activity of the supernatant is very low, and might almost represent vestigial DNAase II activity under the assay conditions since for the most part it is about 5% of the DNAase II activity of this fraction. An alternative possibility is that it represents leakage of enzyme from the particles. If this were so, it would represent rupture of about half the particles concerned. Since it is unlikely that DNAase II would remain completely inactive under the conditions employed for DNAaseI assay, the actual percentage of DNAaseI-containing organelles which have undergone rupture in the preparative procedure is likely to be lower than this figure. The distribution of DNAase I between the two particulate fractions favours the microsomes. If a unique intracellular site for this enzyme is sought, it is unlikely to be the microsomes, since the degree of microsomal contamination of the mitochondrial preparation observed on electron microscopy could not account for the presence of such activity as was found in this fraction. The only organelles that could be distributed between the two fractions in anything like the ratio found for their DNAase I activities are light mitochondria or lysosomes. The latter are unlikely sources of DNAase I since in most tissues their enzymic potential is restricted to acid hydrolases including DNAase II (De Duve et al., 1955; 1962; Beaufay et al., 1957). It is of course possible that both mitochondria and microsomes contain DNAase I but that

it is present in higher concentration in the latter.

One other possibility remains. If one were to make the assumption that only one DNAase is present, and that it resembles DNAase II in requirements, it is not difficult to accept the DNAase I activity in the various fractions which is so very much less than their DNAase II activities as being due to DNAase II activity under the unfavourable conditions employed for DNAase I assay. To account for the DNAase I specific activity of the supernatant being so much lower than that of the particles, one could postulate the existence in this fraction of a DNAase I inhibitor. This suggestion is quite hypothetical but at least has the virtue of being amenable to experimental verification since it should not be difficult to test for the existence of such an inhibitor, or to seek means of establishing by purification procedures whether the two DNAase activities are due to the same enzyme protein.

Turning to DNAase II, and considering for the time being the identity of the enzyme activity measured in the particle fractions, the question once more resolves itself into two alternatives: either mitochondria and microsomes contain the same enzyme; or the activity measured in these fractions derives from an organelle which is common to both. The possibility that the latter might be the lysosome is more acceptable here than it was when DNAase I was under consideration, in view of the known properties of lysosomes (see above references). It is logical to enquire whether the supernatant enzyme could also derive from breakdown of the (allegedly) fragile lysosomes. On two counts the answer must be in the negative. The first is theoretical; as many as 85-90% of the total lysosomes of the homogenate would need to be ruptured during the preparation

of the fractions to account for the activity assayed in the supernatant; indeed, in three of the five groups of thyroid samples examined (normal, carcinoma and thyroiditis) the specific activity of the supernatant exceeded that of both particle fractions. The second is practical; where homogenates were freshly prepared within 30 minutes of removal of the gland, no diminution in the DNAase II activity located in the supernatant was found.

Comparison of Results in Series I with those of Series II

The conditions employed in the preparation of the supernatant in Series II differed from those of Series I in that homogenisation was prolonged from three mimutes to five; 0.15M-KCl was used in place of 0.25M-sucrose; the concentration of the homogenate was 10% (w/v) instead of 20% (w/v); and post-mortem specimens served as normal controls in place of non-affected areas of glands removed surgically. It is therefore only to be expected that some differences between the results obtained in the two series would arise, but the magnitude of these differences is in some instancds surprising and demands explanation.

The principal changes to be expected on theoretical grounds arising from the modifications adopted in Series II are increased destruction of nuclei and cytoplasmic particles and leaching of soluble protein even from those structures maintaining their

integrity. Since inhibitors of RNAase and of DNAase tend to be more labile than the enzymes themselves (Roth, 1956a; 1962; Roth et al., 1958a; Shortman, 1961; Loiselle and Carrier, 1963; Lindberg, 1964; Zalite and Roth, 1964), an increase in the activity of the control samples might be expected if such inhibitors are normally present to any extent. It might be expected that the increased volume of the medium and the prolongation of the time of homogenisation would achieve a more homogeneous preparation which in turn would lessen one of the sources of technical error. That this may have occurred is suggested by the lower variance associated with most of the mean enzyme activities in this Series.

With these points in mind, it is interesting that the activity of DNAase I (which of all the nuclease studied is present in a relatively greater concentration in the particles than in the supernatant) is increased in Series II to a degree that can be explained on the basis of release of enzyme from particles to supernatant. As might be expected the activity of the supernatants of the three types of thyroid tissue studied in this Series does not exceed that of the whole cytoplasm as calculated by summation of the values for all three fractions in Series I. The moderate increase in adenosine deaminase activity per g. wet weight in normal and toxic tissue in Series II can be explained in the same way since, although the enzyme is almost absent from cytoplasmic particles (Schneider and

Hogeboom, 1952 ; de Lamirande et al., 1958 ; De Duve et al., 1962), considerable activity may be found in the nuclei of certain tissues (Stern, Allfrey, Mirsky and Lactren, 1952 ; Stern and Mirsky, 1953 ; Jordan et al., 1959).

With alk. and acid RNAase, the activity of the supernatant in Series II is about 20% higher than that which would be predicted on the basis of the results obtained in Series I if it were assumed that the entire cytoplasmic activity for normal and thyrotoxic tissues in this Series is liberated from cytoplasmic particles by homogenisation. Two sources for this increment might be mentioned. The first is the nuclei which undoubtedly have RNAase activity associated with histone protein components (Leslie, 1961 ; Martin et al., 1963). The second is the activation of RNAase by the KCl employed as the medium for homogenisation. No electrolyte was added to the incubation mixture in the standard assay procedure, but with the usual aliquot of supernatant, the final concentration of K^+ and of Cl^- ions in the mixture would be around 0.005M so that some activation would be expected (McDonald, 1955; Dickman et al., 1956 ; Anfinsen and White, 1961). The uniformity in percentage increase encountered in normal and toxic tissue for both RNAases is in accord with the elevation being due to a procedural change. It might profitably be mentioned at this juncture that the increased ratio of acid:alk. RNAase found in the supernatant of toxic thyroid tissue compared with normal thyroid tissue was

encountered once again in this Series.

The DNAase II activity in the normal glands studied in Series II is 40% higher, and in the case of the toxic glands 70% higher than can be accounted for by complete extraction of particulate enzyme into the supernatant on the basis of the data obtained in Series I. There is no doubt that the presence of K^+ and of Cl^- ions would have an activating effect upon DNAase II (Koerner and Sinsheimer, 1957a; Kurnick and Sandeen, 1959 ; Laskowski, 1961); the final concentration of both ions in the incubation mixture would be 0.025M. Furthermore, the presence of this enzyme in the nuclei of many tissues has been well documented, the amount comprising as much as 25% of the activity of the whole homogenate in some instances (Allfrey and Mirsky, 1952; Brown, Jacobs and Laskowski, 1952; de Lamirande et al., 1954; Roth and Hilton, 1963; Siebert, 1963; Swingle and Cole, 1964). Indeed in the Landschutz ascites tumour, the specific activity of DNAase II in the nuclei is double that of the pooled mitochondria and microsomes (Keir and Aird, 1962).

Apart from these considerations, there appears to be a disproportionate increase in the respect that the activity of the toxic tissue is raised by 70% while that of the normals has undergone a mere 40% elevation. It is very unlikely that the nuclei of thyrotoxic tissue contain so much more DNAase II than those of normal thyroid tissue that they could account for this discrepancy; nor does it seem plausible that DNAase II of toxic thyroid tissue can be subject to a greater degree of activation

by monovalent anions than its counterpart enzyme from normal thyroid tissue. Two alternatives remain. The first is that the statistical sample of five thyrotoxic glands arbitrarily chosen for this study is not representative of thyrotoxic tissue generally and that it constitutes an extreme sample from the thyrotoxic population having a higher level of DNAase II activity than the group as a whole. The second possibility is that the 'normals' in the two series are not really comparable. On the one hand, the fact that the 'normals' in the second series were post mortem specimens would entail the possibility of considerable change of enzyme activity between the time of death and collection of the specimens even though this was limited to six hours. With RNAases and DNAase I, enzymes for which the existence of inhibitors has been widely demonstrated, this would not be of such great consequence since decay of inhibitors would precede that of active enzyme and might actually result in increase in measurable nett activity (Roth, 1954; Henstell and Freedman, 1952). No such inhibitors of DNAase II have ever been identified in animal tissues although an inhibitory factor exists in human urine (Kowlessar et al., 1957). Furthermore, a study of nucleases in normal and neoplastic tissues suggested that DNAase II is a comparatively labile enzyme (Maver and Greco, 1956).

Although the stability studies described in Results: Methodological Appraisal of Enzyme Assay Methods were carried out at an early stage of this work, and only one sample of

thyrotoxic supernatant was examined before and after storage in the deep freeze with comparable results, the existence of an inhibitor of DNAase II, which is especially concentrated in the supernatant of toxic thyroid and destroyed by freezing the supernatant thereby accounting for the increase in normal and toxic supernatants in Series II with a disproportionately greater increase in the latter, cannot be entirely excluded, although such a possibility could very readily be put to the test. On the other hand, it must be recalled that the 'normal' samples in Series I were removed from glands affected by cancer and benign neoplasia although the tissue selected was not involved in the pathological process. This had the merit of allowing direct comparison between normal and abnormal tissue from the same patient; the danger is that such material may not be representative of truly normal thyroid tissue, and that elevation of DNAase II activity might be one such difference between the two. Changes in tissues not involved in the neoplastic process have been reported in the cancer-bearing host. Leakage of glycolytic enzymes from normal cells has been held responsible for the increased levels of these enzymes in the serum of cancer patients (White, 1958 ; Kabakow, Antopol, Albaum, Blinick, Ginzburg and Young, 1962). On the other hand, the activities of three enzymes concerned in glucose metabolism were increased in the livers of patients with carcinomata of the gastro-intestinal tract but without hepatic secondaries.* The levels of certain enzymes of amino acid metabolism were on the whole lower than

* (Dacha, Catterina and Fornaini, 1963)

normal in the livers of rats distant from the site of hepatoma transplants (Pitot, Peraino, Bottomley and Morris, 1963). Studies on DAB feeding have shown that, as a general rule, the activity of nucleases is increased in the liver adjacent to primary tumours, though these changes are probably related to changes in cell population and to the metabolism of the dye (Schneider et al., 1953; de Lamirande et al., 1954; Reid and Lotz, 1958; Roth, 1963). The essential theme underlying these proposals is the possibility that had the materials studied in Series I been subjected to the procedures employed in Series II, they might have displayed an increase in DNAase II more in keeping with that found for the thyrotoxic tissue in Series II. Thus the discrepancy between the results in the two Series may not be so great as would at first sight appear to be the case.

So far as the benign neoplasms studied in Series II are concerned, much of what has already been suggested in connection with the normal and toxic tissues would apply to them also. The activities of both DNAases and of adenosine deaminase do not differ greatly from the mean values obtained for similar tumours in Series I. However, RNAase activity is apparently much higher in the adenomata of Series II. It could be that liberation of enzyme from other loci in the cell and destruction of inhibitors as outlined above might account for part of this increase. Beyond this, there is little doubt that most of the discrepancy can be accounted for by sampling error. Adenomata were taken in sequence as they presented, with no

attempt to restrict the selection to a specific type. Consequently, a very wide range of histological types was present in both series so that the two are not directly comparable. Each series included one specimen from which two distinct areas were chosen for analysis. Even in these two cases, where the material was drawn from a single lesion, the differences between the activities of some enzymes were four-fold or more.

Relation Between Enzyme Change and Tissue Pathology

One of the intriguing problems raised by this work is how best to express enzyme activity so that the figure obtained will be as meaningful as possible. This is especially so in thyroid, a tissue which normally stores vast quantities of extracellular 'Colloid' - a substance consisting almost exclusively of thyroglobulin together with small amounts of other iodine-containing proteins (Rall, Robbins and Edelhoch, 1960; Shulman and Witebsky, 1960). Since the amount of 'Colloid' storage varies from gland to gland in the normal, and especially so in pathological states (Cappell, 1964), there is a grave danger that measurement of enzyme activity with refrence to protein content and to weight of the tissue will merely reflect changes in this substance which accounts for such a preponderant share of the protein content and the weight of the normal gland. Happily, this difficulty is likely to be restricted to the supernatant fraction of thyroid glands since 'Colloid' makes

no contribution to the particulate fractions except in so far as it will influence the weight of tissue upon which the activity per g. of weight is calculated. Although the presence of thyroglobulin has been demonstrated in well-washed thyroid microsomes (Roitt, Ling and Doniach, 1964), this is almost certainly due to synthesis of the protein in these organelles prior to storage in 'Colloid'.

In animal experiments, it is frequently, though not always, possible to estimate enzyme activity in a whole organ so that changes in other tissue constituents will not influence the assessment. This cannot be accomplished with most human organs, and in any case it is not applicable to conditions affecting only part of an organ - a category into which most human diseases and especially neoplasms naturally fall. The use of DNA as a reference material whereby the cellularity of a tissue preparation might be expressed is well-established (Thomson, Heagy, Hutchison and Davidson, 1953; Vendrely, 1955; Mirsky and Osawa, 1961).

Where pleomorphism, polyploidy, and multinucleated cells exist in large numbers, comparison of enzyme activity based upon DNA content of the tissue may lead to erroneous conclusions regarding the activity per cell in the tissue studied. There is no doubt that carcinomata generally have nuclear aberrations associated with a higher content of DNA per cell than the tissue of origin (Oberling and Bernhard, 1961; Le Breton and

Moule, 1961). Even in non-malignant diseases of the thyroid gland including adenomata, thyrotoxicosis and thyroiditis, multinucleated giant cells may be found in considerable numbers (Tremblay and Pearse, 1960; Oberling and Bernhard, 1961). The errors involved in employing DNA estimations as a measure of cellularity are greater for carcinomata and thyroiditis than for the other two conditions; for this reason, as well as by virtue of their rarity, it was not considered worth while to include them in that part of the study concerned with the relationship between enzyme activity and DNA content in the thyroid gland.

