Nucleic Acid Studies on Cell Fractions

by

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DEDICATION.

I wish to dedicate this work to my mother in appreciation of all she has done for me.

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General Introduction.

1. Cytological Aspects.

(a) Historical.

Since the discovery, in 1665 by Hooke, of the cellular nature of cork, the study of Cytology has progressed far. Today, all that remains to remind us of the work carried out by that early investigator is the term which he introduced, the name "cell". Although similar observations were carried out on other plant tissues by Grew and Malpighi, later in the seventeenth century, it was not until the beginning of the nineteenth century that man was able to look beyond the cell membrane into the cell itself.

One of the most important advances was the discovery of the nucleus, in the "cellular juice" of the orchid, by Brown in 1831. This was followed by the observation of Dujardin in 1835, that the cells of lower organisms, such as Rhizopoda and Foraminifera contain "a gelatinous substance, perfectly homogeneous, elastic, contractile, diaphanous, insoluble in water and without traces of organization". This substance, which Dujardin described so precisely, was given the name "Protoplasm" by Purkinji and von Mohl.

It was only with the coming of improved optical instruments and improved techniques that Altmann (1896) was able to show that the cytoplasm, that part of the cell surrounding the nucleus and bounded by the cell membrane, contains granules. It was thus that the large rod-shaped particles, now known as Mitochondria, were observed to be present in the cytoplasm of all cells. In animal cells other cytoplasmic elements, such as the reticular apparatus -- the Golgi Body, were also observed.

The introduction of dark field illumination made visible extremely small bodies which were highly refringent. These small particles are now termed Microsomes.

Subsequent studies on the structure of the cell were mainly carried out with the aid of dyestuffs. Thus in 1900, Michaelis introduced the use of Janus Green B, as a specific stain for mitochondria. Mitochondria stained by this dye are green-blue in colour. On incubating the stained mitochondria at 37°C, the colour changes to red and then complete decolorization occurs. This test is used to identify mitochondria, and it has been suggested by Lazarow and his associates (Lazarow & Cooperstein 1953; Cooperstein, Lazarow & Patterson 1953; and Cooperstein & Lazarow 1953) that it is based on the fact that the dye is specifically re-oxidised by cytochrome oxidase, an enzyme found only in

mitochondria. The ability of the cell components to absorb basic dyes (Berg 1934) has been used to differentiate the cell contents.

One important contribution was the development by Feulgen and Rossenbeck in 1924, of a staining technique, now known as the Feulgen reaction. In this reaction, the nucleus is stained bright purple, while the remainder of the cell is unstained. The exact nature of this reaction is still controversial. Some workers (Brachet 1946, 1947; Stowell 1946, 1947; Di Stefano 1948a, 1948b; Barber & Callan 1949; Caspersson 1949; Overend & Stacey 1949) believe that the Deoxyribonucleic Acid (DNA) of the nucleus is partially hydrolysed by the dilute mineral acid and the hydrolysis products formed restore the colour of basic fuchsin which has been decolourized with sulphurous acid (Schiff's reagent). On the other hand, Stedman & Stedman (1947a, 1947b, 1950) claim that the dye produced by the interaction of hydrolysed DNA and the fuchsin reagent is water soluble and is strongly adsorbed by the non-histone protein, chromosomin, of the nucleus. Thus, although the dye is only produced in the presence of DNA, the structures stained by it consist of protein rather than of DNA, which is destroyed during the hydrolysis procedure. No matter which interpretation is taken of the mechanism involved in

the Feulgen reaction, it still demonstrates that the chemical composition of the nucleus differs from that of the cytoplasmic particles.

A great part of our present knowledge of the chemical composition of the nucleus comes from the classical chemical studies of Miescher (1897) and Kossel (1888) on pus cells, fish spermatozoa, haemolysed bird erythrocytes and on leucocytes and cells of the thymus and liver submitted to peptic digestion. From such tissues, these workers isolated an acidic compound which is now recognised as the nucleic acid, deoxyribonucleic acid (DNA). It was subsequently shown that nucleic acids were normal constituents of all cells and tissues.

Hydrolysis of the nucleic acid, obtained from thymus, was found to yield a mixture of the purine bases, adenine and guanine, the pyrimidine bases cytosine and thymine, a sugar, which was eventually shown by Levene and Mori (1929) to be D(-)2-deoxyribose, and phosphoric acid.

The nucleic acid obtained from plant sources, namely yeast, was found to give different products on hydrolysis. The bases obtained were adenine, guanine, cytosine and the pyrimidine uracil, a pentose sugar, which was eventually identified as D(-) ribose (Barker & Gulland 1943; Barker, Farrar & Gulland 1947) and phosphoric acid.

The different composition of the two nucleic acids, obtained from plant and animal sources, gave rise to the belief that the nucleic acid in animal cells was of the deoxyribose type, while that from plants was of the ribose type. However, it was not long before many workers found evidence to the contrary, among them Hammarsten (1894), and Jorpes (1924, 1928 & 1934). From the evidence put forward by these and other workers, it became evident that ribonucleic acids (RNA) were normal constituents of animal as well as plant tissues. About the same time, the presence of DNA in plant cells was being demonstrated (Feulgen & Rossenbeck 1924; Kiesel & Belozerski 1934; Belozerski 1936, 1939). Jones and Perkins (1924-5) expressed the view that "the distinction between plant and animal nucleic acids will in future not be so definitely drawn". Recent work has confirmed this view completely and it is now clear that both DNA and RNA are abundant in all cells.

For some time it was assumed that nucleic acids were essentially nuclear constituents, but Caspersson & Schultz (1938, 1939) demonstrated that the cytoplasm of certain rapidly proliferating cells contained large amounts of RNA. The cytoplasm of these rapidly growing cells when examined in ultraviolet light showed absorption in the region of

260 mμ, which is characteristic of the nucleic acids. Application of the Feulgen test gave negative results, demonstrating the absence of DNA. The presence of RNA in the cytoplasm was confirmed by Brachet (1940), using a mixture of basic dyestuffs. This mixture of basic dyestuffs, known as Unna Pappenheim stain, contains pyronine, which stains RNA red, and methyl green, which stains DNA green. Using this staining technique in conjunction with the enzyme ribonuclease, first isolated from pancreas by Jones (1920), Brachet (1940, 1941) showed that when tissue sections were incubated with the enzyme and subsequently stained, the basophilic granules had lost their capacity to take up the pyronine, whereas the chromatin of the nucleus still had the ability to take up the methyl green. He, therefore, concluded from this that the RNA of the cell was located mainly in the cytoplasmic particles and with a small amount in the nucleolus, while the DNA was entirely present in the nucleus of the cell.

While histochemical studies give indications of the location of the nucleic acids in the cell, it is not possible by these methods to determine accurately the nucleic acid content of the cell or its component parts. However, modifications of the histochemical technique, whereby the amount of visible light absorbed by the stained tissue is

used to estimate the amount of nucleic acid present, have been developed. Using this microspectrophotometric technique, Pollister and his associates (Pollister 1950, 1952; Pollister, Himes & Ornstein 1951; Pollister, Swift & Albert 1951) and Mirsky and Ris (1951) have determined the amount of DNA in cell nuclei stained by the Feulgen procedure. Caspersson (1936, 1940, 1947, & 1950) has developed and used quantitative microspectrophotometry employing ultraviolet light. With this technique, the section of tissue does not require to be previously stained, and the amount of nucleic acid present is estimated by the amount of ultraviolet light absorbed. The disadvantage of microspectrophotometry using visible light is that it assumes that the intensity of the staining is proportional to the amount of nucleic acid present. Ultraviolet microspectrophotometry requires an elaborate quartz optical system. It is impossible by this method to distinguish between free bases, nucleosides or nucleotides, nor can it differentiate between DNA and RNA. The amounts of nucleic acids present in the cell and its component parts can only be determined with accuracy by chemical means on whole tissue or the isolated cell particles themselves.

(b) Isolation of cell components.

I. Nuclei

I. Nuclei.

The preparation of nuclei on a large scale, by chemical or physicochemical methods, dates back to the work of Miescher (1871), who isolated pus cell nuclei by a rather drastic method, which included the use of a pepsin-hydrochloric acid mixture to digest away the cytoplasm. Miescher also used dilute acid alone to liberate nuclei of fish sperm.

Behrens (1932, 1935) obtained nuclei by subjecting ground dried tissue to specific gravity flotation and differential centrifugation in organic solvents. Feulgen, Behrens and Mahdihassan (1937) and Behrens (1938) obtained isolated nuclei from plant as well as animal tissue, and were the first to show that plant nuclei, so isolated, contained DNA.

In 1937, Crossman observed that strong citric acid solutions liberated muscle cell nuclei in a microscopic preparation. Stoneburg (1937) made use of this to isolate cell nuclei from muscle on a large scale. In this procedure, however, use was also made of pepsin-hydrochloric acid digestion.

Many other workers, among them Marshak (1941), Haven and Levy (1942) and Mirsky and Pollister (1946), developed procedures for isolating nuclei using citric acid. A

method of isolating nuclei suitable for enzyme studies was developed by Dounce (1943a, 1943b, 1950), who based his method upon the previously developed citric acid techniques. Dounce (1943a, 1943b) found that nuclei could be liberated and isolated from liver cells in distilled water adjusted accurately to pH 6.0 with dilute citric acid. Schneider and Hogeboom (1951), in their review of cell fractionation techniques, claim that the nuclei, isolated by the Dounce (1943a, 1943b) procedure, are not morphologically intact. Schneider and Hogeboom (1951) claim that nuclei isolated in either isotonic or hypertonic sucrose, as described by Hogeboom, Schneider and Palade (1948), Ris and Mirsky (1949), and Kurnick (1950), are morphologically intact. Peterman and Schneider (1950, 1951) found that better yields of nuclei were obtained when a small amount of calcium chloride was added to the sucrose solution, to prevent the disruption of the nuclei during homogenising.

Analysis of the nuclei isolated by these various methods all show the presence of DNA and RNA, although the amounts found depend on the isolating technique used. Pollister and Leuchtenberger (1949) found that the ratio of protein to DNA estimated on nuclei isolated in citric acid was lower than that found in tissue sections. Dounce, Tishkoff, Barnett and Freer (1950), using nuclei isolated by the Behrens (1932) method, observed that these nuclei had a

much higher protein-DNA ratio than did nuclei obtained using citric acid. However, nuclei isolated by the Behrens (1932) procedure were peculiar in that their RNA content was greater than their DNA content.

Investigations on the enzymes contained in isolated rat liver nuclei, carried out by Lan (1943, 1944) and Dounce (1943b) have shown the presence of aldolase, d-amino oxidase, arginase, alkaline phosphatase and uricase.

II. Cytoplasm.

The isolation of cytoplasmic particles dates back to the year 1934, when Bensley and Hoerr reported the isolation of mitochondria from frozen dried material. This initial success was followed by the development of the differential centrifugation technique in which Claude (1940a, 1943a, 1943b, 1946) and Lazarow (1943) were the fore-runners.

Claude (1940a, 1943a, 1943b, 1946) made use of alkaline 0.85% (w/v) sodium chloride as homogenising medium, but this method often gave non-uniform, contaminated and damaged particles. The main objection to this method is that there is agglutination of the cytoplasmic particles; consequently agglutinated mitochondria are sedimented with the nuclear fraction and agglutinated microsomes are sedimented with the mitochondrial fraction. The mitochondria obtained by this procedure are not morphologically the same as those

of the intact cell (Hogeboom et al. 1948), since they are spherical and not rod-like in shape and do not stain with Janus Green B as do those of intact cells.

In 1948, Hogeboom et al. found that a preparation of mitochondria, uniform and fairly unaffected in the morphological and cytological sense, could be obtained if the tissue was homogenised in 0.88 M.- sucrose solution. The mitochondria, obtained by this procedure, retain their rod-like appearance and their ability to stain with Janus Green B. This method, however, requires very high centrifugal forces to separate the cytoplasmic particles. Moreover, the high concentration of sucrose used partially inhibits some of the enzyme systems present in the cytoplasmic fractions.

A number of more or less extensive modifications have subsequently been made in the basic method of Hogeboom et al. (1948). The most important variant of the sucrose method, now widely used, is that of Schneider (1948), in which 0.25 M.- (isotonic) sucrose replaces the 0.88 M.- sucrose previously utilised. This renders the preparation easier, in that lower centrifugal fields are required to cause sedimentation of the particles. Furthermore, it eliminates the inhibitory effect of high sucrose concentrations on certain enzyme systems and thus facilitates

biochemical studies (Schneider & Hogeboom 1950).

IIa. Mitochondria.

Mitochondria, in the narrower meaning of the term, are cytoplasmic particles of a magnitude between 0.5 - 1.0 μ, and stainable with Janus Green B.