Again, changes in the ratio of nucleus to cytoplasm might result in an enzyme becoming highly concentrated in a cell although the actual amount per cell may be unaltered; the retention of an enzyme by a cell under circumstances where cytoplasmic volume and protein content are reduced argues for the importance of this enzyme in the overall economy and function of the cell. Whatever method of expression is chosen, or even where a combination of methods is employed, one of the supreme difficulties in the study of human tissues is the presence of several different cell types in normal samples; disease processes may bring about a distortion in the normal ratio of the various cell types without affecting their metabolic behaviour, yet the characteristics of the tissue when examined as a homogenate will have undergone a profound change. Even where abnormal

cells are present as a result of a pathological process, these may be derived from cells represented in small numbers in the normal tissue; under these circumstances, comparison of the normal tissue with the abnormal may be quite invalid. Even in experimental tumours, such as transplantable hepatomas and DAB-induced neoplasms, it is likely that the tumour arises from the bile-duct epithelium and not from the hepatic parenchymal cell in many instances (Schneider et al., 1953; de Lamirande et al., 1954; Pitot, 1960; Jones, 1963) and yet the metabolism of the tumour is compared with that of normal liver in most published reports. Further complications exist in the form of inflammatory infiltrates which may comprise more than half the cells in a given lesion and yet derive from tissues, the reticulo-endothelial and haemopoietic systems, which are utterly dissimilar to the one under study. With these points in mind, the significance of the results obtained with the thyroid samples in the present work can be examined in relation to the underlying pathology.

For most of the supernatant enzymes studied, there was a fairly general increase in specific activity relative to protein in the following order: normal, adenoma, toxic, cancer, and thyroiditis. This could be due to a progressive increase in actual intracellular enzyme protein in these conditions, or to a steady decrease in non-enzyme protein, especially protein of extracellular origin. The evidence available is very much in

favour of the latter explanation, although certain exceptions are apparent. In the first place, the tissues do in fact display a decrease in protein content very roughly corresponding with the suggested order (Table F). Secondly, this progression is for the greater part eliminated when activity is measured relative to the weight of the tissue. Thirdly, when activity is expressed in a manner designed to take account of the relative cellularity of the tissues, the differences between the normal and the toxic gland all but disappear. The significant increase in specific activities of acid RNAase, DNAase II and adenosine deaminase in the supernatant of thyrotoxic glands, as well as the significant increase in acid RNAase per g. wet weight in Series I, and the increased DNAase II and adenosine deaminase activities found relative to both weight and protein content in Series II, are most probably due to dilution of the enzymically active protein in the normal gland by large quantities of extracellular 'Colloid'. The increased ratio in the activity of acid to alk. RNAase stands as the only change in the supernatant fraction of toxic thyroid tissue which may have a connection with the pathological processes in the gland other than those resulting in 'Colloid' depletion.

The adenomata display marked heterogeneity, some being associated with myxoedema and others with hyperfunction of the thyroid; in many, cystic and degenerate areas were present; furthermore, it is a difficult matter to distinguish between

a true benign neoplasm of the thyroid and hyperplastic or hypertrophic areas of glandular tissue (Warren and Meissner, 1953). In so far as they can be considered as a class, they show no differences from the normal gland with respect to supernatant enzyme activity per mg.protein or per g. wet weight in either Series. However, the alk. RNAase activity, when related to DNA content of the tissue, was significantly higher than that of the normal gland. In view of the wide discrepancy in the RNAase activities of adenomata between the two series, it would be wise to seek confirmation of this finding before it can be accepted as a characteristic of benign neoplasia of the thyroid.

The extent to which variations in extracellular 'Colloid' could affect the activity of the particulate enzymes would most probably be limited to an effect upon the weight of the gland. Both fractions were washed once after collection, and the colour of the washings usually indicated the presence of only small amounts of contaminating supernatant in the original pellet; for this reason, a second wash was not carried out. However, the ability of the constituents of 'Colloid' to bind with cytoplasmic particles is not known. It is not to be expected that the binding capacity of the particles or of the 'Colloid' would alter sufficiently with various disease processes to introduce serious errors in the comparison of the enzyme activity of the particles by means of their protein contents; nor is it likely

that an increase in the amount of 'Colloid' trapped in the particles which might conceivably follow an increase in the 'Colloid' content of the gland could be responsible for any but minor variations in specific activity of the particles. By the standards of most mammalian endocrine tissues, the amount of protein located in these fractions in relation to the weight of the gland and to the protein content of the cytoplasm is not large, and there is no suggestion that they might be artificially raised as a consequence of contamination.

It therefore seems that the increased specific activity of acid RNAase in the microsomes, of DNAase I in the mitochondria, and of DNAase II in both mitochondria and microsomes of thyrotoxic thyroid tissue can be accepted as genuine findings. Before they can be visualised as features associated either primarily or secondarily with the metabolic effects of thyrotoxicosis, it is necessary to rule out the possibility that they might be related to drug-treatment or to changes in the activity of other endocrine glands. In this connection, the ability of azo dyes to raise the level of RNAase activity in kidney and liver should be borne in mind (Rabinovitch et al., 1961) as well as the effects of adrenalectomy and hypophysectomy upon nuclease levels in liver and kidney (Stevens and Reid, 1956; Reid and Stevens, 1958). The influence of dietary protein upon RNAase activity (Zigman and Allison, 1959) is yet

one more example of the sensitivity shown by this enzyme towards external factors, and it would not be unexpected were the enzyme level to increase in conditions associated with negative nitrogen balance.

The cytoplasmic particles of thyroid adenomata do not appear to differ in their enzymic properties from those of the normal gland.

The activity of all the enzymes studied, with the exception of acid RNAase, are significantly higher in the supernatant of the carcinomata relative to protein than those of the corresponding enzymes of the normal gland; yet no differences were evident when activity was compared on the basis of the weights of the tissues. Significant differences between the specific activities of nucleases in the particulate fractions of normal and carcinoma, except for DNAase II which was elevated in cancer mitochondria, were not apparent in this study. The activities of DNAase I and II in the "Mitochondrial" and "Microsomal" fractions of the carcinomata were significantly increased relative to the weight of the tissue. So far as the distribution of the enzymes in thyroid carcinomata is concerned, there is a shift in acid RNAase and DNAase II from supernatant to particles compared with the distribution of these enzymes in the normal gland.

For reasons which have already been given, it is difficult to interpret the changes in activity of the supernatant enzymes

in the carcinomata because the cell mass of the samples was not determined. Much of the increase in specific activity must be due to diminution in the amount of 'Colloid' in the samples which is reflected in the very low protein content of the carcinoma supernatants. It is surprising that no differences are apparent relative to the weight of the tissue, since 'Colloid' must account for a considerable proportion of the weight of the normal gland. On the other hand, carcinomata contain increased fibrous and stromal elements as well as degenerative non-cellular areas; besides, changes in weight of the nuclei would tend to limit the weight of true intracellular cytoplasm present in a given weight of intact cancer tissue. The important question whether the concentrations of these enzymes are actually increased per cell is unlikely to be answered for thyroid carcinomata until examination of pure cultures of malignant cells becomes possible. At a rough estimate, the activities of the supernatant enzymes are doubled whereas the concentration of protein is halved, so that if the protein content of normal thyroid supernatant consists approximately of intra- and extra-cellular protein on an equal basis, there is little difference between the specific activities of the various enzymes in the actual cytoplasm of the normal and malignant thyroid cell. In fact, this assumption is not altogether valid because it has been calculated that the thyroglobulin content of the thyroid approximates to

70% of the soluble protein of the gland (Rall, Robbins and Edelhoch, 1960; Shulman and Witebsky, 1960), and most of this is likely to be extracellular. Against this, one has to set the variable, but on the whole lower amounts of 'Colloid' that may be present in carcinomata. One approach towards a resolution of this problem is suggested by the fact that thyroglobulin has a sharp range of insolubility in ammonium sulphate and in potassium phosphate buffers (Derrien, Michel and Roche, 1948). Conditions permitting the maximum precipitation of thyroglobulin with the minimum loss of enzyme activity should be sought by thyroid enzymologists of the future, and might offer the most meaningful basis for comparing the activities in various types of thyroid disease.

The increased DNAase II specific activity in the "Mitochondrial" fraction of thyroid carcinomata is a significant finding which is not open to the objections applicable to the supernatant enzymes. This would appear to be genuinely associated with the thyroid cancer cell and is in accord with the increased activity of this enzyme found in the cytoplasmic particles of breast and cervical carcinomata studied in this work. Since the activity located in the "Mitochondrial" fraction is such a small percentage of that in the entire cytoplasm, this would not seem to be an important change. However the recent demonstration that mitochondria can and do contain DNA (Nass and Nass, 1963; Kalf, 1964; Luck and Reich, 1964), lends some point to this observation. The increased activity

of particulate DNAases relative to the weight of cancer tissue is probably a reflection of the increased protein content of these fractions together with the moderate increase in specific activity of the enzymes, neither increase being individually significant in this study, but when their effects are summated as they are when the activity of a given fraction is expressed relative to the weight of the tissue, quite striking increases are found. Certainly, there is little support here for the view, derived from study of animal tumours, that malignancy is necessarily associated with loss of formed particles from the cytoplasm (Allard, de Lamirande and Cantero, 1953 ; Fiala, 1953 ; 1961 ; Schneider et al., 1953 ; de Lamirande and Allard, 1959a; Fiala and Fiala, 1959). The shift in activity of acid RNAase and of DNAase II so that the percentage found in the supernatant is decreased whereas that found in the particles is increased also conflicts with the situation found in rat liver tumours (de Lamirande, Allard and Cantero, 1954 ; Allard et al., 1957 ; Reid and Lotz, 1958 ; Roth et al., 1964).

In almost every respect examined, the samples of thyroiditis contain higher enzyme activities than the normal tissue. The specific activity of DNAase II is increased three-fold in the supernatant, while for the other four enzymes studied the increases were five- to ten-fold or more in this fraction. The protein content of the supernatant in this condition relative to the weight of tissue is only a third of the value for the

normal gland. The increased enzyme activity, with the possible exception of DNAase II, is thus much greater than can be accounted for on the bads of loss of extracellular 'Colloid' and the conclusion is inescapable that these changes are the result of increased concentration of these enzymes in the cytoplasm of the cells in thyroiditis; this must be especially so with alk. RNAase and adenosine deaminase, dramatic increases of which were recorded when the activity was measured relative to the weight of the tissue. The increased specific activity of alk. and acid RNAase in both particulate fractions is also very striking and can be described as a definite characteristic of the cells present in the thyroid in thyroiditis; even though the protein content per g. of tissue of these fractions is somewhat lower than the corresponding value for normal thyroid the activity of the fractions relative to the weight of tissue is 3-4 times that of the normal gland. No significant changes in the activity of DNAases were found in the particle fractions of thyroiditis tissue although they show a fairly general tendency to be higher than the normal. Like the samples of carcinomata, thyroiditis tissue contains a higher percentage of the cytoplasmic protein in the particulate fractions, and the tissue is similar to the carcinomata in having a higher percentage of the cytoplasmic acid RNAase and DNAase II in these fractions. This is in harmony with histological findings which indicate the presence of mitochondrion-rich cells in the

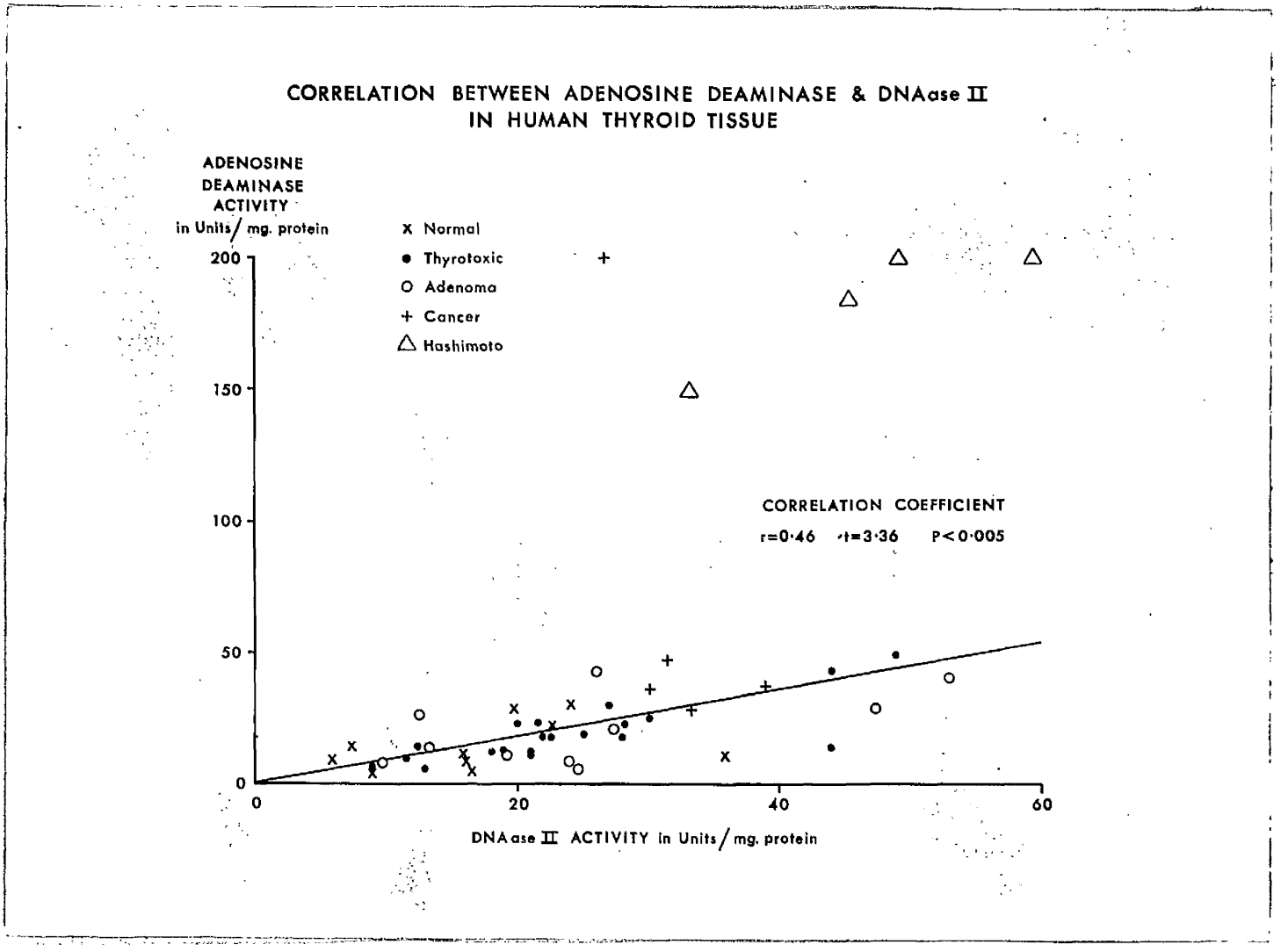

FIGURE 26

CORRELATION BETWEEN SPECIFIC ACTIVITIES OF DNAase 11 AND OF ADENOSINE DEAMINASE IN SUPERNATANT OF HUMAN THYROID TISSUES

thyroid in Hashimoto's thyroiditis (Tremblay and Pearse, 1960; Irvine and Muir, 1963).

One of the most interesting features of thyroiditis tissue, and in this respect it differs from the carcinomata as a group, is the fact that RNAases and adenosine deaminase show greatly increased activity whereas DNAases are increased to a very much lesser extent. An aspect of the data which made an impression when the results on individual cases were scrutinised very carefully was the fact that increased specific activity of DNAase II was generally accompanied by increased specific activity of adenosine deaminase in the supernatant fraction, except in thyroiditis and the highly anaplastic carcinoma. This relationship was analysed in the form of a graph where the two activities are plotted for each tissue (Figure 26).

All the points were grouped in a manner suggesting a linear relationship except for the four samples of Hashimoto's thyroiditis and the anaplastic carcinoma. The correlation coefficient $r = 0.46$ was calculated excluding these five points and was highly significant ($t=3.36$; $p=0.005$). Two points arise from this relationship. Firstly, it would appear that a common factor is operating to increase the specific activity of both enzymes; this could be either a progressive loss of extracellular protein or a specific concentration of both enzymes in the cytoplasm arising from the pathological changes in the tissues as previously discussed. The second is the possibility that those samples not falling on the line are populated by

cells not deriving from the epithelium of the thyroid. On other grounds, particularly in their high content of mitochondria and microsomes, there is reason to suspect that a very large number of cells present in Hashimoto's thyroiditis are not related to thyroid epithelium, and such a view is in accord with the findings of investigators who have examined this question from a histological standpoint (Parmley and Hellwig, 1946; Irvine and Muir, 1963). It is tempting to suggest that this relationship might assist in distinguishing between anaplastic tumours derived from thyroid epithelium, and tumours of the lymphoma group which are derived from lymphoid tissue. These tumours cannot readily be distinguished by histological criteria, yet on prognostic grounds it is important that the distinction be made (Warren and Meissner, 1953).

BREAST

Summary of Significant Findings

With the exception of DNAase I, all five enzymes studied were elevated in the supernatant fraction of human breast carcinoma relative to the activities in normal breast tissue whether activity was referred to the protein content of the fraction or to the weight of the sample. In a group of samples from cases of cystic mastopathy, the specific activities of DNAase II and of adenosine deaminase were increased in the supernatant relative to the normal tissue. The activities of both RNAases and of adenosine deaminase relative to both weight and protein content were increased significantly in the carcinoma group above the levels reached in the supernatant fraction from the cystic mastopathy samples; the content of DNAase II relative to the weight of tissue was higher in the carcinoma group than in mastopathy; but such differences as were found in activity of DNAase I in the two conditions were not remarkable. While the level of activity of these enzymes tended for the most part to be elevated in fibroadenomata compared with normal tissue, these were not significant in any instance (Table H).

The Nature of the Enzyme Activity

By and large, the ratio of acid to alk. RNAase activity in the supernatant of human breast is fairly constant

irrespective of the nature of the tissue, although the ratio for tissues in the mastopathy group is perhaps somewhat lower than that of the other groups; at levels of activity as low as 0.13 units per mg. protein, the mean acid RNAase activity for the mastopathy group, the assay method is not sufficiently precise to permit of proper evaluation. The limitations involved in obtaining data on cytoplasmic particles from breast tissue have been fully described; clearly, such scanty data must be interpreted with caution. Nevertheless, if one compares the ratio of acid to alk. RNAase in the cytoplasm with that in the particles, there is some evidence for a relative concentration of the acid RNAase in the former. Thus, in the non-malignant tissues, the ratio is 1:2 in the supernatant and 1:3 in the particles; in the malignant tissues, the corresponding ratios are 2:3 and 2:5 respectively (Tables H, 53, 54). Very tentatively, it may be suggested that these results are compatible with the presence of an acid RNAase in the cytoplasm and an alk. RNAase in the mitochondria. The specific activity of alk. RNAase in the particles of the non-malignant tissues is of the same order as that in the supernatant, while in the malignant tissues, the particles have somewhat higher specific activity than that of the supernatant. With acid RNAase, on the other hand, the specific activity of the particles is considerably below that of the supernatant in the non-malignant tissues, and a little below in the "Microsomal" fraction of

the malignant tissues - the "Mitochondrial" fraction of the latter having specific activity of acid RNAase approximately equal to that found in the supernatant. These observations, limited as they are to a few samples, would support the distribution suggested above. In view of the repeated freezing and thawing to which the tissues were subjected during the preparation of the homogenate, it is unlikely that many mitochondria could have survived intact, and it would not be surprising if the bulk of mitochondrial enzymes were liberated into the supernatant. This would also apply to lysosomal enzymes, so that if such structures are present in human breast, it is conceivable that the acid RNAase activity measured in the supernatant in fact derives from this source. Evidence for the location of acid RNAase in lysosomes of rat mammary gland has been presented (Greenbaum et al., 1960; Slater, 1961).

As was the case with thyroid tissue, it would appear that the distribution of the two DNAases between the cytoplasmic fractions is dissimilar, although this observation is based upon only 7 individual samples of "Mitochondrial" and "Microsomal" fractions in the entire series. The specific activity of DNAase I in these fractions, was greater than that of the corresponding supernatant while the specific activity of DNAase II was invariably less. The low DNAase I activity of the supernatant could conceivably arise from activity of DNAase II under the unfavourable conditions of

assay whereas this is less credible for the particles because of the increased activity of the former enzyme and the decreased activity of the latter. There are thus at least two distinct DNAases in the homogenate; an enzyme akin to DNAase I which is concentrated in the particle fractions, presumably in light mitochondria which would probably be distributed between both "Mitochondrial" and "Microsomal" fractions as obtained in these studies; and an enzyme akin to DNAase II which is concentrated in the supernatant but could be lysosomal in origin. The rather different behaviour shown by alk. RNAase and by DNAase I would suggest that if they are present in the same particles, then the former enzyme might be located in the sap and the latter in the membrane or cristae, in order to account for the relatively higher concentration of alk. RNAase in the supernatant. Other factors which would account for the observed behaviour could be differences in the levels of inhibitors of the two enzymes in the supernatant, or in its intrinsic content of the two enzymes. Until techniques become available for the disruption of breast tissue by bland procedures which do not lead to destruction of the cytoplasmic particles, this problem is likely to remain unsolved.

The data for the protein content of human breast (Tables 55 and 56) show values which are well below the levels recorded for the various fractions of thyroid tissue. This is especially so with the supernatant. It is clear that collagen and other insoluble proteins must constitute the

bulk of the proteins present in breast tissue. Although the high fat content of this tissue would cause a reduction of the protein content when measured relative to the weight of the sample, this is only one factor accounting for the reduction. Failure to achieve complete disruption of the cells is another possibility which perhaps ought to have been checked histologically. But it is the author's impression that the greater part of the insoluble residue precipitated by spinning at 500g for 10 min. consisted of dense collagen fibres, and it is likely that in all samples fibrous tissue cells outnumbered acinar cells quite considerably. Because of all these problems, extensive trapping of cytoplasmic particles must have occurred. For this reason the results for particulate enzyme activity and protein content per g. wet weight are not worthy of serious comment although the specific activities of the enzymes in these fractions are probably unaffected, and remain valid.

Relation Between Enzyme Change and Tissue Pathology

For all the enzymes studied, the breast tissues showed a general increase in supernatant activity relative to protein content of the fraction and weight of the tissue in the following order: normal, fibroadenoma, mastopathy, and carcinoma. One or two minor exceptions, in truth, can be found; for example, the activity of acid RNAase is below the normal level in the mastopathy group while the DNAase I activity of this tissue

is higher than that of the carcinomata. But these deviations from the overall trend are relatively trivial and do not detract from its validity. It will be recalled that a similar pattern of specific activity was for the most part obtained with the various types of thyroid lesions, so that in the two tissues, nucleic acid degrading enzymes were increased in the following relative order: normal, benign neoplasm, hyperplasia, and malignant neoplasm. However, the picture in thyroid was confused by the reduction in content of extracellular enzymically inactive protein occurring in these pathological states.

No such explanation is possible for the breast, since, in the non-lactating organ, almost all the protein will be intracellular. On the other hand, one difficulty in interpreting the results lies in the fact that at least two distinct cell types are present in large amounts in human breast. The characteristics of the acinar cells are almost certainly quite dissimilar to those of the cell constituents of fibrous and connective tissue. Although fatty tissue visible to the naked eye was removed, some fat cells would undoubtedly persist and represent a third cell type. It is certain that, in the samples of carcinomata, the proportion of cells of acinar origin would be greatly increased. On the other hand, this would not be expected of fibroadenomata and mastopathy where proliferation of both elements takes place, and while variations from case to case might occur, the ratio between the two would not differ to any great extent from the normal when represent-

ative groups are examined. Even if a preponderance of inert fibrous tissue were present, the resistance of this material to disruption would result in its exerting little influence upon the specific activities measured in the cytoplasmic fractions studied in this work. If differences, sufficiently great to influence the specific activity, between the ratio of acinar to non-acinar cells had existed in these tissues, then this should have been reflected in the protein content of the supernatants, tissues with an unusually high percentage of acinar cells containing more supernatant protein per unit weight. In fact, no significant differences were found in this respect.* The mean protein content of the supernatant in the fibroadenomata and mastopathy groups was actually somewhat lower than that of the normal samples, while the carcinomata had a mean protein content only 25% higher than that of the normals. In thyroid, the increased activities found in the supernatant fraction of the carcinomata compared with normal thyroid when protein content was the parameter against which activity was measured were eliminated when the comparison was based upon the weight of the tissues. In the present series of breast samples, the superiority of carcinomata over the normal is actually enhanced when comparison is made on the basis of tissue weight, although the percentage increase in the activity of the carcinomata over the normal is fairly constant by the two methods of expression. The range of divergence was as follows: DNAase I increased in carcinomata by 104% relative

* except that mastopathy group lower than normals (Page 154)

to protein and by 119% relative to tissue weight; adenosine deaminase increased by 479% relative to protein content and by 611% relative to tissue weight.

It would thus appear to be indisputable that the supernatant activities measured are a true reflection of the activity found in acinar breast tissue in the various conditions studied and that they give an accurate picture of changes in the enzymic composition of the cells in these conditions. Where obtained, data on cytoplasmic particles supported the trend observed in the comparison of activities in the malignant and non-malignant supernatants. The view is therefore advanced that a progressive increase in the activity of nucleases and of adenosine deaminase in the human acinar breast cell takes place during the transition from the normal state to hyperplastic and neoplastic states, this increase being greatest when the stage of invasive carcinoma is attained. No opinion can be expressed on the question whether these are primary or secondary features of such changes. The present results are completely opposed to those reported by Daoust and Amano (1963) using histochemical techniques. The methods employed by these authors must be open to criticism, and this conflict should be settled by further independent investigators in this field.

A correlation between the activities of DNAase II and adenosine deaminase in the supernatant of the various thyroid samples has already been described. This possibility was

examined in the samples of human breast. The data yielded a correlation coefficient $r = 0.38$ which was not significant ($t = 1.74$). To a large extent, the failure of this correlation to show significance was due to the relatively small number of samples examined, since a smaller value for r was obtained in a similar analysis of the cervix data, and yet was significant at the 5% level. However, it is probably also true to say that heterogeneity in the cell type present in the samples, as has already been explained, would contribute to the failure to show a significant correlation between these two enzymes.

CERVIX

Summary of the Significant Findings

Summary of the Significant Findings. s studied were significantly elevated in the supernatant fraction of cervical carcinomata, no matter whether they were related to the protein content of the fraction or to the weight of the tissue (Table I). The activities of both RNAases and of DNAase II in the cytoplasmic particles prepared from cervical carcinomata were greatly in excess of those found in particles prepared from samples of normal cervix, (Table J). The distribution of these same enzymes was altered in the carcinomata to the extent that the particles accounted for a higher percentage of the total cytoplasmic activity than was the case with the normal cervix. So far as enzyme content was concerned no significant differences between the "Mitochondrial" and "Microsomal" fractions of the carcinomata could be detected. The protein content of the supernatant and particulate fractions of the carcinomata exceeded the values for the corresponding fractions of the normal cervix, and a greater percentage of the cytoplasmic protein of the carcinomata was present in the particles (Table K).

Irradiation of the carcinomata brought about significant increase in the supernatant activity of a lk. RNAase relative to protein, and significant decrease in DNAase II and adenosine deaminase in this fraction relative to the weight of the tissue.

In the "Mitochondrial" fraction, both RNAases were increased after irradiation irrespective of the method of expression. In the "Microsomal" fraction, irradiation increased the activity of both RNAases per mg. protein and decreased the activity of both DNAases per g. wet weight. A dramatic fall in protein content of the supernatant followed irradiation together with a fall in the percentage of cytoplasmic protein in this fraction; this was accompanied by a rise in the percentage of cytoplasmic protein in the "Mitochondrial" fraction. Some changes in the distribution of enzyme activity between the fractions occurred as a consequence of irradiation, and may be seen by reference to Tables L, M, N and O.

The Nature of the Enzyme Activity

In all fractions of normal and malignant samples, the activity of acid RNAase was almost invariably a little over half the value found for alk. RNAase. Again, when the results in the various fractions of the carcinomata are compared before and after radiation, it will be seen that, as a broad approximation, the activity of both RNAases are increased by the same percentage in each fraction. There is little to suggest that two distinct enzymes are responsible for the activities measured at the two pH values in human cervix.