The mitochondrial content of the cells varies greatly from tissue to tissue. Mitochondria, prepared from homogenates of rat liver in sucrose, accounted for 23-26% of the total nitrogen (Hogeboom et al. 1948; Schneider 1948; Schneider & Hogeboom 1950; Schneider & Potter 1949) and 30-33% of the total protein (Price, Miller & Miller 1948; Price, Miller, Miller & Weber 1949) of the original homogenate. The latter value is also obtained by calculation on the basis of succinoxidase activity. This enzyme, which is totally bound to the mitochondria, affords a suitable index of yield when mitochondria are isolated by centrifugation.

Chemically, mitochondria consist mainly of lipoprotein; in addition they contain small amounts of RNA and low-molecular weight components. Proteins, lipids and nucleic acid together account for 92% of the dry weight of the mitochondria (Ada 1949).

In the estimation of the RNA content of mitochondria, both cytologists and chemists have been confronted by the

same difficulty, namely to make observations on the relatively large mitochondria poor in nucleic acid, uninfluenced by the small, but nucleic acid rich microsomes.

Meyer (1920) was probably the first to show that mitochondria contained nucleic acid. Chantrenne (1947), and subsequently Jeener (1948), in their studies on the chemical and enzymic heterogeneity of the cytoplasmic particles, found that the fraction with the highest sedimentation rate, namely that which represented the purest possible mitochondrial fraction, showed the lowest RNA content. In terms of the dry weight, this content of RNA was about 0.5%.

Hogeboom et al. (1948) found that the RNA content of mitochondria decreased with increased washing of the preparation, until it finally attained a constant value. The value of the RNA content of mitochondria obtained by Hogeboom et al. (1948) agreed well with the findings of other workers (Schneider 1946a, 1948; Hogeboom 1949; Schneider & Potter 1949; Muntewyler, Seifter & Harkness 1950) and had a mean value of 11µg. RNA-phosphorus per mg. total nitrogen, which corresponds approximately to the percentage value obtained by Chantrenne (1947) and Jeener (1948).

Mitochondria have been shown by Opie and Lavin (1946)

to be Feulgen-negative, which indicates that they contain no DNA.

The lipid content, mainly phospholipid, of mitochondria has been estimated by several authors (Claude 1946; Schneider 1946a, 1946b; Barnum & Huseby 1948; Ada 1949) and was found to account for 25-30% of their dry weight.

Ada (1949) showed that the protein content of mitochondria may be taken as 65-70% of the dry weight. From the purely chemical point of view, the mitochondrial protein has not been the object of detailed study. The main interest in this protein fraction has centred round its enzymic properties. The earliest discovered enzymic property of mitochondria was their content of cytochrome oxidase and certain related respiratory enzyme systems (Hogeboom, Claude & Hotchkiss 1946). In 1948, Green, Loomis & Auerbach showed that mitochondrial preparations were capable of oxidising pyruvic acid, fatty acids and amino acids to CO_2 and H_2O through the citric acid cycle. The presence of ribonuclease and deoxyribonuclease was demonstrated by Schneider and Hogeboom (1952b), while Schneider, Claude and Hogeboom (1948) showed that cytochrome c was also to be found in the mitochondria. Uricase has been shown to be present by many workers (Schein, Podber &

Novikoff 1951; Schneider & Hogeboom 1952a; Novikoff, Podber, Ryan & Noe 1953).

IIb. Microsomes.

Microsomes are strongly basophilic, Feulgen-negative, submicroscopic particles of about 50-150 mμ in diameter (Claude 1940b). They are stained by iron haematoxylin (Vendrely 1950). The basophilic and siderophilic properties of microsomes are explained by their high content of RNA.

The microsomes constitute a very large proportion of the cytoplasm. In studies of rat liver fractions (Hogeboom et al. 1948; Schneider 1948; Schneider & Potter 1949), 18-20% of the total nitrogen of the whole tissue was accounted for by submicroscopic particulate material. The most striking chemical characteristic of the microsomes is their high content of RNA and lipids. The high proportion of RNA was first observed by Claude (1944, 1946). In later studies (Hogeboom et al. 1948; Schneider 1948; Schneider & Potter 1949; Schneider, Hogeboom & Ross 1950), it has been shown that approximately 50% of the RNA of whole rat or mouse liver is present in the microsomal fraction. When RNA phosphorus is expressed relative to nitrogen, a value of 63 μg. phosphorus per mg. nitrogen is obtained for the microsomal fraction compared with 27 μg. phosphorus per mg. nitrogen for the tissue as a whole.

The total lipid content of the microsomes is about 43% of the dry weight and 21% of the total lipid content of the cytoplasm (Claude 1946; Ada 1949; Huseby & Barnum 1950; Levin & Chargaff 1952). 30% of the dry weight consists of phospholipids, which corresponds to 65% of the total cytoplasmic phospholipids.

At present few enzymes are known to be located in the microsomes and still fewer appear to be specifically concentrated there. There appears to be no doubt of the presence of DPN-cytochrome c reductase (Hogeboom 1949; Hogeboom & Schneider 1950b) and TPN-cytochrome c reductase (Hogeboom & Schneider 1950a), the former being found in higher concentrations in the microsomes than in any other fraction. Hers, Berthet, Berthet and DeDuve (1951) have shown that the specific phosphatase, glucose-6-phosphatase, in liver and in kidney, as well as alkaline phosphatase of kidney and intestinal mucosa, are located in the microsomes. So marked is this concentration, that glucose-6-phosphatase activity has been used by DeDuve, Appelmans and Wattiaux (1952) as a test for microsomes.

IIc. Cell sap.

This fraction of the cytoplasm contains all the non-sedimentable or soluble material present in the cell, as well as any material, such as lipid droplets, which migrate

centripetally due to its low density. It is to be noted, however, that the preparation of this fraction is usually an arbitrary one, depending on the centrifugal force available. The conditions employed by Schneider and Hogeboom (1950) to prepare microsomes, sedimented particles as small as 50 mμ in diameter. Thus, cell sap, or supernatant, contained all the particles smaller than 50mμ. or, in terms of molecular weight, all particles having molecular weights less than about 100,000,000.

The soluble fraction comprises a considerable proportion of the tissue in terms of total nitrogen or protein. 32-44% of the total nitrogen of rat liver (Hogeboom et al. 1948; Schneider 1948; Schneider & Hogeboom 1950) and 49% of rabbit liver (LePage & Schneider 1948) have been recovered in this fraction.

RNA is also present in the cell sap, although generally its concentration in this fraction is lower than in the whole tissue. Hogeboom et al. (1948) and Schneider (1948) reported that 21-23% of the total RNA present in rat liver is found in the cell sap fraction.

The main component of the cell sap appears to be protein in nature. Thus, Price et al. (1949) reported that 39% of the total protein of rat liver was to be found in the cell sap. Among the enzymes, which have been reported present in the cell sap of rat and mouse liver, are cytochrome c

(Schneider et al. 1948; Schneider & Hogeboom 1950), isocitric dehydrogenase (Hogeboom & Schneider 1950a) and acid and alkaline phosphatases (Novikoff, Podber & Ryan 1950).

Julen, Snellman & Sylven (1950) fractionated mast cells with a view to investigating the intracellular distribution of heparin. They ground ox liver with isotonic phosphate buffer and fractionated the resulting homogenate into mitochondria, microsomes and a final supernatant, which they obtained by centrifuging for 5-6 hours at 60,000 g. The supernatant, which was so obtained, was found to contain 82% of the total heparin present in the homogenate. Micrographs of this material showed the presence of particles with an estimated diameter of less than 10 mμ. Electrophoretic and ultracentrifugal studies indicated that the heparin was bound to these particles in the form of a protein complex.

2. Estimation of Nucleic Acids.

Various methods have been described for estimation of nucleic acids in whole tissue and in the components of cells. The chemical methods for determining the nucleic acid content of a tissue may be based on (a) the phosphorus content; (b) the sugar content; and (c) the amount of purine(or pyrimidine) present. Whichever method of estimation is employed, it is

first necessary to remove from the tissue any interfering substances, such as simple nucleotides or nucleosides. It is, therefore, usual to first extract the tissue with acid, e.g. trichloracetic acid (TCA), to remove acid soluble phosphorus compounds, such as mono- and di-nucleotides, and then with lipid solvents to remove lipid phosphorus (LP). The residue contains acid-insoluble-non-lipid phosphorus (AINLP), which is protein bound and made up mainly of ribonucleic acid phosphorus (RNA-P)and deoxyribonucleic acid phosphorus (DNA-P) along with phosphoprotein phosphorus (PP), and small amounts of other phosphorus compounds.

In the fractionation scheme described by Schmidt and Thannhauser (1945), the extracted tissue residue, containing AINLP, is incubated overnight with warm dilute alkali which hydrolyses the RNA to acid soluble ribose mono-nucleotides, without affecting DNA in any way. When the alkaline digest is acidified, the DNA is precipitated along with the degraded protein, and may be determined as DNA-P. The soluble matter present in the supernatant fluid contains the nucleotides cytidylic acid, adenylic acid, guanylic acid and uridylic acid, derived from the RNA, which may be estimated as RNA-P. Any phosphoprotein phosphorus present may be determined by precipitation of the inorganic phosphorus from the acid supernatant fluid. The difference between the inorganic

phosphate and the total phosphate represents mainly the amount of RNA-P.

In the procedure of Schneider (1945), the nucleic acids are extracted from the AINLP with dilute TCA at 90°C for 15 minutes. The nucleic acids are split off as soluble products in the acid extract, which can then be determined by their ribose and deoxyribose content. Schneider at al. (1950) and Ogur, Minkler, Lindegren and Lindegren (1952) have modified the Schneider (1945) procedure by using perchloric acid in place of TCA.

It has been shown by Davidson, Gardner, Hutchison, McIndoe, Raymond and Shaw (1949) and Davidson, Frazer and Hutchison (1950) that the nucleic acid fractions obtained by these methods are contaminated with inorganic phosphate and that the phosphoprotein obtained by the Schneider (1945) method was always greater in amount than that obtained by the Schmidt and Thannhauser (1945) procedure. When the amount of RNA, obtained from the AINLP by the Schmidt and Thannhauser (1945) method is calculated from ribose estimations, and when it is fractionated by the method of ionophoresis, described by Davidson and Smellie (1952a) more phosphorus is found to be present than can be accounted for by the four nucleotides alone. Davidson and Smellie (1952b) have shown that in liver tissue only 80% of the phosphorus of the A2S,

obtained by the Schmidt and Thannhauser (1945) procedure, is nucleotide phosphorus, the remainder being derived from other phosphate esters.

The procedure described by Ogur and Rosen (1950), by which the AINLP residue is treated with N-perchloric acid for 18 hours at 4°C to remove RNA, and the residue, remaining after N-perchloric acid, is then treated with 0.5 N-perchloric acid for 20 minutes at 70°C to remove the DNA, was originally designed for plant tissues. With animal tissues, it is recommended that the DNA be extracted with N-perchloric acid at 80°C for 30 minutes. Ogur et al. (1952) have shown that with this procedure some of the DNA is extracted with the first acid extraction, and appears in the RNA fraction.

The methods of estimating nucleic acids by their sugar content are dependent on colour reactions of the pentose and deoxypentose sugars. Most of the techniques of determining pentose depend upon the liberation of furfural when the pentose-containing material is heated with hydrochloric acid. Reeves and Munro (1940) have described a procedure whereby the furfural formed is trapped in xylene and allowed to react with aniline acetate to form a red colour. This method has been used by Davidson and Waymouth (1944) for the estimation of RNA. Another method of estimating pentoses is dependent on their reaction with orcinol (Mejbaum 1939). This technique

has also been used by Davidson and Waymouth (1944) for the estimation of RNA. The most commonly employed method for deoxypentose estimation is the Dische (1930) diphenylamine reaction. This reaction is dependent upon the formation of a blue colour when DNA is heated with diphenylamine in acid solution. More recently, Ceriotti (1952) described a quantitative colour reaction with indole, which can be used for determining DNA.

The usual method of estimating nucleic acids based on their purine content is that of Graff and Maculla (1935), in which the purines are precipitated as copper complexes. Vendrely (1947) has modified this technique so that micro amounts of purine nitrogen may be determined. The main disadvantages of methods based on the purine content of nucleic acids, are that they are tedious to carry out and that they do not distinguish between RNA and DNA.

After the separation of RNA and DNA from the AILNP residues, by the method of Schmidt and Thannhauser (1945) or that of Ogur and Rosen (1950), the amount of nucleic acid in each fraction may readily be determined by the extinction of the solution in the ultraviolet in the quartz spectrophotometer (Ogur & Rosen 1950; Logan, Mannell & Rossiter 1952; Patterson & Dackerman 1952). When the extinction of the solution in the ultraviolet is to be used as a means of

determining the amount of nucleic acid in the solution, perchloric acid is a useful extracting and precipitating agent, since it has a lower extinction in the ultraviolet than does TCA, which absorbs strongly at 260 mμ.