The situation is quite the reverse with DNAase I and II. As in the other tissues studied, the data point to at least two

enzymes being present, their distribution varying in the supernatant and in the particles. Thus, the specific activity of DNAase I in the supernatant is less than that of the particles. The specific activity of DNAase II in the supernatant on the other hand exceeds that of the particles. Again, radiation appears to affect the activities of these enzymes in different ways. For example, in the supernatant fraction of the carcinomata, the specific activity of DNAase I is slightly increased while that of DNAase II is moderately decreased by radiation. As was the case with both thyroid and breast, DNAase I is distributed between the supernatant and particles in such a way that while the greater part of the cytoplasmic activity is present in the former fraction, the specific activity of the latter fractions exceed that of the supernatant; and DNAase II is distributed with even more of the cytoplasmic activity located in the supernatant, the specific activity of which exceeds that of the particle fractions. As before, the smallest number of distinct enzymes which can account for these facts would be a mitochondrial DNAase I distributed between the two particle fractions and liberated into the supernatant by widespread rupture, a state of affairs which might not be unexpected in view of the harsh treatment meted out to the tissue during the preparative procedure; and a DNAase II present in a different particle, possibly belonging to the lysosomes, more fragile than the mitochondria so that much more of the enzyme

becomes solubilised, and forming only a minority of the particulate material in the "Mitochondrial" and "Microsomal" fractions. Until it becomes possible to prepare fractions from human cervix by mild procedures, it will remain an open question whether this interpretation is correct, or whether each of the fractions contains its own intrinsic complement of these enzymes.

Differences in Enzyme Content of Normal and Malignant Cervix Uteri

The activities of all five enzymes studied were significantly elevated in the supernatant of cervical carcinomata relative to both the protein content of the fraction and weight of the sample (Table 1). These differences between normal and carcinomata are in some instances so great as to suggest the possibility of their being employed for the purpose of cancer diagnosis. With DNAase I, the results in the two groups overlap to a considerable extent; and with adenosine deaminase the cancer series is weighted by several samples having activity well beyond the range for the group as a whole (See Figures 24 and 25). With DNAase II, the range of co-incidence between the normal and cancer groups is very limited, but it is arguable whether the degree of separation is sufficiently precise to permit its formulation for the basis of a diagnostic test. With the two RNAases, the separation between the two groups is well-nigh complete. As it is not by any means certain that the activity is due to two distinct enzymes, it would seem more

appropriate to concentrate upon the activity at pH 7.4 in view of the fact that the activity at this pH is much greater than that at pH 5.6 and would lend itself to more precise measurement.

In recent years, the enzymic activity of vaginal fluid has been proposed as the basis for a series of tests designed for screening large populations with a view to detecting pre-clinical cases of cervical carcinoma. The enzymes which have been studied in this regard include β-glucuronidase (Odell and Burt, 1950; Lawson, 1959), α-mannosidase (Lawson, 1960) and 6-phosphogluconate dehydrogenase (Bonham and Gibbs, 1962; Bonham, 1964). Automated methods have become available for measuring this latter enzyme (Cameron and Husain, 1965) but earlier reports claiming that the technique gives no false-negative results and only a handful of false positives have not been confirmed (Muir, Canti and Williams, 1964). In view of the degree of skill that must be lavished upon the time-consuming techniques of exfoliative cytology, there is an urgent need for a simple laboratory procedure which can be carried out on a very large scale, preferably using automated techniques. There can be little doubt on the basis of the results presented in this study that alk. RNAase activity of vaginal fluid ought to be evaluated for this purpose. The mean activity in the supernatant fraction of the carcinomata per mg. protein was more than ten times the mean value for the normal samples, while the mean activity of the carcinomata per g. wet weight was 15 times the mean for the normals. In a parallel study on the

case material employed in this work, the activity of 6-phosphogluconate dehydrogenase in the carcinomata was elevated three-fold per mg. protein and five-fold per g. wet weight of tissue compared with the normal samples (Ayre, 1965). There are thus a priori grounds for the belief that alk. RNAase activity of vaginal fluid might offer a better basis for cancer screening than any of the enzymes evaluated hitherto. It would clearly be unwise to take too optimistic a view at this stage, since the samples of carcinomata were all fairly well established and at the present there are no data to indicate whether the elevated RNAase content of these tumours is early or late, primary or secondary. Furthermore, no systematic attempt has been made to ascertain the effect of non-malignant lesions upon the cervix uteri. However, two points can be made in this connection. The first is that the so-called "normal" samples were not by any means free of changes such as epithelialisation, proliferation of secretory glands, and inflammatory infiltration such as would be expected of non-malignant cervical lesions like chronic cervicitis and cervical erosion. Secondly, a sample obtained from a case diagnosed as carcinoma on the basis of exfoliative cytology but subsequently ammended to a diagnosis of florid chronic cervicitis was actually analysed, although the results have not previously been included in the Tables. In this sample, the activity of both RNAases fell below the levels recorded in any of the carcinomata, being well within the range encountered in the normals. Adenosine deaminase

activity in this sample was at the upper limit of the normal range, and DNAase II activity fractionally above the highest level recorded in a normal sample. An additional point which is relevant to this discussion in view of the fact that several samples in the "normal" series showed increased keratinisation and epithelialisation is this: normal human skin, which functionally and histologically is very close to the surface tissue of the cervix uteri, has activity of nucleic acid-splitting enzymes present in the epidermis and not in the dermis (Santoianni and Rothman, 1961); moreover, lesions such as psoriasis which are associated with increased keratinisation are accompanied by considerable increases in nuclease activity (Steigleder and Raab, 1962 ; Liss and Lever, 1962).

The question arises whether the results obtained reflect genuine differences between the enzyme content of cervical carcinomata and the lining epithelium of normal cervix from which the carcinomata are presumed to originate. The parallel here is with breast rather than with thyroid. While it is unlikely that intracellular enzymes will be diluted to any extent with soluble extracellular protein, the samples of normal cervix inevitably included a fair amount of underlying fibrous tissue, whereas in the tumour samples there was proportionately much less contamination from this source. Fortunately, in the homogeniser, the fibrous material seemed to escape disruption so that it was either trapped when the homogenate was filtered,

or precipitated with the nuclei during the preliminary centrifugation at 500g. It is therefore unlikely to have contributed either protein or enzyme activity to the fractions analysed in amounts that would invalidate the results obtained. The extent of this contribution can be calculated very roughly from the fact that the protein content of the supernatant per g. wet weight of tissue in the normal samples was only half that of the carcinomata. If we assume that fibrous contamination in the carcinomata was negligible, we can hazard a guess that in the normal samples, epithelium was contaminated with approximately its own weight of fibrous tissue. Even though this calculation is only very approximate, it does indicate that contamination from this source cannot explain the very large differences in enzyme activity (three-fold to fifteen-fold) between the carcinomata and the normals. It is felt that these elevations are meaningful in terms of tumour development, though it is impossible to say whether these are secondary changes brought about as a consequence of more fundamental alterations in tumour metabolism.

One other finding should be mentioned at this point. A study of the relationship between DNAase II and adenosine deaminase in the supernatant of normal and malignant samples of cervical tissue produced a result for the correlation coefficient $r = 0.322$ ($t = 2.11$; $p < 0.05$). Although this result was significant, the degree of correlation was clearly less than that found with breast and thyroid. This could be

explained either as a feature of the cervical epithelial cells themselves, or as an expression of variability in the contribution of the epithelium and stroma to the soluble protein of the homogenate.

Because of difficulty in obtaining mitochondria and microsomes from normal cervix in sufficient yield, the particles were for the most part combined and compared with data derived by re-calculation of the activities measured in the "Mitochondrial" and "Microsomal" fractions of the carcinomata. For this purpose, the activities of each enzyme in both fractions of each carcinoma measured per g. of tissue were added, and divided by the sum of the protein content of both fractions per g. of tissue. The results were thus strictly comparable in both series and showed values for the specific activities of all four nucleases in the carcinomata, with the exception of DNAase I, which were significantly higher than the values obtained in the normal samples (Table J). It is hard to see how these results can be interpreted other than as fundamental differences between carcinomata and their tissue of origin, though this does not mean that they are necessarily involved in the mechanism of malignant transformation. The data relating to differences in particulate activity based upon weight of the sample and to the percentage of the total cytoplasmic activity present in the particles are much more questionable, as are the data giving the protein content of the particles per g. of tissue, since comparison is valid

only if it can be established that the yield of particles was quantitative, or represented the same percentage of the total in both normal and carcinoma samples. Such was certainly not the case. It is probable that many mitochondria and microsomes became enmeshed in the network of collagen fibres and were dragged down during the first centrifugation of the normal samples at 500g. Because less collagen was present in the samples of carcinomata, particles are very unlikely to have been lost to anything approaching the same extent. An additional factor which has to be taken into consideration is the likelihood that little particulate material is present in the fibrous tissue which, as has been estimated, might account for half the weight of the normal samples. The mean protein content of the combined particles was 2.6 mg. per g. wet weight for the normals and 17.3 mg. per g. wet weight for the carcinomata. Even if allowance is made for 50% of the weight of the normal samples being non-epithelial, there is still a difference of more than three-fold between the normals and the carcinomata. It cannot be stated with any certainty whether all of this difference is due to differences in the recovery of the particles, or whether it reflects, in addition, the presence of cytoplasmic particles in larger numbers in cervical carcinomata than in the normal cervix. However, the present findings are supported by the results of an electron-microscope study of cervical carcinomata which left the authors in no

doubt that relative to the normal cervix, the carcinomata have many more mitochondria per cell; furthermore, the typical cancer mitochondrion was larger than its counterpart in the normal cell (Luibel, Sanders and Ashworth, 1960). It can at least be said with some measure of confidence that there is little evidence here to support the view derived from studies on animal tumours that malignancy is necessarily associated with diminished content of mitochondria and of endoplasmic reticulum (Allard, de Lamirande and Cantero, 1953; Fiala, 1953; 1961; Schneider et al., 1953; de Lamirande and Allard, 1959a; Fiala and Fiala, 1959).

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a) Effects Upon the Tumour

Effects of Radiation upon Patients with Cervical Carcinomata

a) Effects Upon the Tumour

The most consistent change seen in this work was the decrease in supernatant protein after radiation, the mean content per g. wet weight of tissue dropping from 59.9 mg. to 32.9 mg. Decrease in protein content of the tissues is a fairly general effect of ionising radiation (Holmes, 1957), and is frequently accompanied by evidence of increased protein catabolism. Swelling of the cells surviving therapeutic radiation of human cervical carcinomata is an established histological observation (Sugimori, 1963 ; Koga, Yamada and Tanaku, 1963), and it is known that profound shifts in water and electrolytes accompany irradiation of the rat intestine (Baker and Mitchell, 1963). Accumulation of water in the

tissues could thus be another factor responsible for the lower protein content per unit weight of tissue encountered after radiation in the present work. The finding that the 'Mitochondrial' and 'Microsomal' fractions are for all practical purposes unaffected is somewhat unexpected, since it has been suggested by several workers that solubilisation of particle-bound protein is one of the sequelae of tissue irradiation and could account for increase in supernatant enzyme activities which they observed (Roth, 1956b; Okada and Peachey, 1957; Roth and Eichel, 1958; Roth and Hilton, 1963). However, a clear-cut demonstration of particulate destruction by ionising radiations is lacking. Much attention has been paid to lysosomes in this respect. From a study of the effect of whole-body radiation upon the activity of several hydrolases reputedly associated with lysosomes, no uniform change could be demonstrated, and it was concluded that radiation-sensitivity is restricted to a relatively small number of these structures containing certain enzymes, such as RNAase, in very high concentration (Roth, Bukovsky and Eichel, 1962). Other experiments have shown that, while release of enzymes from lysosomes seems to occur after irradiation of the whole animal or tissue slices, direct irradiation of homogenates or relatively pure lysosomal preparations induces no such change, and it has been suggested, with a certain amount of supporting experimental evidence, that hormonal factors and not irradiation per se are responsible for these changes (Rahman, 1963; Sottocasa, Glass and de Bernard,

1965). The present results, based as they are upon techniques which are not wholly satisfactory for the quantitative preparation of cytoplasmic particles, do not suggest that loss of particulate enzymes into the supernatant occurs with therapeutic dœs of radiation in man. As a consequence of the fall in supernatant protein unaccompanied by any change in the protein content of the particles, the former, expressed as a percentage of the total cytoplasmic protein, falls significantly, while the latter rises - significantly in the case of the "Mitochondrial" fraction. A similar fall in the supernatant enzyme activities expressed as a percentage of those for the whole cytoplasm also follows from this situation, though the fall in this parameter is significant only with alk. RNAase.

A rise in the specific activity of both RNAases takes place in every fraction; this elevation is highly significant in every instance with the exception of acid RNAase activity of the supernatant. When the enzyme activities of the various fractions are related to the weight of the tissue, little change is observed in the supernatant, a significant elevation occurs for both RNAases in the "Mitochondrial" fraction, and both are moderately elevated in the "Microsomal" fraction. These changes are difficult to interpret. Since the specific activity of the supernatant rises whereas the total activity does not, the simplest explanation is that the decrease in protein content of this fraction is accompanied by a selective retention of the enzyme within the cell. Increased liberation from particle-

bound enzyme cannot be a factor, since in that event the activity of these fractions would be diminished. Nor does it appear that destruction of inhibitors can be the sole explanation since this would be expected to lead to an increase in the total RNAase of the fraction as well as to an increase in its specific activity. It is of course possible that RNAase is not selectively retained but that a combination of events including general protein loss and specific destruction of RNAase inhibitor could account for the twin facts of increased specific activity and unchanged total activity. The point could very readily be settled by assay for RNAase inhibitors in the samples before and after radiation. In so far as the particles are concerned, a genuine increase in the activity of both RNAases appears to have taken place. This could be due to destruction of inhibitors, but it is well to mention that those RNAase inhibitors which have been characterised so far appear to be concentrated in the supernatant (Roth, 1956a; Roth et al., 1958a), although binding of the inhibitors to microsomes occurs to a certain extent (Dickman and Trupin, 1958; Dickman and Morrill, 1959; Roth, 1960; 1962). It could of course be that normally a considerable amount of inhibitor is bound or adsorbed by the particles, this being destroyed by radiation. Again, this possibility can be tested experimentally. Two other possibilities can be mentioned at this stage, namely, profound changes in the cell population with retention or influx of cells with different enzymic properties from those present before radiation,

though it is unlikely that such cells could be normal constituents of cervical epithelium in view of the low content of RNAases found in such tissue; and de novo synthesis of enzyme. The time interval of one week elapsing before radiation and collection of the post-radiation sample was such as would permit both possibilities.

The present findings, indicating a general increase in RNAase specific activity subsequent to tissue irradiation, are more in accord with events occurring in radio-sensitive tissues such as spleen and thymus where activity is increased (Weymouth, 1958; Maor and Alexander, 1963) than in radio-resistant tissues such as liver where activity is variable but on the whole decreased (Roth et al., 1953; Roth, 1956b). On the other hand, the shift of activity from particles to supernatant which has been reported to follow irradiation of the spleen (Roth and Eichel, 1958) does not seem to occur in human cervical carcinomata.

When activities are related to the weight of the tissue, DNAase II is decreased significantly in the supernatant and both DNAases are significantly diminished in the "Microsomal" fraction. There would appear to be no specific effect of ionising radiation upon DNAase I activity, since the activity of the supernatant is diminished pari passu with the loss of soluble protein from this fraction, and such changes as there are in the particles are more in keeping with a redistribution

of the activity between the "Mitochondrial" and "Microsomal" fractions. Thus the percentage of the total cytoplasmic activity of this enzyme present in the former is significantly increased, while the percentage present in the latter fraction is significantly decreased. On the assumption that the activity in both fractions is due to their complement of light mitochondria, it is possible that ionising radiation brings about a subtle change in the size and sedimentation properties of these particles, or causes aggregation and clumping to take place. In every fraction, the activity of DNAase II per mg. protein is diminished. This must stand as suggesting a specific lesion caused by ionising radiation in this tissue, although it must be made quite clear that the reduction was not significant in any fraction. Even were this finding established on a statistically sound basis, it would not follow that this reduction in activity is in any way connected with the initial and primary effects of radiation. It may simply be a manifestation of reduced protein synthesis with failure of enzyme renewal, or it may be a reflection of changes in cell population with reduction in the number of cells endowed with high DNAase II activity.

The reports of those investigators who have examined the effect of whole-body irradiation upon the DNAase I levels of tissues are somewhat contradictory; all agree however that such changes as occur are small, with a tendency for liver to

show somewhat increased activity and spleen somewhat decreased activity (Douglass et al., 1954 ; Fellas et al., 1954 ; Kurnick et al., 1958). The present findings occupy a position midway between the above, since supernatant specific activity is marginally increased, and particulate activity taken as a whole somewhat decreased. On the other hand, it is agreed almost with unanimity that whereas DNAase II activity of liver and kidney is unchanged by irradiation, the activity of the spleen and thymus is greatly increased (Douglass et al., 1954 ; Fellas et al., 1954 ; Douglass and Day, 1955 ; Okada et al., 1957 ; 1958 ; Weymouth, 1958 ; Kurnick et al., 1958 ; 1959). No such finding was obtained in the present study. The change which took place was in the direction of decreased activity; and while this was not significant when measured relative to tissue protein content, it was highly significant when measured relative to the weight of the sample, although the more general changes likely to be responsible for this phenomenon have earlier been discussed. It is well to remember that the nature of the radiation, which was strictly localized by means of refined techniques designed specifically for this very purpose; the dosage, which was far below that employed in the animal studies cited and administered over a much longer period; and the time interval at which the post-radiation specimen was examined, were all so very different from the corresponding factors which characterised previous published work in this

field, that little surprise attaches to the discrepancies noted. Added to this, it is very probable that the tissues of the human cervix uteri are naturally radio-resistant, since they have virtually no morphological features in common with those tissues such as spleen, thymus and bone-marrow which are the arch-types of radio-sensitive tissues. In addition, it is probable that the response to radiation would show variability in the different tumours, and until a prospective study on the present case material separates those showing a favourable response from the non-responders, it cannot be ascertained whether the changes reported are a cause, a result, or an indirect reflection of tumour ablation, and whether they offer any prognostic indication to the therapist concerned with assessing the progress of the patient.

It is possible nevertheless to reconcile the results of the present work with those reported from animal investigations. It was suggested by Weymouth (1958), and subsequently demonstated by histochemical techniques (Aldridge et al., 1960), that increased specific activity of DNAase II in lymphoid tissues is due to loss of radio-sensitive cells poor in the enzyme with accumulation of radio-resistant cells possessed of a high content of DNAase II. This view has, moreover, been confirmed by Kurnick et al., (1960). It has been conclusively shown in the present investigations that the cells which make up the cervical carcinoma are richer in DNAase II than the

cells of the normal cervix. The effect of radiation upon the cervix will bring about death of the sensitive cancer cells while leaving the insensitive stromal elements unimpaired. This will induce a dimimution of enzyme activity by a population change which is the inverse of that occurring in spleen and thymus.

The activity of adenosine deaminase was reduced in the supernatant relative to both protein content of the fraction and weight of the tissue, only the latter reduction being statistically significant, and almost certainly a result of non-specific loss of soluble protein from the fraction. The only published works relating to the effect of radiation upon this enzyme have shown it to be increased in spleen though only relative to the protein content of the organ (Eichel and Roth, 1960 ; Roth et al., 1964), unchanged in liver (Eichel, 1955), and decreased in thymus (Roth et al., 1964). Since a correlation between these enzymes has been established in this work, the population changes suggested above to be responsible for diminished DNAase II activity are likely to lie at the root of the fall in adenosine deaminase content of the cervical carcinomata subsequent to irradiation.

b) Effect of Ionising Radiation Upon Serum Nucleases

The first point to emerge from this study was the fact that no differences were apparent between the serum levels of all four nucleases in the cases of cervical carcinoma and those found in a small series of healthy normal subject (Tables 82 and 83). In truth, the levels of both DNAases are somewhat below those of the normal subjects as is to be expected from the reports of Wroblewski and Bodansky (1950), of Kurnick (1953) and of Gavosto et al., (1959), while the level of RNAase activity is almost identical in the two groups, in agreement with the findings of several investigators (Metais and Mandel, 1955 ; Houck and Berman, 1958 ; Levy and Rottino, 1960) but in contrast with the reports of Migliarese (1958a; 1958b). The present study differs from those which formed the basis of the above publications in that cases were restricted to one type of cancer only, and a proper control group was not selected.

It should be pointed out that the level of RNAase activity in the serum at both acid and alkaline pH is very much greater than the level of DNAases. Not only are the units of DNAase activity presented in Table 82 multiplied by a factor of 10^3 with respect to those describing RNAase activity, but the acid-soluble material formed by action of the latter enzyme was estimated after an incubation of 30 min. whereas with the former enzyme the incubation period was 4 hours; no allowance for this difference in time of incubation was made in defining

the units employed in this work (Materials and Methods, Page 91). It is noteworthy that Gupta and Herriott (1963) found that the activity of RNAase was 100 times that of DNAase in human blood serum. In view of the very low results obtained for DNAase I estimations in human serum, several samples of blood drawn from dogs and from rabbits were analysed with results that demonstrated activity 25-50 times as high as that usually encountered in human serum; this is in agreement with the results of Kurnick (1953) who found serum DNAase I activity to be lower in man than in any other of a large series of mammals investigated. The DNAase II activity of serum measured in the present work was so low as to be undetectable in many instances. Kowlessar et al. (1955) found a similar situation to obtain in the normal rat. Although Gavosto et al. (1959) claimed to measure significant activity of this enzyme in human serum, their method of assay ommitted versene from the incubation mixture, and it is probable that inhibition of DNAase I was incomplete under these conditions.

The administration of therapeutic radiation to the ten subjects was without significant effect upon the level of nucleases in the group as a whole. Although clear evidence for the existence in serum of an RNAase with acid pH optimum could not be obtained (Page 110), the assay was carried out at pH 5.6 in all specimens so that if ionising radiations brought about a release of acid RNAase from tissues rich in this enzyme, the effect would not be missed.

A brief comment to the effect that serum RNAase activity tends to fall subsequent to therapeutic radiation appears in the reports of Metais and Mandel (1955) and of Migliarese (1958b). No details regarding either the subjects studied or the results obtained were included by these authors, and no mention was made of statistical analysis, so that it is not possible to make a critical evaluation of their findings. It was reported by Kowlessar et al. (1955) that large increases in the activities of DNAase I and DNAase II occurred in the plasma of rats exposed to ionising radiation over a period extending from 18 hours to 6 days after radiation; unfortunately they did not specify the dose, but if it was in line with that employed in parallel experiments by the same group of investigators, it would have been 700r administered as whole body radiation. No doubt, differences in dose, in the form of administration, and in the species irradiated adequately account for the failure to observe similar changes subsequent to therapeutic radiation in the human subjects studied during the present work. The principal fact to emerge is that the estimation of serum nucleases do not offer any guidance as to the reaction of a patient subjected to localized pelvic irradiation.

c) Effect of Ionising Radiation Upon Urine Nucleases

At the outset a serious difficulty was encountered in deciding how best to express urinary enzyme activity. From

many points of view, describing activity as a concentration is unsatisfactory, since wide fluctuations in water excretion occur from patient to patient, and in the same patient from time to time and from day to day. The most satisfactory measure is the total 24-hour output, but there were certain difficulties here also. The most troublesome is the problem in the female subject of avoiding loss of urine during bowel movement. Some of the patients had attacks of sharp diarrhoea during part of their treatment; under these circumstances, it would have been surprising if losses had not occurred; and yet intelligent subjects were not always able to say whether involuntary micturition had taken place, and if so, to what extent this was likely to interfere with the validity of the 24-hour urine collection. Where diarrhoea was pronounced and serious losses admitted, the collection was not included, but where losses were said to be minor, the evidence of the patient was accepted, since it is almost certain that patients with normal bowel movements will also lose some urine during evacuation. Special difficulty was encountered in obtaining collections from patients during radium implantation. During this period, the patient usually feels off-colour and tends not to drink; minor degrees of dehydration are common with a consequent reduction of urine flow-rate. Added to this, the pack which is placed in the vaginal vault commonly presses on the bladder and may even constrict the urethra; while these mechanical problems are not important clinically since they remit as soon as the pack

is with-drawn, they may seriously lower the excretion of urine which is pooled in a distended bladder above the level of the radium pack. Finally, in this particular series, the majority of the patients were resident at one hospital (The Royal Beatson) and travelled to another hospital (The Western Infirmary) for supervoltage treatment. Delays frequently occurred in transport or in therapy so that the patient on occasions spent several hours away from the base hospital. Although they were instructed to bring the winchester used for the collection with them on each visit to the treatment centre, it was ascertained by the investigator that this was not always done. A desire not to offend must be a potent factor in inducing a patient to withhold the information that a collection is faulty. For all these reasons, while a 24-hour urine collection is the ideal, it is not always that it can be obtained in practise, and worse still, it is not always that the investigator is made aware of anomalies in the collection. Catheterisation was carried out where permission was obtained, but on ethical grounds many clinicians object to this procedure in view of the risk of urinary infection it entails. *

To overcome the limitations attaching to measurement of the concentration of a constituent in the urine and the difficulty of obtaining complete 24-hour specimens, many investigators have chosen to express their results in relation to the excretion of urinary creatinine, which is little affected by diet, exercise, or variation in urine volume but remains constant for a given

* Only during radium implantation

individual in health and is determined chiefly by his muscle mass (Peters and Van Slyke, 1946). In company with other nitrogenous constituents, the excretion of creatinine is increased following whole-body radiation (Haberland, Schreier, Bruns, Altman and Hempelman, 1955; Holmes, 1957). The effect of localized tumour radiation upon urinary creatinine output is however not known. Even if it were increased it might be expected that this would prove to be an effect of general tissue irradiation against which a specific anti-tumour effect might profitably be measured. Thus, it was argued that an increased excretion of nucleases, which have been shown to be present in higher concentration in cervical carcinomata than in the normal tissue, might take place relative to the excretion of creatinine which would be present in much higher concentration in uterine and pelvic muscle tissue than in the carcinoma. If this were so, it would provide an index of tumour dissolution which could then be evaluated against the actual response as assessed clinically. Two possible fallacies in this reasoning must however be borne in mind. The first is the assumption, and it is far from established, that urinary nucleases are derived from systemic sources by renal filtration rather than from the lower renal tract itself. The second is the premise that creatinine and the nucleases are handled in an identical manner by the renal excretory apparatus.

Regarding the first assumption, a considerable amount of circumstantial evidence is available in the case of ribonucleases,

and it is almost certain that urinary alk. RNAase activity is derived in some measure by renal excretion of RNAase in the systemic body fluids. In the first place, serum RNAase activity increases after bilateral nephrectomy in the rat (Rabinovitch and Dohi, 1956) and high values are found in the serum of uraemic human patients (Rabinovitch, Liberman and Fauto, 1959 ; Connolly et al., 1962). Experimental evidence from work in rats and dogs points to a mechanism whereby the enzyme is filtered at the renal glomerulus and partially re-absorbed by the tubules (Rabinovitch, 1959 ; Dohi et al., 1959). Both acid and alk. RNAases have been identified in human urine (Hakim, 1959a; 1959b; Hakim and Pappas, 1959). More recently, two enzymes have been purified from human urine by Delaney (1963); one has been shown to resemble pancreatic RNAase and the other splenic RNAase. If both of these enzymes reach the urine by means of filtration from the blood, it is surprising that definite evidence for an acid-optimal RNAase in human serum has not yet been obtained. Although as in the case of serum, the identity of a separate RNAase with an acid-optimal pH in normal human urine was not demonstrated conclusively in the early methodological work, it was still considered worth while to carry out the assay at pH 5.6 so as not to miss a rise in this component if preferential release of acid RNAase from damaged tissues happened to take place as a consequence of therapeutic radiation.