Of all the methods available for the determination of the nucleic acid content of tissues, the Schmidt and Thannhauser (1945) procedure, in conjunction with the ionophoresis technique of Davidson and Smellie (1952a), remains the simplest and most convenient method of estimating the RNA content. The Schmidt and Thannhauser (1945) procedure alone, while it gives satisfactory results for DNA-P content, has a tendency to give high values for the RNA-P content. However, if the concomitant phosphorus compounds are removed from the ribonucleotides by ionophoresis, or the amount of RNA is determined by a method other than by phosphorus content, the Schmidt and Thannhauser (1945) procedure gives good results for the RNA content of the material.

Smellie, Kay, Humphrey and Davidson (1955) have recently employed a modification of the Kay, Simmon and Dounce (1952) method of isolation of DNA from tissues using the detergent sodium dodecyl sulphate (Empicol) to obtain a purified DNA fraction from the acid insoluble residue after alkaline digestion of the AINLP fraction.

Some of the results obtained for the nucleic

Table 1.

Nucleic Acid Content of Various Tissues.

(Method of Schmidt & Thannhauser 1945)

Tissue	Species	mg.P/100 g.fresh tissue		
		RNA-P	DNA-P	Ratio R/D
Liver	Rat (200-240g.)	77-110	21-25	4.0
	Rat (60-80g.)	106-122	28-37	3.6
	Rat (pregnant female)	110-118	21-23	5.2
	Rat (embryo)	87-134	35-65	2.2
	Rabbit	44-76	16-29	2.7
	Rabbit (pregnant female)	67-138	14-17	6.8
	Rabbit (embryo)	87-105	61-84	1.3
	Man	37-74	16-25	3.0
Pancreas	Rabbit	108-130	44-61	2.3
	Ox	170-185	21-22	8.1
	Man (one sample)	42	31	3.6
Kidney	Rat	25-47	33-43	0.7
Brain	Rat	20-33	15-19	1.5
Spleen	Rat (200-240g.)	63-86	76-85	0.9
	Rat (60-80g.)	70-82	68-78	1.0
	Rabbit	67-79	81-96	0.8
	Man (one sample)	36	77	0.5
Thymus	Rat (200-240g.)	87-116	181-242	0.5
	Rat (60-80g.)	114-135	181-261	0.6
	Rabbit	89-99	181-250	0.4
	Calf	80-100	224-250	0.4

Table 2.

Concentration of Nucleic Acids in Normal and Malignant Tissues as Determined by the Method of Schneider(1945) (Schneider & KLug 1946).

Tissue	mg. phosphorus per 100 g. fresh tissue	
	RNA-P	DNA-P
Rat skeletal muscle	4.7-10.4	3.6-9.4
Rat cardiac muscle	9.6-15.3	10.8-19.7
Rat brain	14.7-19.8	11.9-12.9
Rat lung	11.7-24.4	46.6-80.7
Rat kidney	24.9-30.2	33.1-42.6
Rat thymus	28.6-47.2	240-309
Rat spleen	36.7-53.7	115-135
Rat pancreas	147-234	37.2-48.2
Rat liver	55.0-71.5	20.8-31.3
Rat hepatoma	32.9-69.1	48.0-84.9
Flexner Jobling rat hepatoma	35.2-64.0	47.2-62.6
Jensen rat sarcoma	47.0-59.2	52.0-75.9
Walker No. 256 rat carcinosarcoma	52.4-65.0	61.3-72.1

acid content of tissues, using the Schmidt and Thannhauser (1945) procedure are given in Table 1. Results obtained by Schneider and Klug (1946), using the Schneider (1945) method of determining the nucleic acid content of tissues are given in Table 2. It can be seen that the straightforward Schmidt and Thannhauser (1945) procedure gives much higher RNA values than does the Schneider (1945) procedure.

With the aid of the cell fractionation techniques and using these methods of estimating nucleic acids several workers have studied the distribution of nucleic acids in the nuclei and cytoplasmic components of many tissues. Some of the results obtained are listed in Table 3. These results show that DNA is to be found exclusively in the nuclei, as was shown by histochemical techniques. The distribution of RNA in the cell is to be noted. The nuclear RNA appears to account for approximately 15 per cent of the total RNA of the cell. In the cytoplasm, the microsomes would appear to be richest in RNA, accounting for nearly 50 per cent of the cytoplasmic RNA, while the mitochondria contain only a small amount of RNA.

3. <u>The Biological Activity of the Nucleic Acids</u>.

The metabolism of the nucleic acids has been extensively studied by several groups of workers, using the isotope

Table 3.

The Distribution of RNA and DNA in Liver Cell Fractions.

Fraction	mg./100g. fresh tissue DNA-P	RNA-P	Tissue	Method of Isolation	Reference
Homogenate	22.6	65.2	Rat liver	Saline	Schneider (1946a)
Nuclear fraction	23.4	4.9			
Mitochondrial fraction	-	11.4			
Residue	-	47.6			
Homogenate	26.0	76.3	Rat liver	0.88M - Sucrose	Hogeboom et.al.(1948)
Cytoplasmic extract	-	59.3			
Nuclear fraction	25.8	14.4			
Mitochondrial fraction	-	13.6			
Microsome fraction †	-	26.5			
Supernatant fraction †	-	20.5			
Nuclear fraction		29.0	Rat liver	0.14M-KCl	Price et al. (1948)
Mitochondrial fraction		17.0			
Microsome fraction		31.0			
Supernatant fraction		9.0			
Nuclear fraction		9.0	Rat liver	0.88M-Sucrose	Price et al. (1948)
Mitochondrial fraction		33.0			
Microsome fraction		26.0			
Supernatant fraction		20.0			

Table 3 (cont'd).

Homogenate	27.1	82.8	Rat liver	0.25M - Sucrose	Schneider (1948)
Nuclear fraction	26.8	11.4			
Mitochondrial fraction	-	6.0			
Microsome fraction	-	43.5			
Supernatant fraction	-	19.2			
Homogenate	22.4	47.0	Rabbit liver	0.25M - Sucrose	LePage & Schneider (1948)
Nuclear fraction	22.3	13.1			
Mitochondrial fraction	-	4.6			
Microsome fraction	-	15.2			
Supernatant fraction	-	14.8			
Cytoplasmic extract		38.2	Rat liver	0.88M - Sucrose	Hogeboom (1949)
Mitochondrial fraction		10.4			
Microsome fraction		21.8			
Supernatant fraction		5.3			
Homogenate	28.5	78.9	Rat liver	0.25M - Sucrose	Schneider & Potter (1949)
Nuclear fraction	28.7	9.3			
Cytoplasmic extract	-	70.1			
Mitochondrial fraction	-	8.2			
Microsome fraction	-	36.3			
Supernatant fraction	-	26.2			
Homogenate	26.8	83.9	Rat liver	0.25M- Sucrose	Muntwyler et al. (1950)
Nuclear fraction	25.2	11.3			
Mitochondrial fraction	-	4.3			
Microsome fraction	-	41.6			
Supernatant fraction	-	30.4			

Table 3 (cont'd).

Cytoplasmic extract		69.5	Mouse liver	Saline	Huseby & Barnum (1950)
Mitochondrial fraction		8.6			
Microsome fraction		30.3			
Supernatant fraction *		18.0			
Homogenate	27.9	92.9	Mouse liver	0.25M - Sucrose	Schneider Hogeboom & Ross(1950)
Nuclear fraction	22.4	10.2			
Mitochondrial fraction	-	15.6			
Microsome fraction	-	48.7			
Supernatant fraction	-	15.3			

† The authors carry out separate estimations on the washings from the mitochondrial fraction and microsome fraction. For the sake of uniformity, the content of RNAP in these has been added on to the subsequent fraction in each case.

* The authors obtain a further particulate fraction from the microsomal supernatant by exceedingly high speed centrifugation. For the sake of uniformity, this has been included with the supernatant fraction.

techniques which have become available in recent years. The problem of nucleic acid turnover may be investigated by the administration of radioactive phosphorus (^{32}P), in the form of inorganic phosphate, to the experimental animal, or by administering compounds labelled with radioactive carbon (^{14}C) or heavy nitrogen (^{15}N). It is possible to study the metabolic activity of the nucleic acids, by determining the incorporation of these labelled compounds into the nucleic acids of the tissue.

It has been possible to examine the turnover rates of the bases of both RNA and DNA of various tissues, by using precursors of nucleic acids labelled with either ^{14}C or ^{15}N. Davidson and Raymond (1948) fed ammonium citrate labelled with ^{15}N to pigeons and rats, and found that the isotope was incorporated into the RNA of the liver, but that there was negligible incorporation into the DNA of the liver. In 1944, Plentl and Schoenheimer showed, with the aid of labelled guanine, that guanine did not act as a precursor of tissue nucleic acid purines or pyrimidines. Brown, Roll, Plentl and Cavlieiri (1948) found that labelled adenine was incorporated into the adenine, and to a lesser extent into the guanine, of tissue nucleic acids. Brown, Petermann and Furst (1948) fed labelled adenine to rats and found that in the RNA fraction of the viscera 15.9 per cent

of the adenine and 9.1 per cent of the guanine was derived from the dietary adenine. In the DNA fraction, the corresponding amounts were only 0.55 per cent and 0.32 per cent. LePage and Heidelberger (1951) obtained rapid incorporation of ^{14}C-glycine into the purines of both RNA and DNA of rat liver and tumour.

Several workers, among them Marshak and Calvet (1949), Jeener (1949a), Barnum and Huseby (1950), Jeener and Szafarz (1950) and McIndoe and Davidson (1952), have found that ^{32}P is incorporated much more rapidly into the nuclear RNA than into the RNA of the cytoplasmic components.

Davidson et al. (1951) showed that in experiments using ^{32}P it was not merely sufficient to separate the various phosphorus components of the tissues by some procedure, such as that of Schmidt and Thannhauser (1945), since the fractions containing RNA and DNA also contain other phosphorus compounds which have much higher activities than those of the nucleic acid fractions.

Jeener (1949a, 1949b) had recognised the dangers of contamination of the nucleic acid fractions, as prepared by the method of Schmidt and Thannhauser (1945), with compounds highly labelled with ^{32}P. To remove these active phosphorus compounds, Jeener (1949a, 1949b) added

carrier inorganic phosphate to the wash solutions used in preparing the nucleic acid fractions.

The ionophoretic procedure of Davidson and Smellie (1952a)has been shown to be invaluable in eliminating these active phosphorus compounds and for the purification of the RNA nucleotides for specific activity measurements.

The object of this work was to study the ribonucleic acids of the cell and to investigate the possible mechanisms by which the RNA of the different cell particles may be synthesised.

The initial approach to this investigation was made by studying the rate of incorporation of ^{32}P into the RNA of the cellular fractions in relation to time. This was done so that any differences in the rates of incorporation of the isotope into the various cytoplasmic particles and into the nuclei of liver cells might be determined. The physiological state of the experimental animal was then studied in relation to the incorporation of ^{32}P into the nucleic acids of the cytological components of liver cells.

Since <u>in vivo</u> studies did not appear to give any indications of the method of synthesis of the RNA of the cell, an attempt was made to establish an <u>in vitro</u> system whereby the synthesis of RNA might be studied.

PART ONE.

A Study of the Metabolism of the Phosphorus Compounds in Liver in vivo, Investigated with the Aid of Radioactive Phosphorus.

1. Introduction.

It has been shown by Marshak (1948), Marshak and Calvet (1949), Barnum and Huseby (1950) and Jeener and Szafarz (1950) that the incorporation of ^{32}P into the RNA of the cell nucleus is much more rapid than into the RNA of any cytoplasmic fraction.

Jeener (1949a), using mouse embryo, chick embryo and pigeon crop gland, has shown that the greatest incorporation of ^{32}P into the cytoplasmic RNA fractions was to be found in the RNA of the cell sap, with the incorporation into the RNA of the microsomes being the lowest of all. Jeener and Szafarz (1950), however, found that in resting rat liver, the greatest incorporation of ^{32}P into the nucleic acid fraction was to be found in the RNA of the microsomes. Barnum and Huseby (1950), on the other hand, found that with mouse liver the incorporation of ^{32}P into the RNA of the mitochondrial and microsomal fractions was approximately the same, while the incorporation into the cell sap RNA was markedly higher.

In the following work, the uptake of ^{32}P into the phosphorus compounds, and in particular the nucleic acids, of the nuclei and cytoplasmic fractions of rabbit liver have been investigated with relation to time. The effect of the physiological state of the experimental animal on

the uptake of ^{32}P into the phosphorus fraction of the liver have also been investigated, using fowls.

2. Methods.

(a) Biological.

In the experiments, in which the incorporation of radioactive phosphorus (^{32}P) at different times after administration of the isotope, was studied, the animals used were adult female chinchilla rabbits, bred in the department. The weights of these animals were between 2 and 3 kg. Throughout the experimental period, they were fed stock diet ad lib.

In the experiments, in which the effect of the physiological state of the animals in relation to the incorporation of ^{32}P was studied, the animals used were fowls. Birds of differing physiological states, e.g. cock, laying hen, hen at the end of its laying period and non-laying hen, were used. A non-laying hen bearing a GRCH 15 tumour (Peacock 1933) was also examined.

In both series of experiments the animals received 50 µc. ^{32}P/100 g. body weight, as carrier-free inorganic phosphate, by intramuscular injection.

In the rabbit experiments, in order that comparison

might be made between two animals receiving ^{32}P at different times and to eliminate differences in the amount of isotope injected, blood was withdrawn from the ear vein 2 hours after the injection of the isotope. The blood was collected in a beaker containing potassium oxalate, to prevent clotting, and set aside in the cold, before separating out the blood acid soluble inorganic phosphate fraction.

In the fowl experiments, it was not found practical to withdraw blood at 2 hours after the injection of the isotope, therefore a portion of the isotope solution injected was set aside so that the number of counts injected might be estimated.

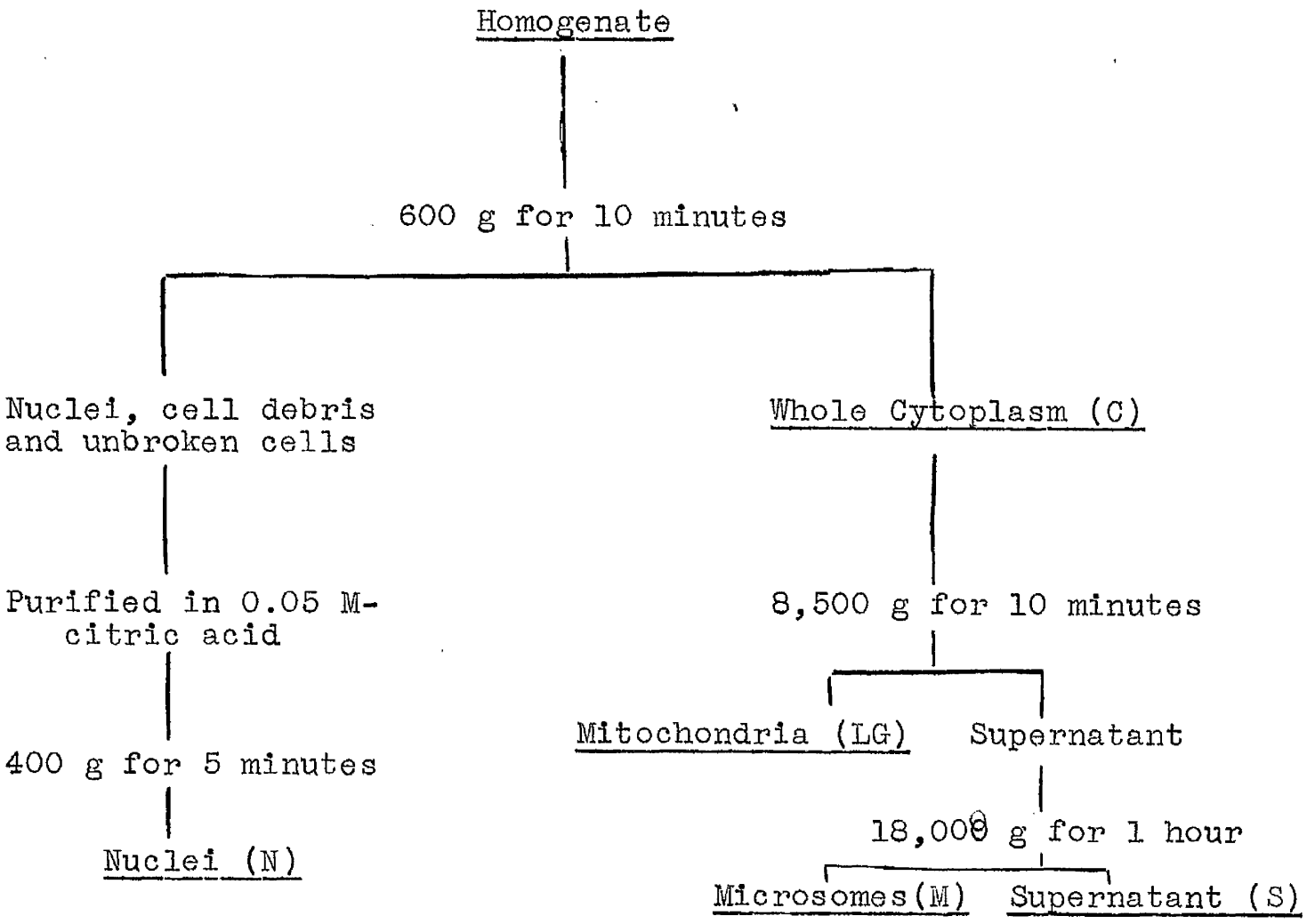
Rabbits were killed by cervical dislocation, at intervals of 2, 18, 24, 30, 37 or 48 hours after the injection of the ^{32}P. Fowls were killed 4 hours after the injection of the isotope. The abdomen was quickly opened and a sample of blood taken by opening the heart and collecting the blood into a beaker containing potassium oxalate.

The livers were perfused with 0.9% (w/v) sodium chloride solution through the portal vein, and were then rapidly excised, extraneous tissue removed, weighed and chilled in ice.

The liver tissue was finely minced with scissors and

FIGURE 1.

The Fraction of Cytoplasmic Fractions by the Schneider (1948) Procedure.



homogenised in 0.25 M.- sucrose, in the proportion of 1 g. of tissue to 4 volumes of sucrose solution. The homogenisation was carried out in a M.S.E. Nelco blendor (Measuring and Scientific Equipment Ltd. London, S.W.1) with the blades replaced by a paddle. This process was controlled microscopically and continued until optimal cellular breakdown with minimal nuclear breakdown was observed.

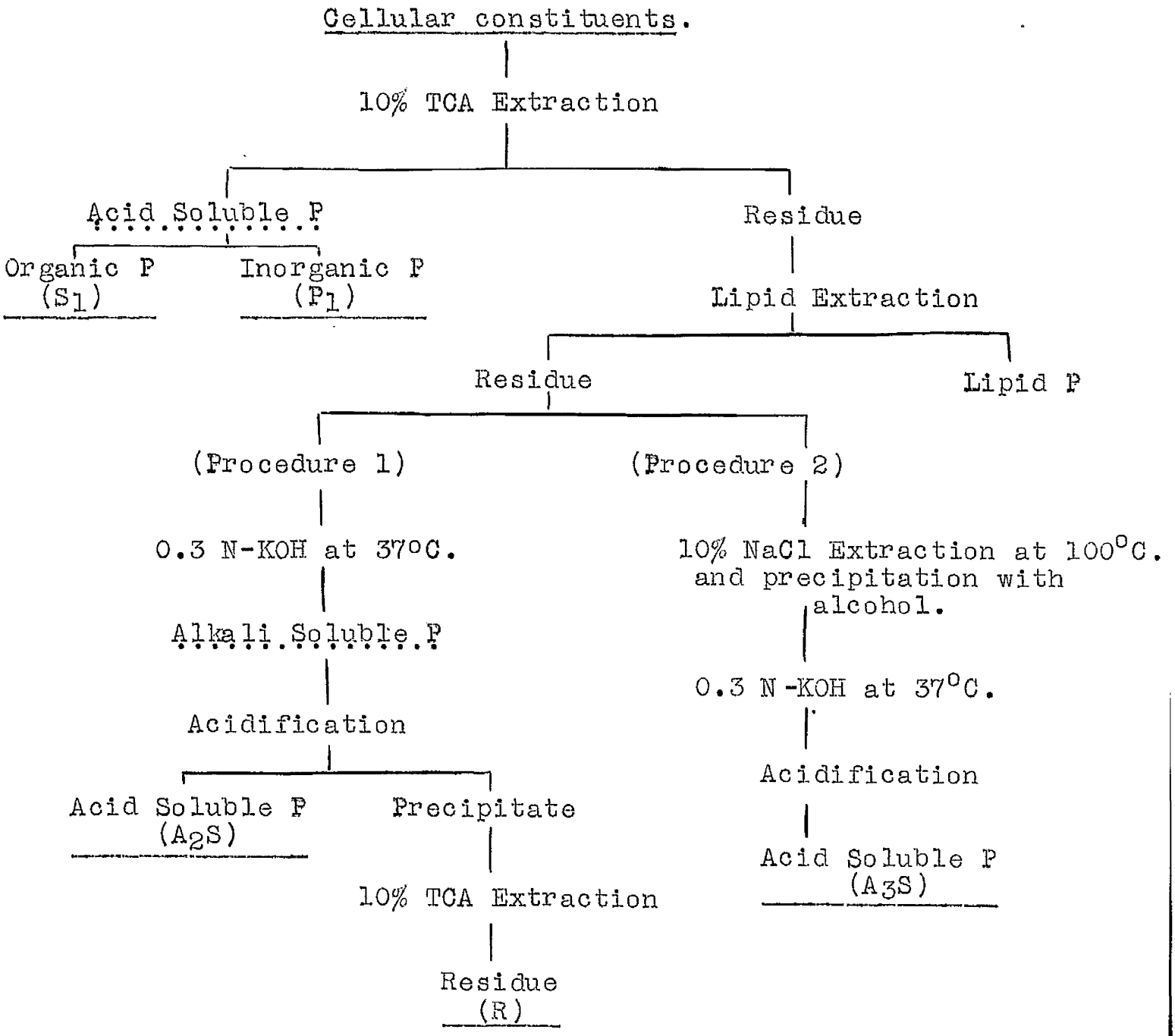
The homogenate, so obtained, was strained through gauze and fractionation (Schneider 1948) carried out as follows (Figure 1). The homogenate was centrifuged for 10 minutes at 600 g (I. E. C. refrigerated centrifuge, horizontal yoke). The supernatant fluid was decanted and the sediment washed twice with one volume of 0.25 M.- sucrose. The original supernatant and washings were combined and an aliquot taken for whole cytoplasm (C) analysis.

The sediment, which contained the nuclei, unbroken cells and cellular debris, was given to Dr. W.M. McIndoe, who isolated nuclei from it. The sediment was rehomogenised in 0.05 M.- citric acid, and the homogenate,so obtained,was centrifuged at 400 g (I. E. C.refrigerated centrifuge, horizontal yoke). The sediment was washed with 0.01 M.- citric acid, until microscopic examination showed that the nuclei were free from extraneous material. The isolated

FIGURE 2.

Scheme of Separation of Phosphorus Compounds in Tissues.

(Modified Schmidt & Thannhauser (1945) procedure).



nuclei (N) were then analysed.

The remainder of the sucrose suspension was then centrifuged for 10 minutes at 8,500 g (10,000 r.p.m.; I.E.C. multispeed attachment). The supernatant was decanted and the sediment collected. The sediment (LG) contained the large granules (mitochondria).

The supernatant, after sedimentation of the large granules, was centrifuged for one hour at 18,000 g (18,000 r.p.m.; I.E.C. multispeed attachment).

The supernatant (S), corresponding to the cell sap, was decanted and the sediment (M), containing the microsomes, collected.

The nuclei (N) and the cytoplasmic constituents (C, LG, M and S) were then submitted to a modification of the Schmidt and Thannhauser (1945) method of fractionation shown in Figure 2.

(b) Chemical.

The cellular fractions were suspended in distilled water, treated with 0.5 volume of 30% trichloracetic acid (TCA), and allowed to stand for 30 minutes in ice. The resulting precipitate was centrifuged down and the precipitate washed twice with ice cold 10% TCA. The sample of whole cytoplasm (C) was likewise treated with TCA and the acid extract and

washings combined and set aside for further fractionation into acid soluble inorganic (P_1) and acid soluble organic (S_1) phosphorus fractions.

The material precipitated by the TCA from the nuclei and all four cytoplasmic fractions were then extracted successively with acetone, ethanol, ethanol-$CHCl_3$(3:1), ethanol-ether (3:1) (twice) and ether. The extracts were collected, combined and taken to dryness on a hot water bath. The dry residue was extracted with $CHCl_3$ to provide the lipid fraction (Lipid P).

From the acid-insoluble-non-lipid (AINLP) residue RNA was extracted. Two different procedures were used to obtain RNA from this AINLP residue.

Procedure 1.

The method used to extract RNA from the AINLP obtained from the nuclei from both the rabbit and fowl liver tissue and from the cytoplasmic material from the rabbit tissue was as follows. The dry residue was suspended in distilled water (1 ml./150 mg. dry residue), 5 N-KOH added to a final concentration of 0.3 N-KOH, and incubated overnight at 37°C. The digest was acidified to pH 1 with 10 N-perchloric acid in the cold, and the precipitate centrifuged off. The extract was adjusted to pH 3.6 with 5 N-KOH and the precipitate of potassium perchlorate removed by centrifugation.