One or two comments are in order regarding the levels of nuclease activity found in the urine of human subjects in the

course of the present work. The activity of both RNAases was increased in urine compared with serum, alk. RNAase by a factor of three and acid RNAase by a factor of about four. In the only direct comparison between serum and urine of which the author is aware, Levy and Rottino stated that the RNAase activity of human urine is considerably greater than that of serum though they do not report actual values for urine RNAase activity in their paper (Levy and Rottino, 1959). Earlier, Houck (1958) had found the activity of urine RNAase to be higher than that of serum in a single patient studied. An even greater increase in DNAase activity over the levels found in serum is evident in human urine, this increase being of the order of fifty-fold for DNAase I. The increase in DNAase II activity is nothing like so great. Indeed, the disparity between the two enzymes raises the possibility that DNAase II activity of urine as measured in the present work really derives from DNAase I under unfavourable conditions of pH and magnesium concentration. Hakim (1959) has already reported that the activity of urinary DNAase I greatly exceeds that of DNAase II. On the other hand, enzymes akin to DNAase I and DNAase II have been purified from human urine by ammonium sulphate fractionation (Koszalka et al., 1954). It is probable that DNAase II activity in urine is not capable of expression unless it is separated from the specific inhibitor identified by Kowlessar et al. (1957). The opinion of the present author on the status of urinary DNAases will become clear when the results of this work are

interpreted.

Comparison of the values obtained in the group of carcinoma patients with those found in a group of healthy subjects revealed no significant differences between the two series. (Tables 84 - 7). Aleksandrowicz et al. (1958) claimed to detect elevated levels of RNAase measured at pH 5.6 in acetate buffer in the urines of patients with chronic granulocytic leukaemia. Hakim (1959) reported that DNAase activity was increased in the urines of early cancer subjects, but low levels were found in advanced cases.

The concentration of both RNAases rose after radium implantation, significantly so in the case of acid RNAase. A return to base-line concentrations accompanied the first two weeks of supervoltage therapy, but in the last two weeks, the concentration rose once again to significantly elevated levels. No significant changes were found in the 24-hour output of these enzymes, but the excretion of both relative to that of creatinine rose significantly in the last two weeks of therapy.

The most likely explanation for the increase in concentration of urinary RNAases during the last two weeks of therapy, which was accompanied by increased excretion relative to creatinine output but not relative to the total 24-hour volume, would be destruction of RNAase inhibitors in the urine with no actual increase in enzyme protein. It has already been established that the tumour tissue does not lose RNAase during radium implantation. Whether this enzyme is lost from the tumour

during the later stages of supervoltage therapy has not been conclusively decided, but it is highly unlikely in view of the data obtained with three subjects in whom the second insertion followed supervoltage therapy, thereby offering an opportunity for a second biopsy at that stage of treatment. The results in these patients were in line with those obtained in the remaining subjects, that is to say an increase in the specific activity of RNAase was accompanied by no change in the content of these enzymes in the tumour per g. wet weight. Conclusive evidence for the existence of two distinct RNAases corresponding to the properties of pancreatic and splenic RNAases has been presented (Delaney, 1963). On the other hand, while both RNAases followed the same pattern in the urine as a consequence of radiotherapy, those RNAase inhibitors which have been characterised in mammalian tissues seem to be specific for alk. RNAase. If destruction of inhibitors is the reason for the changes observed, then there must be a different type of inhibitor in human urine which inactivates both enzymes, or there must be a separate inhibitor for each. It has been fully established that RNAase inhibitors are fairly labile compounds (Roth et al., 1958a; Shortman, 1961; Roth, 1962). and their destruction by radiation is very likely. On the other hand, in view of the time interval involved, it is feasible that the inhibitors are reduced in the urine more by virtue of interference with their renewal than as a consequence of their physical destruction.

In a general way, the changes in urinary RNAases show a resemblance to those encountered in the carcinoma itself following radiation, where the concentration of the enzymes in the tissue was significantly raised per mg. protein whereas the total amount per g. wet weight was unaltered.

DNAase II activity, which was in any case so low in the urines examined as to be absent entirely in many instances, showed no significant change in any respect. But DNAase I output per 24-hours and per mg. creatinine was lowered during the implantation of radium.

It is not an easy matter to interpret these findings.
It is not an easy matter to interpret these findings.

One thing that seems clear is that the excretion of urinary DNAases has no connection whatever with the corresponding enzymes in the tumour tissue. In the tumour, the level of DNAase II is 10 times that of DNAase I, whereas in the urine the level of DNAase II is less than one-twentieth that of DNAase I. The reduced output of urinary DNAase I during radium implantation would appear to be a consequence of three possible factors. The first could be a direct destruction by radiation of the urinary enzyme which may be more sensitive to radiation damage than the corresponding tumour enzyme. The second could be an increase in the excretion of DNAase I inhibitors from damaged tissue; urinary DNAases have been shown to exist in the form of complexes with other urinary constituents thereby masking their activity (Hakim, 1959c; 1959d) while an inhibitor of DNAase II in urine has been reported (Kowlessar

et al., 1957). In any case, both these possibilities could be tested experimentally. The third possibility is not so amenable to an experimental approach; it is that, on the unlikely assumption that urinary DNAases are derived from body tissues and excreted by the kidney, the clearance of the enzyme is reduced by radiation either as a consequence of alteration to the physico-chemical properties of the enzyme, or as a result of minimal damage to the renal excretory apparatus involved - either tubular or glomerular. Thus, while the clearance of small molecules such as creatinine is not affected, the excretion of large molecules such as proteins may be impaired. It can be said that the failure to demonstrate an elevation of serum DNAase I activity during this study is a major difficulty in the way of accepting this suggestion.

Certainly, it appears that the excretion of RNAases is not impaired whereas there is a possibility that interference with renal excretion of DNAase I might occur. There is nothing paradoxical in this situation in view of the difference in molecular weight between the two enzymes, pancreatic RNAase having a molecular weight of about 14,000 (Schmidt, 1955) while pancreatic DNAase I has a molecular weight of 61,566 (Gehrmann and Okada, 1957). It should also be mentioned that Delaney (1963) found values of 13,500 and 18,500 for the pancreatic-type and splenic-type RNAases of human urine respectively. It has thus not been possible to repeat the work of Kowlessar et al. (1953 ; 1954) who reported that administration of 350 r

and 700 r whole-body radiation to rats produced marked increases in urinary excretion of DNAase I and DNAase II. Here, as with the serum and tissue studies, it appears that localized pelvic irradiation in the human produces different sequelae from those following whole-body irradiation in the rodent, besides which the relative dose administered in the animal experiments would far exceed any dosage practised therapeutically in man.

d) Effect of Ionising Radiation Upon Urinary Deoxymucleotide Excretion

The excretion of deoxymucleotides measured prior to radiotherapy in the cancer subjects did not differ significantly from the values obtained in the group of normal subjects, although the mean excretion of the former group was somewhat higher than that of the latter (Tables 84 and 88). Using the reaction of Stumpf (1947), a wide range of deoxymucleotide excretion was found in the urine of subjects in health and disease, and there was no diagnostic use to which the estimation could be put (Kosyakov, Libikova and Merkina, 1962).

The excretion of deoxymucleotides as measured by the Stumpf reaction has not shown any significant change following radiation therapy in the present series with the exception of the output relative to creatinine during the period of radium implantation which is significantly reduced compared with the output occurring in the later weeks of therapy but not compared with the control period. This observation in itself is not

	Creatinine Output g/24 hrs
Pre Radiation	1.02
1st Insertion Radium	1.08
1st Withdrawal	1.11
2nd Insertion Radium	1.07 *
2nd Withdrawal	1.20 *
First Week	1.04
Second Week	1.00
Third Week	0.96
Fourth Week	0.98

Deep X-Ray Therapy

TABLE 90.

EXCRETION OF CREATININE IN PATIENTS WITH CARCINOMA OF CERVIX DURING SUPERVOLTAGE RADIATION THERAPY

All results mean of 10 subjects
except for those with asterisk
which are mean of 6 subjects

of great importance. It seems that during radium implantation some reduction of the output does in fact occur as seen in the values for the 24-hour output of deoxynucleotides, while the excretion of creatinine is unaffected. This could be due to radiation having a more pronounced effect upon those catabolic reactions leading to the formation of creatinine in tissue than upon those reactions leading to liberation of deoxynucleotides; or it could be that whereas excretion of creatinine takes place from the glomeruli only (Peters and Van Slyke)*, excretion of deoxynucleotides might take place from the tubules predominantly or in addition to glomerular excretion, and that tubular excretion but not glomerular excretion is impaired by radiation. It might be of interest to state that the data for 24 hour creatinine output in the subjects showed remarkably little change in the group as a whole, although individual variations were encountered, some of which were almost certainly due to erratic urine collections. The mean value for each period of therapy is presented in Table 90 and analysis of variance showed that none of the changes observed are significant, as would indeed be expected from casual inspection of the data.

Conclusions Regarding Urinary Constituents and Radiation Response

Until it becomes possible to separate those patients showing a favourable response from those who respond poorly to therapeutic radiation in the present series, no evaluation

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of the prognostic usefulness of the tests carried out on this case material can be made. The prospects for assay of deoxynucleotides and DNAases do not look good, and while it might be possible to establish a correlation between response to radiation and excretion of RNAases, prognostic changes would require to occur at an early stage of therapy such as during the insertion of radium in order to provide an index of the patient's responsiveness in the light of which the rest of the therapeutic course can be rationally planned. Since deoxycytidine is quantitatively the most important deoxyriboside in rat urine (Parizek et al., 1958; Rotherham and Schneider, 1960) and is excreted after radiation in amounts proportional to the dose administered (Parizek, 1960), it is possible that this estimation might offer a more satisfactory basis for a laboratory test. However, the analytical methods available at present would not lend themselves to routine use on the scale that would be required. While Berry et al. (1963) have demonstrated increased excretion of deoxycytidine after whole-body radiation in two human subjects, the increase was very transitory, and there is no guarantee that localised pelvic irradiation would produce anything like the same response. Experience with cytological smear techniques has shown that the cytological response to radiation does not always correlate with the clinical response (Feiner and Garin, 1963) and while it has been claimed that histological examination of post-radiation biopsy specimens provides a reliable indication of

the prognosis (Walter, Harrison, Glucksmann and Cherry, 1964), a simple laboratory procedure such as those employed in this work would be preferable, both to the patient and to her clinical attendents; no efforts should be spared in continuing the search for such a test.

INTRACELLULAR LOCATION OF ENZYMES

The discussion of this point, so far as adenosine deaminase is concerned, can be brief, since the activity of this enzyme was studied only in the supernatant fraction of the tissues examined. It has already been made quite clear that this enzyme is primarily located in the supernatant (Schneider and Hogeboom, 1952; De Duve et al., 1962) although in some tissues the nuclei contain significant activity (Stern et al., 1952; Stern and Mirsky, 1963; Jordan et al., 1959).

Turning to the nucleases, the situation is very much more complex. In all the tissues examined in this work, the activity of both RNAases and of DNAase II was present predominantly in the supernatant, although the specific activities of these enzymes relative to protein were on the whole quite similar in all three cytoplasmic fractions of each tissue. DNAase I, on the other hand, showed a different distribution; the particle fractions contained a higher percentage of the cytoplasmic activity than was the case with the other nucleases, and the specific activities of these fractions were higher - in many cases very much higher - than the specific activity of the supernatant.

Further discussion of this topic must take account of the extent to which the RNAase activities measured can be ascribed to two separate enzymes. This question has already been considered for each of the organs in turn in an earlier part of

this Section, when it was concluded that there was good evidence for two distinct enzymes in thyroid, some evidence for this in breast (notwithstanding the failure to demonstrate two peaks of activity with varying pH in the supernatant of normal breast, Page 110), and little evidence in the cervix uteri. A final answer to this question can only emerge from detailed study of the pH-activity curve of every fraction in each of the organs examined. It can however be said that most organs of the rat and the mouse have well-defined and distinct RNAase activities at alkaline and at acid pH (de Lamirande et al., 1954 ; de Lamirande and Allard, 1959a; Ellem et al., 1959 ; Eichel et al., 1961). The presence of similarly distinct RNAases in human urine (Delaney, 1963) and saliva (Eichel et al., 1964) suggests that these may be of general occurrence in human tissues.

The current fashion in cytochemical enzymology insists upon a unique location for each enzyme in the cell. Heterodox views to the contrary are usually dismissed as deriving from ill-conceived or poorly executed experiments in which cross contamination has taken place. A great deal of justification attaches to such criticism, since it is on the whole uncommon to find that those concerned in such investigations have bothered to visualise the ultrastructure of their preparations by means of the electron microscope which is the only satisfactory arbiter in this situation.

One of the principal concepts which has arisen partly as

a result on the 'one enzyme one locus' theory is that of the lysosomes with which the name of De Duve is most prominantly associated (De Duve et al., 1962). According to this concept, a number of hydrolytic enzymes which includes acid RNAase and DNAase II, are confined to a group of special particles with sedimentation properties intermediate between those of mitochondria and microsomes. While the existence of these particles is not in doubt in so far as their morphological appearances are concerned, and while in many tissues they can be shown to contain very high concentrations of hydrolytic enzymes, it is by no means certain that the existence of these enzymes in other parts of the cell can be completely excluded. A more serious criticism which can be levelled against the protagonists of the lysosomal theory is that the methods employed frequently do not distinguish between latency of an enzyme and its compartmentalisation - in other words, the techniques used to liberate enzymes from cytoplasmic particles are in many instances the same as those necessary to free an enzyme in the supernatant or the microsomes from an inhibitor with which it may be bound. Thus one frequently reads of claims for the lysosomal distribution of an enzyme which are founded exclusively upon the fact that its activity in a homogenate is increased by detergent, no real attempt having been made to isolate the postulated particles or to establish the absence of inhibitors in the preparation. These criticisms have been strongly expressed in a recent review (Levvy and Conchie, 1964). Nevertheless

it does appear as though the lysosome has come to stay, although future work may necessitate a modification of current views regarding its properties and distribution.