The supernatant (A2S) was decanted and retained.

The precipitate from the perchloric acid acidification was washed twice with 10% (w/v) TCA and the residue dissolved in N-NaOH. This solution was used to determine the deoxyribonucleic acid phosphorus (DNA-P).

Procedure 2.

It has been shown by Davidson and Smellie (1952b) that the A2S solution, prepared as described above, contains highly labelled phosphorus concomitant compounds, which give erroneous specific activity values for the RNA nucleotides. A modification of the Schmidt and Thannhauser (1945) procedure was therefore used to isolate the ribonucleic acid phosphorus (RNA-P) fraction from the cytoplasmic components of fowl liver.

The dry residue, obtained after extraction of the lipids, was extracted three times at 100°C with 25 ml. of 10% (w/v) NaCl. The NaCl solution removed most of the nucleic acids, together with some protein, from the tissue residue. The nucleic acids were precipitated from the salt extract by the addition of 2 volumes of absolute alcohol, and the precipitate washed with 70% alcohol (v/v), absolute alcohol and ether and dried. The dry powder was then incubated overnight at 37°C with 0.3 N-KOH, in the proportion of 20 mg. dried powder/ml. The hydrolysed material was then

acidified to pH 1 with 10 N-perchloric acid, and the acid extract readjusted to pH 3.6 with 5 N-KOH. The precipitated potassium perchlorate was removed by centrifugation, and the supernatant (A_3S) decanted and retained.

Aliquots of the blood, collected from the ear vein of the rabbits 2 hours after the injection of the isotope, and of the blood, collected at the time of killing of both rabbits and fowl, were treated with 0.5 volume of 30% (w/v) TCA and the precipitate centrifuged off. The TCA extracts of the blood and of whole cytoplasm were then fractionated into acid soluble inorganic phosphate (P_1) and acid soluble organic phosphorus (S_1) fraction as follows.

Mathison's (1909) reagent was added (1 ml./10 ml. extract) and the mixture made alkaline to phenolphthalein with NH_4OH (S.G. 0.88). After standing overnight in the refrigerator, the precipitate was centrifuged down and the supernatant decanted through a Whatman No. 42 paper. The precipitate was washed twice with 10% (v/v) NH_4OH and dissolved in N-HCl. This provided the inorganic phosphate fraction P_1. The mother liquor and washings were taken as acid soluble organic phosphorus fraction S_1.

Suitable portions of the phosphorus containing fractions were taken for determination of P by a modification of Allen's (1940) method. 1.2 ml. 10 N-H_2SO_4 was added to

the phosphorus solution in a 50 ml. micro-Kjeldahl flask and the mixture heated until it was colourless; where necessary a few drops of 100 vol. M.A.R. hydrogen peroxide were added to oxidise any organic matter present. After cooling, the acid solution was diluted with a little water, 1 ml. 8.3% (w/v) ammonium molybdate and 2 ml. Amidol reagent (2 g. Amidol, 40 g. sodium bisulphite and 200 ml. water), and the total volume made up to 25 ml. with water. The blue colour, which developed, was measured between 10 and 30 minutes after the addition of the reagents, on a Spekker absorptiometer, using an Ilford red filter (No. 608). The reading was converted into µg. P from a curve obtained using standard phosphorus solutions.

For the determination of radioactivity, portions of each of the blue solutions, used for the determination of phosphorus content, were pipetted into a liquid counter (Type M6, manufactured by 20th Century Electronics) attached to a conventional probe unit and a scaling unit (Type 200, manufactured by Dynatron Radio, Ltd.). All specific activities (S.A.) were calculated as counts/min/100 µg. P. In the experiments on rabbits, it was felt necessary to have a common denominator to which all specific activities might be related, especially when results obtained at different time intervals were being compared. The specific activity

of the blood P_1 at 2 hours, after the injection of the isotope, was determined and used for the calculation of relative specific activities (R.S.A.). Where fowls were used, since it was not practicable to obtain blood 2 hours after the administration of ^{32}P, 0.1 ml. of the isotope solution administered was diluted to 100 litres and the counts per minute of the administered solution determined. All the specific activities were then calculated relative to an administered dose of 10^{M} counts/min., to give the R.S.A.

The ionophoresis procedure of Davidson and Smellie (1952a) was used to separate the nucleotides of RNA from the A_2S and A_3S solutions. Portions of the A_2S and A_3S solutions were applied to strips of Whatman 3m.m. filter paper(73 cm. x 7 cm.) at a distance of 7 cm.from one end. The solution was applied to the paper, using an Agla micrometer syringe (manufactured by Burroughs Wellcome & Co., London) with a blast of cold air behind the paper to speed drying. The volume of solution applied to the paper was adjusted so that it contained between 60 and 90 µg. P. The paper was then wetted with 0.02 M-citrate buffer pH 3.6(33 ml. M.- citric acid, 11 ml. M-trisodium citrate made up to 2.2 litres). The wet paper was then suspended over a glass rod so that each end dipped 1 cm. into the vessels, which contained 500 ml. citrate buffer, so that the cathode was nearer the spot on the paper. The whole apparatus was enclosed

in a glass case. The electrodes were connected to a D.C. supply, consisting of a valve rectifier capable of a smoothed output of 600 V. and 300mA., but which on a small load delivered about 800 V. The output was controlled by means of a Variac transformer in the input stage, making available a continuous range of output from 100-800 V. The current was then switched on, and the voltage adjusted so that the fall in potential along the paper was approximately 11 V./cm. At the end of 18 hours, the current was switched off and the paper dried by suspending it in fromt of two 250 W. infrared lamps. The nucleotide spots on the dried paper were located in ultra-violet light, by the procedure of Holiday and Johnson (1949). The nucleotides were recovered quantitatively on elution, by the technique of Consden, Gordon and Martin (1947), into graduated tubes and the eluate made up to 5 ml. with distilled water. The radioactivity was determined by pipetting 4 ml. into a liquid counter. The solution was returned to the same tube after the count was completed. The determination of P was then carried out by the modified Allen (1940) method, using one fifth of the amounts of all the reagents.

Results

Rabbit Liver Experiments

In preliminary experiments some difficulty was encountered in relating the specific activity of the various phosphorus fractions to the amount of isotope administered owing to variations in dosage and in adsorption of the ^{32}P from the site of injection. It was therefore necessary to find a suitable factor in terms of which the results of radioactivity measurements might be expressed. The factor chosen was the specific activity of the blood inorganic P (Blood P_1) 2 hours after the administration of the ^{32}P, which was taken as 1,000, and the specific activities of all other phosphorus fractions were calculated in relation to it. By this means, it was hoped to eliminate any differences between the animals and the isotope administered.

The Relation of the Activities of Rabbit Liver Inorganic and Acid Soluble Organic Phosphates with Time.

Table 4 and Figure 3 show the relationship between the time of administration of ^{32}P and the relative specific activities of blood inorganic P (Blood P_1), blood acid soluble organic P (Blood S_1), cytoplasmic inorganic P (Cytoplasmic P_1) and cytoplasmic acid soluble organic P (Cytoplasmic S_1).

The relative specific activity of Blood P_1 falls rapidly from 2 hours to 18 hours after the injection of the

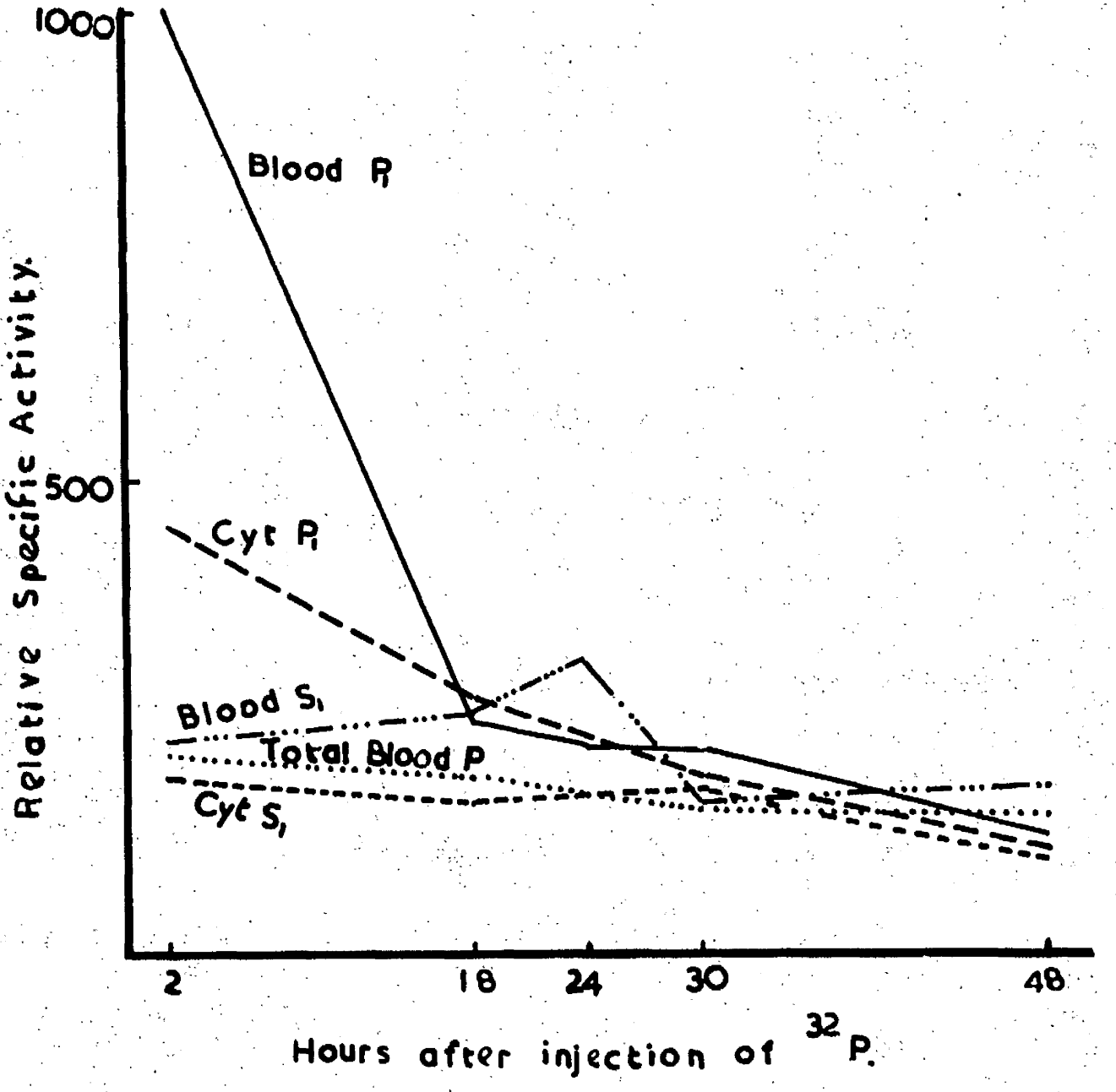
Table 4.

Specific Activities of Total Blood Phosphorus, Blood Acid Soluble Inorganic and Acid Soluble Organic Phosphate and Whole Cytoplasmic Acid Soluble Inorganic and Acid Soluble Organic Phosphate of Rabbits, Related to Blood Inorganic Phosphate at 2 hours, as 1,000.

	Hours after Injection of ^{32}P				
	2	18	24	30	48
Total Blood Phosphate	210	189.5	169	165	134
Blood Acid Soluble Inorganic P	1000	242	217	213	122
Blood Acid Soluble Organic P	220.4	247	304	159.5	173
Cytoplasmic Acid Soluble Inorganic P	458	269	229	187	111.5
Cytoplasmic Acid Soluble Organic P	187	162	174	129.5	101.5

Figure 3

Specific Activities of Blood and Whole Cytoplasm Acid Soluble Phosphorus Compounds, Related to Blood P_i at 2 Hours as 1,000.



isotope. There then appears to be a gradual fall in activity to the lowest value at 48 hours after the injection of ^{32}P.

The relative specific activity of Blood S_1 shows very little increase between 2 hours and 18 hours, where it would appear to have the same relative specific activity as Blood P_1. There is a slight increase in activity to the 24 hours level, from whence it would appear to fall off fairly rapidly to the 30 hour level. Thereafter, the level of activity of Blood S_1 would appear to rise very slightly to the value obtained at 48 hours after the administration of the isotope.

Cytoplasmic P_1 shows a fairly gradual decrease in relative specific activity between 2 hours and 48 hours after the administration of ^{32}P, where the lowest value for Cytoplasmic P_1 was recorded.

Cytoplasmic S_1 shows very little change in relative specific activity between 2 hours and 30 hours, after which it apparently falls to the 48 hour level of activity. The relative specific activity of Cytoplasmic S_1 would appear to have a lower level of activity at all the time intervals investigated, other than at 30 hours after the injection of the isotope.

The Relation of the Activities of the Phospholipid of Rabbit Liver Cytoplasmic Fractions with Time.

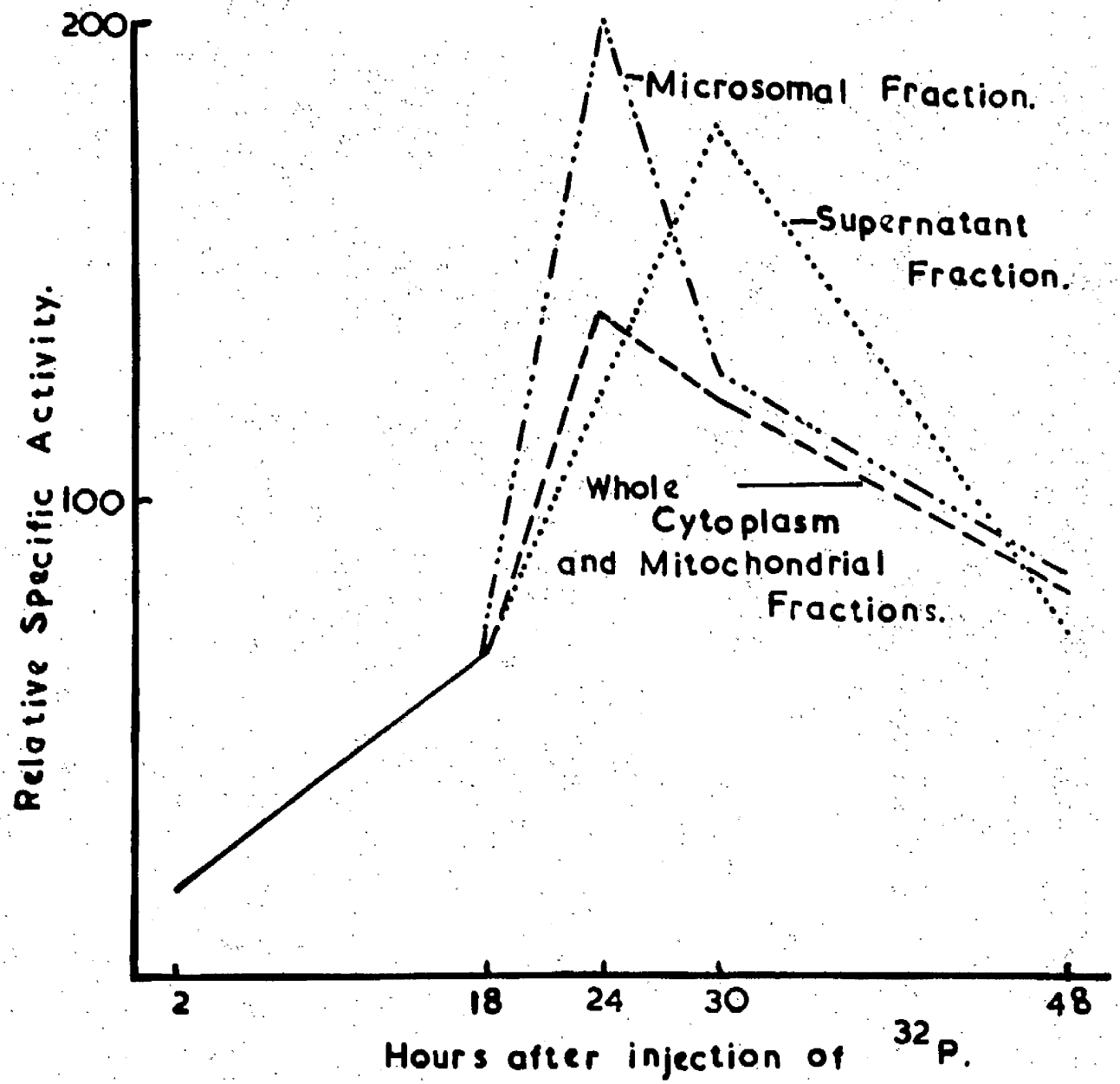
Table 5.

Specific Activities of Phospholipids of Whole Cytoplasm, Mitochondrial, Microsomal and Supernatant Fractions of Rabbit Livers, Related to Blood Inorganic Phosphate at 2 hours as 1,000.

	Hours after Injection of ^{32}P				
	2	18	24	30	48
Whole Cytoplasm	9.2	72	138	122.5	98.4
Mitochondrial Fraction	8.2	66.4	138	119	98.8
Microsomal Fraction	9.95	71.6	197	126.5	101.2
Supernatant Fraction	8.22	66.7	118	175	91.2

Figure 4.

Specific Activities of the Phospholipids of the Cytoplasmic Cellular Fractions, Related to Blood P_i at 2 Hours as 1,000.



During the time interval 2–18 hours, after injection of ^{32}P, all of the cytoplasmic fractions have approximately the same uptake of ^{32}P.

After the sedimentation of the microsomal fraction there was a lipid layer on the surface of the supernatant liquid. The supernatant was decanted without disturbing this lipid layer, which was later collected along with the microsomal fraction. It was not always possible to avoid transferring part of the lipid layer with the supernatant liquid. It is therefore very difficult to state exactly the origin of the lipid material extracted from the various cytoplasmic fractions in the initial stages of the modified Schmidt & Thannhauser (1945) procedure.

Table 5 and Figure 4 show the specific activities of the phospholipids extracted from the whole cytoplasm and the cytoplasmic fractions. These values were again related to Blood P_1 at 2 hours after the injection of ^{32}P.

The rate of uptake of ^{32}P during the time interval 2 to 18 hours after the administration of ^{32}P is fairly constant for all fractions. The Cytoplasmic and Mitochondrial phospholipid levels rise more rapidly to the 24 hour levels of activity, thereafter falling off more gradually to their 48 hour levels of activity. After 18 hours, the rate of uptake of the microsomal phospholipid rises very rapidly to its maximum value at 24 hours, then it appears to fall rapidly to the 30 hour value, after which it fell off at a much less rapid rate to the 48 hour value. The supernatant phospholipid

activity, on the other hand, appears to show a gradual uniform rise to a maximum at 30 hours, before dropping gradually to its 48 hour level of activity.

The Relation of the Nucleic Acids of the Nuclear and Cytoplasmic Fractions of Rabbit Liver with Time.

In order to determine whether any breakdown of nuclei had occurred during homogenation, with the liberation of deoxyribonucleic acid phosphorus (DNA-P), the DNA-P content of each of the cytoplasmic fractions was estimated.

The dry residue obtained by the modified Schmidt & Thannhauser (1945) procedure was digested with 0.3 N-KOH overnight and then acidified with 12 N-perchloric acid. The supernatant (A_2S) was then used to determine the activities of the nucleotides. The precipitate was washed twice with 10% (w/v) TCA and then dissolved in N-NaOH and phosphorus determinations carried out on this alkaline solution as previously described. The phosphorus of this solution is present mainly as DNA-P, but it is possible that other organic phosphorus-containing compounds are also present, as was shown to be the case with bacterial preparations by Mitchell and Moyle (1951). The DNA-P was compared with the phosphorus content of the A_2S solution. The values obtained are given in Table 6. It can be seen that there was very

Table 6.

Average "Deoxyribonucleic Acid Phosphorus" Present in the Ribonucleotide Solution Obtained from the Whole Cytoplasm, Mitochondrial, Microsomal and Supernatant Fractions of Rabbit Liver

	µg "DNA-P"/100µg RNA-P.
Whole Cytoplasm	3.7
Mitochondrial Fraction	5.9
Microsomal Fraction	8.2
Supernatant Fraction	4.4

Table 7.

Specific Activity of the Deoxyribonucleic Acid Present in the Nuclei of Rabbit Liver, Related to Blood Inorganic Phosphate at 2 hours as 1,000.

	Hours after the Injection of ^{32}P.				
	2	18	24	37	48
DNA	3.7	14.6	8.15	7.88	3.17

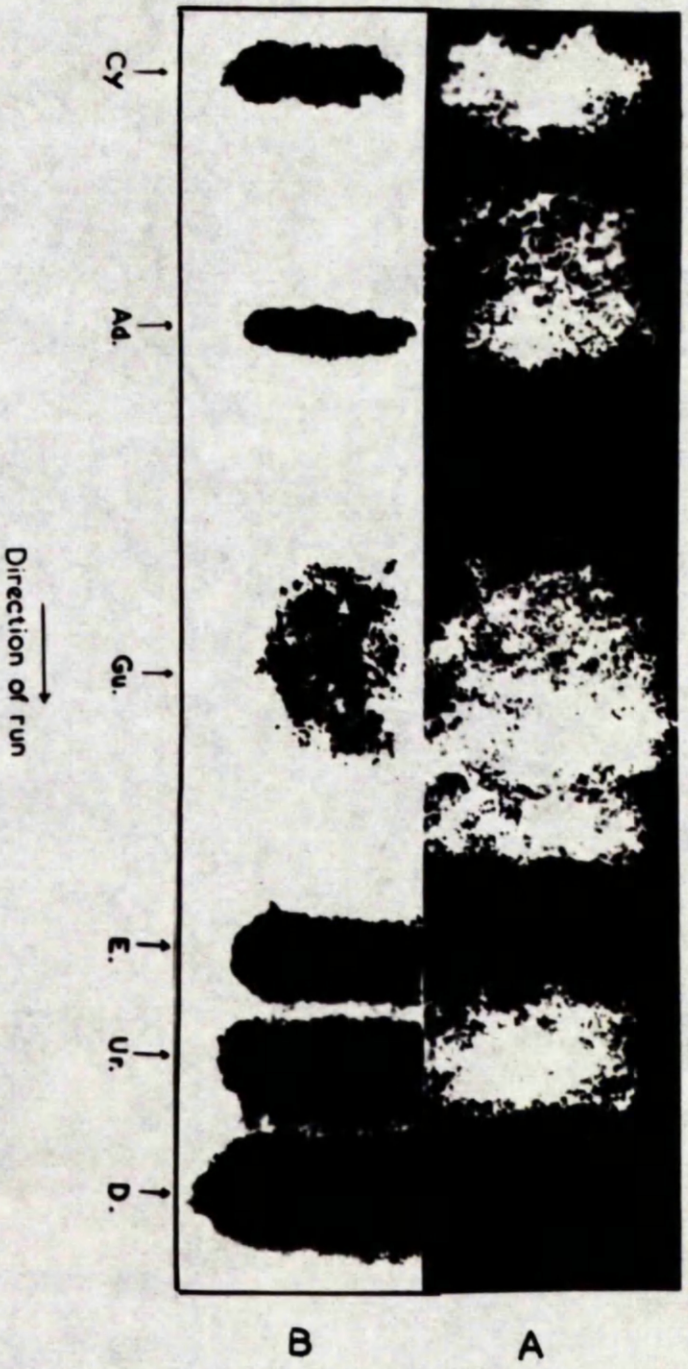
Figure 5.

Ultraviolet print (A) and autoradiograph (B) of the corresponding ionophoretic run of the ribonucleotide fraction of rabbit liver prepared by procedure 1.

Separation of the nucleotides is adequate, but uridylic acid is accompanied by components D and E which appear in the autograph but not in the ultraviolet print.

Cy., cytidylic acid; Ad., adenylic acid;
Gu., guanylic acid ; Ur., uridylic acid.

Figure 5.



little DNA-P present in the A_2S solution of the cytoplasmic fractions, the microsomal fraction containing the most (8.2 µg. DNA-P/100 µg. A_2S-P). Since rabbit liver nuclei have been shown by Davidson and McIndoe (1949) to contain approximately 10% of the total nucleic acid as RNA, there can be little contamination of the cytoplasmic fractions by nuclear RNA(less than 1% nuclear RNA present) due to nuclear breakdown.

Davidson, Gardner, Hutchison, McIndoe, Raymond and Shaw (1949) and Davidson, Frazer and Hutchison (1951) have commented on the contamination of the ribonucleotide (A_2S) fraction obtained by application of the modified Schmidt & Thannhauser (1945) procedure. The A_2S solution was therefore subjected to ionophoresis (Smellie & Davidson 1952a) in order to separate the nucleotides and to eliminate contamination by small amounts of highly active inorganic and organic phosphates. The separation of the nucleotides achieved by this method is illustrated in Figure 5A, (obtained by photographing an ionophoretic strip in ultraviolet light), from which it is apparent that a good resolution of the nucleotides can be obtained. The nucleotides separate in the order cytidylic acid, adenylic acid, guanylic acid and uridylic acid, in increasing order of mobility.