It is difficult to obtain a clear picture of the distribution of RNAases in animal tissues because so few investigators have satisfied rigid criteria regarding preparation and examination of cell fractions, and so little uniformity in the assay of enzyme activities has been observed. There is, moreover, reason to believe that quite marked differences occur in distribution from one tissue to another. In rat liver, it was reported that about 50% of alk. and acid RNAase activity is associated with the mitochondria (de Lamirande et al., 1954; Allard et al., 1957; Roth et al., 1957). De Duve et al., (1955) produced evidence that acid RNAase of rat liver was confined to lysosomes. Reid and Nodes (1959) interpreted their data as indicating that both alk. and acid RNAases of rat liver were lysosomal, but subsequently decided that significant amounts of acid RNAase were present in the cell sap (Reid and Nodes, 1963). Good evidence for the presence of RNAase activity in rat liver microsomes has been presented (Roth, 1960; Morais and de Lamirande, 1965), while the enzyme is also present in guinea-pig liver microsomes (Leslie, 1961; Martin et al., 1963). Most of the activity of mouse pancreas RNAase appears to be in the microsomes, with lesser amounts in the supernatant and the zymogen granules (Dickman and Trupin, 1958; Dickman and

Morrill, 1959 ; Morris and Dickman, 1960). In rat spleen, 37% of acid RNAase was found in the mitochondria and 28% in the supernatant (Roth and Eichel, 1958 ; Eichel and Roth, 1962). Conflicting reports have been published concerning acid RNAase activity of rat kidney which was described as a supernatant enzyme by two independent groups (Reid and Stevens, 1958 ; de Lamirande and Allard, 1959) although it had earlier been demonstrated in intracellular droplets akin to lysosomes present in this tissue (Strauss, 1956 ; 1957). Acid RNAase has been described in lysosome-like particles from rat brain (Beaufay et al., 1957) but in goat brain, both RNAases have been found in ribosomes (Datta et al., 1964). An association of acid RNAase with lysosome-like particles has also been found in rat mammary gland while alk. RNAase was thought to be mitochondrial (Greenbaum et al., 1960 ; Slater, 1961). Acid RNAase has also been reported in association with lysosome-like particles in rabbit leucocytes (Cohn and Hirsch, 1960), rat intestine (Hsu and Tappel, 1964) and skeletal muscle of several species (Zalkin et al., 1961).

DNAase II has been less thoroughly studied than the RNAases in respect of its intracellular distribution. It has been reported that most of the activity is divided between the mitochondria and supernatant in rat liver (de Lamirande et al., 1954) and rat spleen (Roth and Hilton, 1963). Convincing evidence for the presence of this enzyme in rat liver mitochondria carefully prepared and identified by electron microscopy has

been published by Okada and Peachey, (1957). On the other hand, De Duve et al., (1955) consider that it is restricted to lysosomes in rat liver. Significant activity was reported in the supernatant of rat kidney (Reid and Stevens, 1958) whereas Strauss (1956; 1957) found that the greater part of the activity in this organ was present in droplets similar to lysosomes. The enzyme has been reported in association with lysosomes in rat brain (Beaufay et al., 1957) and rabbit leucocytes (Cohn and Hirsch, 1960) but this association could not be demonstrated in rat mammary gland (Greenbaum et al., 1960). In Landschutz ascites tumour cells, the specific activity of the nuclei is twice that of the particles (Keir and Aird, 1962).

Discussion of the results obtained in the present work is difficult, since so many technical problems conspired to prevent a clear-cut answer to many of the questions raised. It does appear, however, as though ribonucleases and DNAase II are present in the supernatant as well as in the particles in all the tissues examined. The evidence for this distribution being natural rather than artificial is strongest in the thyroid where the preparative conditions employed were less severe than for breast and cervix, and where an opportunity was taken to carry out immediate preparation of fresh specimens on several occasions. The distribution of these enzymes has been considered in some detail for each tissue earlier in this section. The smallest number of enzymes required to account for the observed activities of both RNAases in cytoplasmic fractions of human thyroid would

be a n acid RNAase in the supernatant, an acid RNAase in the lysosomes or microsomes (or both), and an alk. RNAase in mitochondria - probably that sub-group of mitochondria requiring a fairly high centrifugal field for complete sedimentation. While little can be said of the distribution in breast because so few samples were fractionated in a satisfactory manner, the same pattern as has been described for the thyroid would fit in with the data obtained, and would in any case be expected in view of the fact that the epithelium of breast and thyroid have many functional and morphological features in common. In the cervix, the evidence for two distinct RNAases is not very great. The minimum number of enzymes required in this tissue would be an alk. RNAase distributed predominantly in the supernatant and subsidiarily in light mitochondria.

Certainly in the thyroid, and probably in the other two organs as well, it seems as if DNAase II is located very largely in the supernatant. It is unlikely that adsorption can account for the activity found in the particles, and most probably some of the enzyme in all three tissues is present in an organelle distributed between both the "Mitochondrial" and "Microsomal" fractions; this could be a light mitochondrion, or a lysosome.

The distribution of DNAase I is fortunately more clear-cut. In all three tissues examined, there are good grounds for describing this enzyme as predominantly particulate and attributing the apparent supernatant activity measured to a) rupture of some of these particles and b) the slight activity manifested

by DNAase II under the assay conditions used for DNAase I. The most likely site of DNAase I location is in the light mitochondria. This would accord with the high concentration of this enzyme reported in purified preparations of mitochondria (Goutier-Pirotte and Oth, 1956 ; Dounce et al., 1957 ; Baudhuin, 1959).

THE FUNCTION OF THE ENZYMES STUDIED

Broadly speaking, the only cellular function of these enzymes which can be examined in the light of the data obtained in the course of this work is cell growth. A detailed account of the views held by others who have examined this question has already been given in the Introduction, and will not be repeated here. It is sufficient to state that at the present time, there is no agreement whether these enzymes have an important role to play in growth and mitosis, or are mainly degradative in function. So far as RNAases are concerned, there is some circumstantial evidence in favour of an anabolic function (Brody and Thorell, 1957 ; Maciejewska-Potapczyk, 1959) and other results pointing to a catabolic function (Jeener, 1959a; Firket et al., 1955); the experiments of Kessler and Engelberg (1962) can be interpreted as indicating that RNAases function in both ways at different phases of cell life. Those who favour a role for DNAase II in mammalian cell growth (Allfrey and Mirsky, 1952; Brody and Thorell, 1957 ; Brody and Balis, 1958 ; 1959 ;

Brody et al., 1959; Chevremont et al., 1959; Goutier and Goutier-Pirotte, 1961; Goutier and Leonard, 1962; Goutier-Pirotte and Goutier, 1962; Tunis and Regelson, 1963), are opposed by the results obtained from studies on metamorphosis and embryonic development in lower animals (Blumenthal, 1957; Coleman, 1962; 1963; Solomon, 1964). An association between DNAase I and optimal function of DNA-synthesising enzymes has been demonstrated (Mantsavinos and Canellakis, 1959b; Sarkar, 1961; Keir et al., 1962; Keir, 1962) and supports the findings of those who claim a relationship between this enzyme and cell growth (Zahn, 1959; Maciejewska-Potapczyk, 1959); other workers have been unable to find such a relationship (Brody and Thorell, 1957; Tunis and Regelson, 1963). While it has been claimed that adenosine deaminase activity increases in the chick embryo during growth (Gordon and Roder, 1953) this observation could not be confirmed (Solomon, 1960; Fisher et al., 1962).

The first point to be made is that the benign neoplasms examined in the present work showed no differences from the corresponding normal tissue in respect of any of the enzymes studied. Although it is not possible to be definite about this, it is probable that their rate of growth exceeds that of the normal tissue, and on this assumption it does not appear as though these enzymes are directly involved in the process of growth <u>per se</u>. Surprisingly, an altogether different conclusion

is reached when the enzyme levels of the hyperplastic tissues are considered. In both thyrotoxicosis and cystic mastopathy, increases in the specific activity of adenosine deaminase and DNAase II were found. It is true that in the former, this was largely attributable to changes in the 'Colloid' content of the organ in this condition, but it must also be remembered that all the subjects were under treatment at the time the gland was removed, so that it might be expected that many changes associated with florid and untreated thyrotoxicosis would be masked. The mere fact that the other enzymes studied were not significantly raised under the same conditions argues for the fact that these two enzymes have a role in the cellular hyperplasia of the thyroid gland in thyrotoxicosis. Once again, it is likely that the rate of cell division is higher in the hyperplastic tissues than in the normal. The present investigation has thus yielded a paradox which cannot be resolved by the data obtained. Clearly much work will require to be done in order to determine the relationship, if any, between nucleases and adenosine deaminase on the one hand, and cellular growth on the other; but already it is apparent that it is an oversimplification to speak of 'cellular growth' as an all-embracing term. Future work will require to take account of different types of growth, and to distinguish between natural, regenerative, hyperplastic, and neoplastic proliferations.

Turning to malignant growth, brief reference may be made

to the Introduction where an account was presented of the findings of those investigators who have measured the activities of nucleases and adenosine deaminase in various tumours. An attempt will be made to sum up the most relevant observations as succinctly as possible. Some workers have reported increased activity of RNAases in rat liver tumours (Schneider et al., 1953 ; Mayer and Greco, 1956 ; Allard et al., 1957); some have adopted an equivocal position (Reid and Lotz, 1958 ; Brody and Balis, 1958 ; Wannamacher et al., 1962 ; Roth et al., 1964); only Daoust and Amano (1963) have stated that RNAase activity is reduced in malignant neoplasms. Many reports of increased DNAase II activity in tumours have been published (Schneider et al., 1953 ; de Lamirande et al., 1954 ; Brody et al., 1959 ; Tunis and Regelson, 1963), but Daoust and Amano, (1963) have reported the opposite to be the case. There is fairly general agreement that adenosine deaminase activity is increased in cancers, (de Lamirande et al., 1958 ; Fodor et al., 1958 ; de Lamirande and Allard, 1959; Fiala and Kasinsky, 1961) although some reports are somewhat more reserved and non-commital (Reid and Lewin, 1957 ; Roth et al., 1963).

With the exception of acid RNAase of thyroid and DNAase I of breast, the specific activities of all five enzymes were higher in the carcinomata than in the corresponding normal tissues in the present work. The significances of these findings have been carefully appraised in relation to the histo-pathology of the individual organs in the earlier part of this section.

It remains to evaluate these results in terms of current concepts regarding the biochemistry of cancer, and to consider whether they justify any fresh approach to this problem.

RELATIONSHIP OF PRESENT WORK TO CURRENT CONCEPTS OF CARCINOGENESIS

It would be presumptuous to expect the handful of results incorporated in this report to shed fresh light on a problem which as yet has not yielded to those who have devoted a lifetime to its study. On the other hand, it is relevant to consider what support the present work lends to those hypotheses concerning the origins of neoplasia which command the widest respect. Those which have a primarily biochemical background include:

a) The Theory of Aerobic Glycolysis

The original theory put forward by Warburg advanced the suggestion that cancer originates as a consequence of respiratory damage to the cell leading in turn to increased glycolysis even under aerobic conditions. The theory has an honourable history and has been defended by Warburg with much new experimental data since its initial promulgation in the 1930's (Warburg, 1956a; 1956b). Various modifications of this theory have been proposed. One view which enjoys considerable support was first suggested by Weinhouse (1955); this stresses the abnormality of the glycolytic process itself in tumours, rather than regarding the abnormality as secondary to damage to the cell's respiratory apparatus.

b) The Convergence Theory (Greenstein, 1954).

The fact that cancer cells derived from a multiplicity

of tissues resembled each other in enzymic composition and were frequently quite dissimilar to their cell of origin led to the suggestion that the pattern of changes necessary to produce a cancer resulted in a metabolic convergence towards a common cell type - namely, 'the cancer cell' - irrespective of the direction in which these changes proceeded.

c) The Theory of Catabolic Deletion (Potter, 1956; 1957; 1958).

In its original form, this theory centred upon loss of enzyme systems concerned with the degradation of nucleic acid precursors especially thymidine and its phosphate derivatives. More recently, however, the emphasis has shifted to lay particular stress upon deletion from the cell of hypothetical systems exercising negative feedback control upon enzymes involved in DNA synthesis and cell division (Potter, 1964).

These views have been critically discussed by Bergel (1961), by Le Breton and Moule (1961) and by Busch (1962) as well as by many other distinguished oncologists too numerous to mention. They have stimulated a great deal of research and inspired many fundamental observations but they have not yet led to a solution of the problem.

So far as the Theory of Aerobic Glycolysis is concerned, the present findings have little of relevance to add, since no study was made of enzymes of the glycolytic pathway or of those involved in oxidative pathways of carbohydrate metabolism. However, scarcity of mitochondria in tumours is frequently

cited in support of the concept of a respiratory block in cancer cells. No such deficiency was found in the tumours studied in the present work; indeed it is probable that they were increased relative to the weight of the tissue and that they formed a higher percentage of the cytoplasmic protein of the cancer cell than of the normal cell. Although technical difficulties precluded a conclusive answer to this question, it would appear as though the normal human breast and cervix are deficient in these organelles. Loss of mitochondria can only take place if the parent cell is mitochondrion-rich, as in the case of the liver cell; and the fact that certain hepatomas are deficient in mitochondria is probably a consequence of de-differentiation rather than a cause of the malignant growth.

The Deletion Hypothesis has gained wide support from studies carried out largely with hepatomas, whether induced by feeding carcinogens or cultured in the form of tumour transplants. While the explanation it offers for the development of malignancy in this context is extremely attractive, there are aspects which cannot be reconciled with the biological properties of other tumours. It is unable to offer a convincing explanation of viral carcinogenesis in which the genetic potential of the affected cell is supplemented rather than diminished (Hilleman, 1963). A more serious stumbling block must be the recent realisation that certain tumours derived from rapidly growing tissues such as those of the gastro-intestinal tract have a lower rate

of DNA synthesis and of mitosis than the tissue from which they originate (Lipkin, 1965). Once again, it appears as though the high rate of mitosis in the hepatoma cell compared with that of the stable liver cell could be a feature of de-differentiation rather than the basic lesion responsible for its malignancy. It is in some ways unfortunate that so much attention has been lavished upon liver tumours. Occurrence of primary liver tumours in man is extremely rare. Moreover, the liver cell is probably richer in enzymes than any other cell in the body. It is therefore only to be expected that such changes as occur in neoplasia will lie in the direction of decreased activity rather than increased activity, except for those enzymes in which the cell is normally deficient. Enzyme deletion is not restricted to neoplasia, since loss of intracellular protein including enzymes is a general feature of cell damage. The majority of human carcinomata arise in tissues far less sophisticated than the liver cell and lacking the complex enzyme apparatus of the latter. Where the parent cell type is poor in enzymes, it is not unreasonable to suppose that such changes as occur are likely to lie in the direction of increased activity.