Preliminary experiments indicated that uridylic acid occurred in association with organic phosphorus compounds of high specific activity. The presence of these organic P

compounds can be demonstrated by placing the filter paper strip, on which the nucleotides have been separated by ionophoresis, in contact with an X-ray film (Standard X-ray film, Code 6, manufactured by Kodak Ltd., London) for 2 weeks. On developing the X-ray film, radioautograph of the ionophoretic strip is obtained (Figure 5B). Radioautographs show the presence of an organic P compound immediately preceding uridylic acid (Component D), and one following immediately after uridylic acid (Component E). Component D was taken as a 3 cm. strip immediately in front of the uridylic acid spot and E was taken as the 2 cm. strip immediately behind the uridylic acid spot, together with 1 cm. of the trailing edge of the uridylic acid spot as seen in ultraviolet light. Even taking these precautions to remove the concomitants from the uridylic acid fraction, the relative specific activities of this fraction were still much higher than were expected, suggesting that there were still concomitants present. Hultin, Slautterback and Wessel (1951) reported that the uridylic acid fraction which they obtained by separating a chicken liver ribonucleotide solution, labelled with ^{32}P, on a Dowex 50 (cation exchange) column was also grossly contaminated.

Table 7 shows the relative specific activity (RSA) values obtained for the DNA of the purified nuclear fraction. Table 8 shows the RSA values obtained for the ribonucleotides

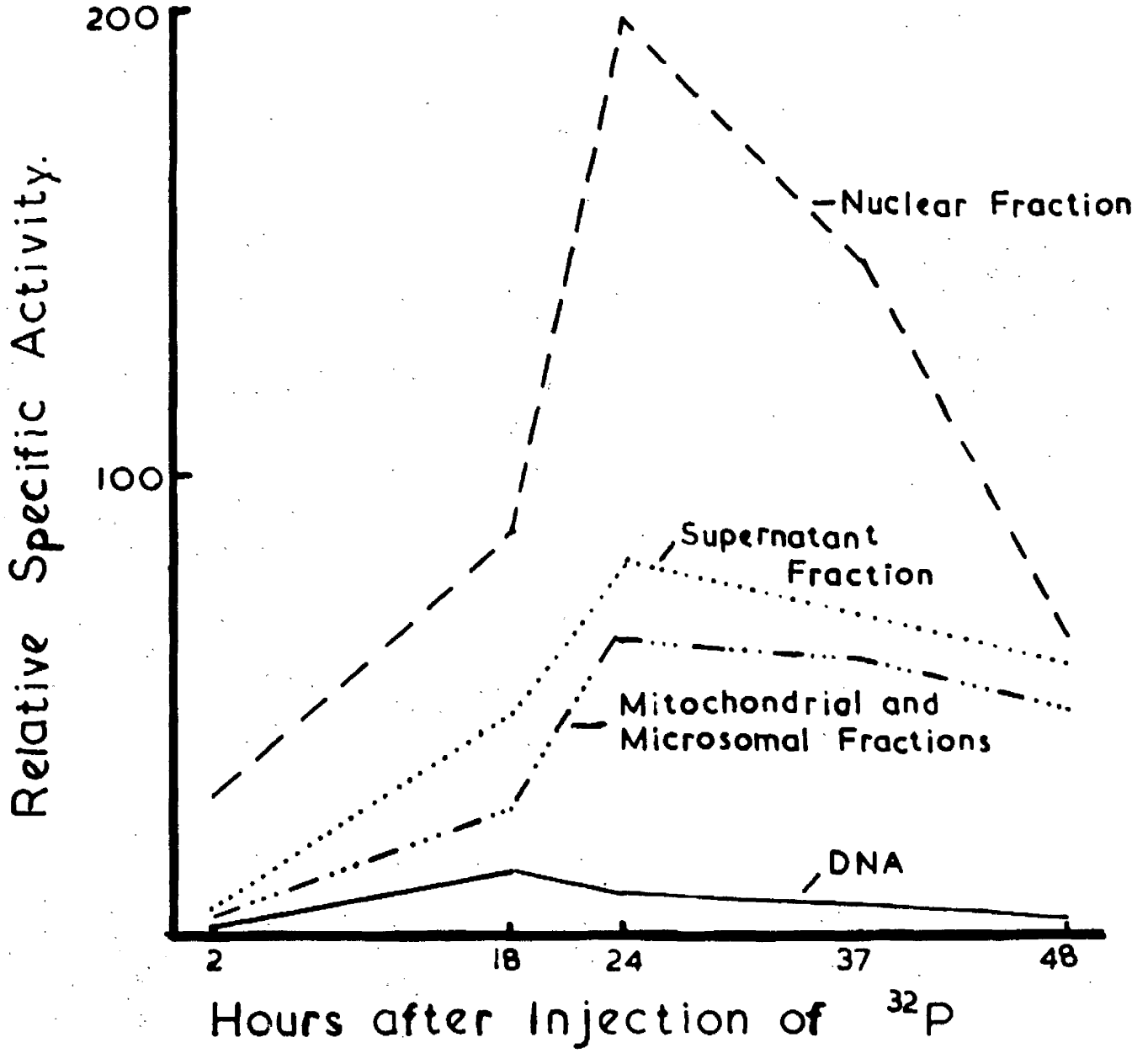
Table 8.

Specific Activities of the Nucleotides from the Ribonucleic Acids of the Nuclei and of the Cytoplasmic Fractions of Rabbit Liver, Related to Blood Inorganic Phosphate at 2 hours as 1,000.

Fraction	Nucleotide	Hours after the Injection of ^{32}P				
		2	18	24	37	48
Nuclear Fraction	Cytidylic Acid	27.3	95.3	194	133	81.9
	Adenylic Acid	30.0	89.6	199	146	65.5
	Guanylic Acid	24.6	76.1	162	137	85.1
	Uridylic Acid	22.0	131.0	166	138	80.5
Whole Cytoplasm	Cytidylic Acid	3.46	25.1	70.5	59.8	53.2
	Adenylic Acid	3.84	24.0	65.5	59.8	63.3
	Guanylic Acid	2.66	23.1	64.5	57.0	48.0
	Uridylic Acid	5.61	22.5	62.0	32.4	49.7
Mitochondrial Fraction	Cytidylic Acid	1.63	23.5	67.2	63.5	54.5
	Adenylic Acid	2.11	26.7	72.0	64.4	49.4
	Guanylic Acid	1.43	25.1	66.8	65.6	52.6
	Uridylic Acid	4.35	20.5	68.2	60.3	56.3
Microsomal Fraction	Cytidylic Acid	0.92	22.9	68.0	60.8	53.0
	Adenylic Acid	1.21	26.3	62.1	57.8	53.2
	Guanylic Acid	1.14	23.9	71.5	49.4	49.8
	Uridylic Acid	2.4	23.5	66.5	57.8	48.3
Supernatant Fraction	Cytidylic Acid	3.88	27.6	68.0	67.7	54.4
	Adenylic Acid	4.82	48.0	69.5	57.6	56.3
	Guanylic Acid	3.01	33.1	69.1	57.2	62.5
	Uridylic Acid	8.1	51.1	79.8	65.8	41.1

Figure 6.

Specific Activities of DNA and of the Adenylic Acids from the RNA of the Nuclear and Cytoplasmic Cellular Components, Relative to Blood P_i at 2 Hours as 1,000.



Throughout the time intervals investigated, the mitochondrial and microsomal fractions have approximately the same Relative Specific Activities.

of the nuclei and cytoplasmic particles. In Figure 6, the RSA of the DNA fraction has been plotted against time. On the same figure has also been plotted the RSA of the adenylic acid fraction of the nuclear and cytoplasmic RNA's. The other three nucleotides give similar patterns.

The relative specific activity-time curves of the nucleotides of Whole Cytoplasm and of the Mitochondrial and Microsomal fractions all follow each other fairly closely and the rate of uptake of ^{32}P is apparently the same in all cases. There is an initial slow rate of incorporation of ^{32}P up to 18 hours, thereafter the rate increases markedly until,24 hours after the administration of the isotope, the maximum is reached. After the 24 hour level of activity is reached, there is a gradual decrease in activity until the 48 hour level is attained. The supernatant nucleotide activity, on the other hand, is slightly higher at all times although it follows the same type of curve.

In all cases the highest level of activity at all times was to be found in the nucleotides derived from nuclear RNA. 2 hours after the administration of the isotope, the nuclear nucleotides show an activity approximately ten times that of any of the cytoplasmic fractions. The activity increases relatively slowly until 18 hours, after which it increases rapidly until its maximum is reached at 24 hours. The activity

then drops rapidly until the 48 hour level of activity is reached.

The activity of the DNA was found to be low at all times. There was a small increase in activity between 2 and 18 hours after the injection of ^{32}P, after which there appeared to be a very gradual decrease in activity until the 48 hour level of activity was reached.

These results have been included in a paper published by Smellie, McIndoe, Logan, Davidson and Dawson (1953).

Fowl Experiments.

In preliminary experiments, using fowls, it was not found to be practicable to withdraw a blood sample from the bird at 2 hours after the administration of the isotope. In order that there should be a common factor between individual experiments, a sample of the injected isotope solution was counted and all the specific activities obtained were calculated relative to this

The Relationship of the Activities of Inorganic and Acid Soluble Organic Phosphates to the Differing Physiological Conditions of the Experimental Birds.

There is a very wide scatter of results, as shown in Table 9 and Figure 7. In the two laying hens studied, only the relative specific activities of the cytoplasmic acid soluble organic P

Table 9.

Specific Activities of Blood Acid Soluble Inorganic Phosphate and Acid Soluble Organic Phosphate and Liver Whole Cytoplasm Acid Soluble Inorganic Phosphate and Acid Soluble Organic Phosphate of Fowls, Related to Counts/minute injected (x 10^7).

	LHI	LHII	C	TBH	T	N.L.H.
Blood Inorganic P	3,400	6,780	47	32.6	-	232
Blood Organic P	228	3,270	278	86	-	4,100
Cytoplasmic Inorganic P	3,640	14,500	1,840	306	285	26,000
Cytoplasmic Organic P	13,000	13,550	1,260	237	215	22,400

LHI - Laying hen at middle of laying period

LHII - Laying hen at end of laying period.

C - Cock

TBH - Hen bearing GRCH 15 tumour

T - GRCH 15 tumour

N.L.H.- Non-laying hen.

Figure 7.

L.H.I. Laying hen at middle of laying period.

L.H.II. Laying hen at end of laying period.

C. Cock

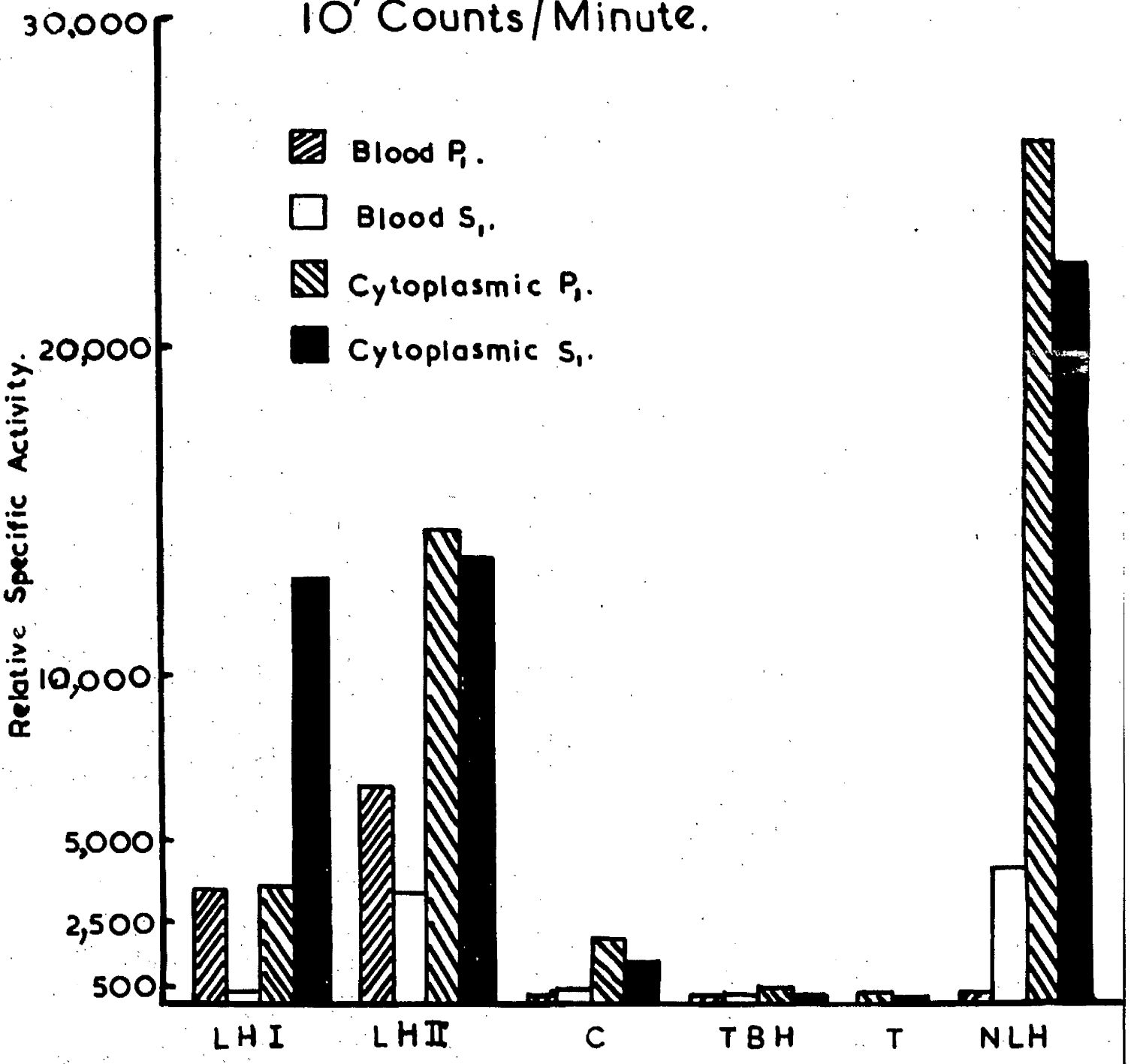
T.B.H. Hen bearing GRCH 15 tumour.