Certainly, the present results do not suggest that enzymes degrading nucleic acids are lost from human cancer cells. In fairness, it must be admitted that Potter has never considered it essential to his theory that nucleases should be deleted,

	THYROID CANCER	BREAST CANCER	CERVICAL CANCER	HASHIMOTO'S THYROIDITIS
Alk. RNAase	0.070 (171)	0.096 (343)	0.910 (1167)	0.338 (824)
Acid RNAase	0.047 (152)	0.064 (356)	0.510 (1545)	0.150 (484)
DNAase I	1.81 (274)	2.30 (105)	7.23 (287)	2.98 (452)
DNAase II	32.8 (187)	39.3 (479)	51.4 (450)	46.7 (267)
Adenosine Deaminase	86.8 (611)	74.3 (415)	163.2 (301)	187.0 (1317)

SUMMARY TABLE P.

SPECIFIC ACTIVITIES OF SUPERNATANT ENZYMES IN CANCER AND THYROIDITIS.

The mean activities for each group per mg. protein are recorded above, and the activities as a percentage of the mean for the corresponding normal group are in brackets below.

since he was primarily concerned with explaining the availability of nucleotides and other precursors for nucleic acid synthesis and postulated that the pathways by which these were normally degraded became impaired with the initiation of neoplasia. Nor did he pay much heed to adenosine deaminase, as he regarded xanthine oxidase as the enzyme controlling the degradation of purine derivatives, and many reports testified to the diminution or even the complete absence of this activity from several types of tumour.

Turning to the Greenstein hypothesis, it must be admitted that this is largely descriptive rather than mechanistic. It informs us that tumours converge towards a common metabolic pattern without providing a clear indication how this process is initiated and what factors are responsible for maintaining its impetus. The results of the present work can, in a very general way, be shown to accord with the idea of a progression towards a common cell type. To begin with, there is some evidence that they are richer in cytoplasmic particles than their cell of origin. Secondly, comparison of the specific activities of the enzymes studied in the three types of carcinoma reveal an increase above those of the normal tissue. This may be seen rather clearly by reference to Table p where the activities are expressed as a percentage of the specific activity of the corresponding normal tissue. Neither the benign or the hyperplastic tissues studied approached the levels

of activity found in the carcinomata, although the latter generally took up a position intermediate between that of the normal and the carcinoma. The only lesion which showed consistently higher activity than the corresponding carcinoma from the same tissue was thyroiditis. This point has bden emphasised by inclusion of the latter along with the carcinomata in Table P . The thyroid involved in Hashimoto's thyroiditis is populated by three main groups of cells - epithelial, stromal, and lymphoid - of which only the last are absent from the normal gland. It is likely that the high enzyme levels encountered in thyroiditis are due to these primitive and undifferentiated cells, and that the similarity between the carcinomas and the thyroditis tissue is a reflection of the progression of the former towards an undifferentiated state.

A word of caution is necessary, because it is unlikely that all the enzymes elevated in carcinomata are relevant to this process of de-differentiation. The DNAase I content of normal breast and cervix is higher than that of thyroid carcinoma, and the alk. RNAase activity of normal cervix is higher than that of thyroid carcinoma; but differences in the degree of differentiation of the normal tissues themselves must also be taken into account. On other grounds, a case can be made out for regarding these two enzymes as irrelevant to the process of malignant neoplasia. In the first place, the range of RNAase activity among the different cancers is enormous, with cervical

carcinomata possessing ten times the specific activity of the other two malignant groups. Secondly, tumour ablation actually resulted in an increased activity of this enzyme, while DNAase I was hardly affected; the only two to show decreased activity after irradiation were DNAase II and adenosine deaminase.

One of the intriguing questions which tends to be forgotten in most theories of carcinogenesis propounded by biochemists is the manner in which malignant neoplasms acquire the capacity to invade neighbouring and distant tissues. The convergence of carcinomas towards a pattern sharing certain features in common with Hashimoto's thyroiditis prompts an exciting speculation. For one of the outstanding properties of cells of the lymphoid series - and it is to these that the enzymological characteristics of thyroiditis are ascribed - is the capacity to invade other tissues in the body. Adequate reference has been made in the appropriate sections of the "Introduction" to the high nuclease content of lymphoid organs such as spleen and thymus, especially DNAase II from which sources the most potent preparations of the enzyme have been obtained, (Koerner and Sinsheimer, 1957a; Shimomura and Laskowski, 1957; Koszalka et al., 1957; Oth and Fredericq, 1958; Hodes and Swenson, 1962; Bernardi, Griffe and Appella, 1963). The question arises whether the high content of nucleases shown to be present in human carcinomas is related to their capacity for invasiveness. A priori, it is to be expected that the ability to degrade vital macromolecules such as those of protein, and of nucleic acids, would be

fundamental requirements of a cell capable of invading other tissues. It has already been shown that tumours have a high content of peptidases (Wu and Bauer, 1963), and many reports testify to the high content of nucleases which develop in cells subjected to viral invasion (Wormser and Pardee, 1957; Korn and Weissbach, 1963; Keir and Gold, 1963; Russell et al., 1964). Moreover, a correlation between virulence and DNAase activity has been established for certain bacteria (Jacobs et al., 1963); and impairment of various metabolic functions, especially protein synthesis and oxidative phosphorylation, has been demonstrated by addition of pancreatic RNAase to cells growing in culture (Firket et al., 1955; Groth, 1956; Hanson, 1959; Jeener, 1959a; 1959b; Jeener et al., 1960). A curious fact which at present has no explanation would take on significance if this speculation were correct; it is the well-nigh total inability of human cancers to form metastatic deposits in the spleen. Here we have an organ in which the cells are endowed with a high content of nucleases and yet have acquired the ability to co-exist in a homeostatic equilibrium. Clearly other cells with high nuclease content would not have a selective advantage in this milieu, whereas in a milieu composed of cells poor in nucleases, this capacity would endow them with properties favourable for invasion.

On this basis, we might visualise a solid tumour as one which has acquired the potential for invasiveness common to primitive undifferentiated cells, while retaining the

cohesiveness of the specialised cells from which they originate. The more malignant the neoplasm, the nearer does the cell of which it is composed resemble morphologically those of the lymphoid series, such that the distinction between a highly anaplastic carcinoma and a malignant lymphoma can scarcely be made (Warren and Meissner, 1953).

While it is necessary to apologise for this speculation in view of the slender basis on which it rests, no apology is necessary for pleading the importance of making a thorough study of the biochemical and enzymological factors which lie at the root of malignant invasion. This aspect has been sadly neglected in the past. One avenue for research suggested by the p_resent work is a thorough comparison of tumour metabolism with that of cells derived from the lymphoid series. Another is a quest for those factors which make the spleen an unpopular site for tumour metastasis, while similar structures such as lymph nodes are among the first to be invaded. Such studies might reveal the true role of nucleases in the economy of the cancer cell.

Finally, in view of the technical challenge which has to be met in conducting investigations in the field of human cancer, attention should be focused upon the importance of expanding the sparse amount of work which has been carried out in this field. In a recent review, Davidson (1963) concluded: "No one is more convinced than I am that the solution to the problem

of cancer will not come at the bedside of patients dying of advanced malignant disease .... but only by the study .... of the basic principles of why cells grow and why they stop growing". While the present author is largely sympathetic to this viewpoint, he feels that the time is nigh when greater emphasis should be laid on the problem of invasiveness rather than of growth, and he regards any theory of carcinogenesis which fails to take account of the characteristics of human cancers as likely to prove sterile in the long run. There are many questions which we can ask of the laboratory animal, but the final and crucial question can only be asked of man himself. There may be no harm in stating that in the field of cancer, as in other researches, "the proper study of mankind is man" (Pope, 1733).

SUMMARY

SUMMARY

1) The activities of four nucleases -- alk. RNAase, acid RNAase, DNAase 1, and DNAase 11 -- were measured in three cytoplasmic fractions prepared by differential centrifugation of homogenates of three human tissues in normal and diseased states: thyroid, breast, and cervix uteri. The activity of adenosine deaminase was measured in the supernatant fraction of these same tissues.

2) The activities found in adenomata of the thyroid did not differ significantly from those in the normal gland.

3) In thyrotoxicosis, the activities of acid RNAase, of DNAase 11, and of adenosine deaminase in the supernatant were significantly elevated, but no differences could be established between the normal and the toxic gland when account was taken of the DNA - phosphorus content of the tissues. It is probable that the differences between the supernatant enzyme content of the normal and the toxic thyroid gland are due to diminished 'Colloid' storage in the latter. The ratio of acid to alk. RNAase was consistently higher in the toxic gland. The specific activities of acid RNAase, of DNAase 1, and of DNAase 11, relative to protein, were elevated in the particulate fractions of the toxic gland, but the relevance of these changes to the underlying pathology is far from clear.

4) Carcinomata of the thyroid have increased activities of alk. RNAase, DNAase 1, DNAase 11, and adenosine deaminase, per mg. protein in the supernatant. These changes are not seen when activity is related to tissue weight; it is probable that 'Colloid' depletion is a factor contributing towards these changes. Per unit weight of tissue, the amount of protein in the particle fractions greatly exceeds that of the normal gland. This has the effect of increasing the activities of alk. RNAase and of both DNAases in one or both of the particle fractions relative to tissue weight, and results in a shift in the distribution of all four nucleases such that an increased percentage of the total cytoplasmic activity is located in the particle fractions.

5) In Hashimoto's thyroiditis, a dramatic increase in the activity of all the enzymes takes place in almost all fractions of the cytoplasm. Moreover, a great increase in the protein content of the particle fractions occurs hand in hand with a decrease in the protein content of the supernatant. Consequently, the distribution of the nucleases is altered, a higher percentage of the cytoplasmic activity being relocated in the particle fractions. It is suggested that these changes are a consequence of changes in cell population of the thyroid occuring in thyroiditis, especially infiltration by cells of the lymphoid series.

6) Fibroadenomata of breast showed little difference from the normal

breast in respect of nuclease activities. Samples from cases of cystic mastopathy had significantly higher specific activities of DNAase II and adenosine deaminase in the supernatant. Carcinomata of breast had very much higher levels of all the enzymes studied, except DNAase I, than any of the other breast tissues examined. Moreover, the particle fractions prepared from carcinomata were richer in nucleases than those derived from non - malignant samples.

7) The activities of all five enzymes were dramatically raised in the supernatant and in the cytoplasmic particles of cervical carcinomata. It would appear that per unit weight of tissue the carcinomata have a higher protein content in the supernatant, and even moreso in the particulate fractions, than the normal tissue. Under the conditions of this study, a higher percentage of the protein content and the nuclease activity of the cytoplasm was located in the particle fractions of the carcinomata.

8) There did not appear to be any correlation between the degree of malignancy as assessed clinically and histologically, and the enzyme activities measured. Nevertheless, since the normal and the malignant groups of cervical specimens could virtually be distinguished on the basis of their DNAase content, it is suggested that this observation might provide the basis for a diagnostic test of gynaecological cancer.

9) Therapeutic irradiation of cervical carcinomata brought about a pronounced fall in the protein content of the supernatant, together with a decrease in the DNAase II and adenosine deaminase of this fraction per unit weight of tissue. On the other hand, the specific activity of both DNAases was consistently elevated in all fractions after irradiation, usually to a significant degree. Changes in other particulate enzymes were generally small, but the increased protein content of the particles relative to the supernatant after irradiation was accompanied by a corresponding shift in the cytoplasmic distribution of the nucleases.

10) By and large, the specific activities of both DNAases and of DNAase II were comparable in all three cytoplasmic fractions of all the tissues examined. The distribution of the enzymes thus paralleled the distribution of the protein, with the supernatant containing the greater part of the cytoplasmic activity. The specific activity of DNAase I was however greater -- in many tissues very much greater -- in the particles than in the supernatant, although as a rule, more than half the total cytoplasmic activity was located in the latter fraction. Malignancy invariably resulted in an increase of particulate protein and nuclease activity relative to that of the supernatant.

11) It would appear from the results of this work that benign neoplasia is not associated with changes in nuclease content of

the tissue. Malignant neoplasia, on the other hand, is accompanied by considerable increases in the levels of nucleases and adenosine deaminase. Hyperplastic growth would appear to occupy a position midway between normal and malignant processes in most respects, and especially so with regard to DNAase II and adenosine deaminase, between which enzymes a correlation has been shown to exist in the present work.

12) Serum nucleases, measured prior to therapeutic radiation in ten subjects with carcinoma of the cervix uteri, did not differ notably from the levels found in a group of ten healthy subjects. No significant changes were noted during radiotherapy.

13) Urine nucleases, measured prior to therapeutic radiation in the above ten subjects did not differ notably from the levels found in the healthy subjects. The concentration of urinary ribonucleases per ml. of urine rose during radium implantation, and rose once more during the last week of supervoltage therapy. During the last two weeks of therapy, an increase in the urinary excretion of RNAases relative to that of creatinine took place. The total 24-hour excretion of RNAases, however, did not show any significant changes. Apart from a reduction of urinary DNAase 1 excretion per 24-hours and per mg. creatinine during radium insertion, no notable changes in urinary DNAases occurred during therapeutic radiation.

14) The excretion of total deoxynucleotides in the ten cancer subjects prior to radiation did not differ from that of the healthy subjects. Changes occurring during radiotherapy were not significantly different from the excretion of deoxynucleotides in the pre-radiation period.

15) The excretion of creatinine was unaltered by therapeutic radiation.

16) Such changes as occurred during irradiation could not be definitively related to the success of therapy and to the extent of the clinical response, though it is hoped that continued follow-up of the present case - material may suggest such a relationship.

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