T. GRCH 15 tumour.

N.L.H. Non-laying hen.

Figure 7.

Specific Activities of Blood and Tissue Acid Soluble Phosphorus Compounds, Relative to an Injected Dose of 10^7 Counts/Minute.



show any agreement. The hen which is in the middle of its laying period shows much lower activities in the Blood P_1, Blood S_1 and Cytoplasmic P_1 in comparison with the values observed for the hen at the end of its laying period. The cock used in this experiment showed a very low uptake of ^{32}P into all of the inorganic and acid soluble fractions of blood and cytoplasm. The lowest incorporation of ^{32}P was observed in the hen bearing a GRCH 15 tumour. In the case of the non-laying hen, the value of the Blood P_1 at 4 hours after the injection of the isotope was low, while that of the Blood S_1 was approximately the same as that recorded for the hen at the end of its laying period. The Cytoplasmic P_1 and S_1 fractions of the non-laying hen were extremely high. In all instances, except in that of the laying hen in the middle of its laying period, the Cytoplasmic S_1 fraction showed a lower activity than that of the Cytoplasmic P_1 fraction, while in all instances both fractions were appreciably higher than the values observed for the Blood phosphorus fractions. The Cytoplasmic P_1 and S_1 fractions of the tumour tissue itself had approximately the same level of activities as those of the liver cytoplasmic phosphorus components obtained from the same bird.

<u>The Relationship of the Activities of the Phospholipids of</u>

Table 10.

Specific Activities of Phospholipids of Whole Cytoplasm, Mitochondrial, Microsomal and Supernatant Fractions of Fowl Livers, Related to Counts/minute injected (x 10^7).

	LHI	LHII	C	TBH	T	N.L.H.
Whole Cytoplasm	6,540	8,600	3,610	134	26.1	10,600
Mitochondrial Fraction	5,720	7,390	3,190	124	-	7,250
Microsomal Fraction	6,250	8,550	5,000	158	-	5,850
Supernatant Fraction	6,380	6,750	4,400	72.9	-	6,250

LHI - Laying hen at middle of laying period.

LHII - Laying hen at end of layingpperiod.

C - Cock

TBH - Hen bearing GRCH15 tumour

T - GRCH15 tumour

N.L.H. - Non-laying hen.

Figure 8.

L.H.I. Laying hen at middle of laying period.

L.H.II. Laying hen at end of laying period.

C. Cock.

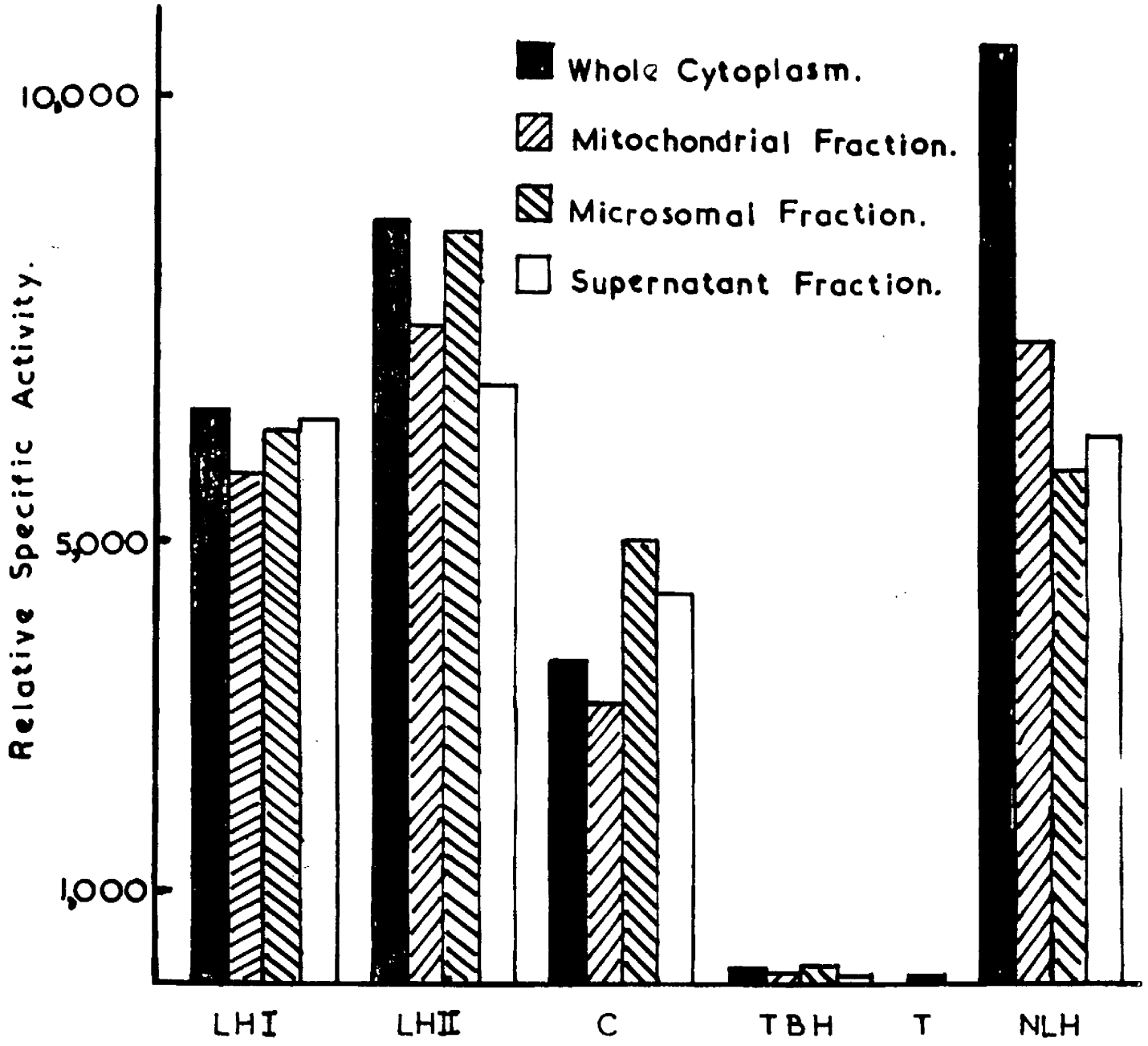
T.B.H. Hen bearing GRCH 15 tumour

T. GRCH 15 tumour.

N.L.H. Non-laying hen.

Figure 8.

Specific Activities of the Phospholipids of the Cytoplasmic Cellular Components, Relative to an Injected Dose of 10^7 Counts/Minute.



the Cytoplasmic Fractions with Respect to the Differing Physiological Conditions of the Experimental Birds.

The phospholipids isolated from the cytoplasmic particles of any one experimental bird show approximately the same level of activity. This is illustrated in Table 10 and Figure 8. The main difference to be observed is that the tumour-bearing bird again shows the lowest levels of activities, while the values obtained from the liver of the cock appear to be slightly lower than those obtained from the livers of the hens, no matter whether they are in lay or not.

The Relationship of the Activities of the Ribonucleotides of the Cytoplasmic Particles with Respect to the Differing Physiological Conditions of the Experimental Birds.

In this series of experiments the second procedure, previously described, was used to prepare the ribonucleotides from the AINLP residue, obtained after extraction of the tissue with TCA and lipid solvents. It has been found in preliminary experiments that extraction of the RNA from the AINLP residue with NaCl, prior to alkaline hydrolysis, removed most of the highly active concomitant phosphorus compounds. The ionophoresis of the nucleotide solution, so obtained, showed only the presence of the four ribonucleotides. A typical separation obtained by this procedure is shown in Figure 9. It can be seen that

Figure 9.

Ultraviolet print (A) and autoradiograph (B) of the corresponding ionophoretic run of the ribonucleotide fraction of fowl liver prepared by procedure 2.

The nucleotides are adequately separated and no concomitants are visible.

Cy., cytidylic acid ; Ad., adenylic acid;
Gu., guanylic acid ; Ur., uridylic acid.

Figure 9.

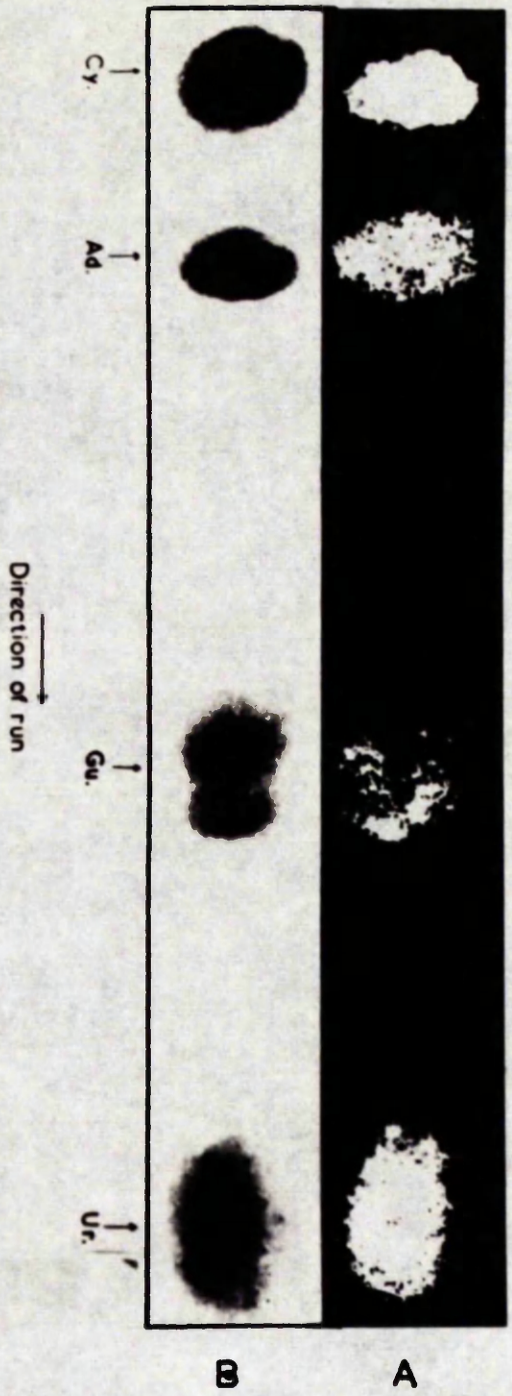


Table 11.

Specific Activities of the Ribonucleotides of the Nuclei and Cytoplasmic Fractions of Fowl Liver and Tumour, Related to the Counts/minute injected ($\times 10^7$).

Fraction	Nucleotide	LHI	LHII	C	TBH	T	NLH
Whole Cytoplasm	Cytidylic Acid	74.2	84.4	72.0	18.6	15.1	1,075
	Adenylic Acid	116.0	85.5	96.5	27.0	18.6	1,490
	Guanylic Acid	66.7	57.8	69.6	16.0	14.4	1,060
	Uridylic Acid	81.5	69.4	71.2	22.3	17.3	1,315
Mitochondrial Fraction	Cytidylic Acid	64.8	60.2	56.7	13.1	-	1,110
	Adenylic Acid	96.4	90.0	72.5	15.7	-	1,385
	Guanylic Acid	62.5	59.8	55.4	12.2	-	925
	Uridylic Acid	69.5	63.2	55.0	13.5	-	1,126
Microsomal Fraction	Cytidylic Acid	48.4	52.5	54.5	13.1	-	756
	Adenylic Acid	66.6	69.3	73.7	17.1	-	1,430
	Guanylic Acid	44.3	38.3	36.8	12.1	-	792
	Uridylic Acid	46.5	48.7	61.0	12.5	-	974
Supernatant Fraction	Cytidylic Acid	91.8	138.8	122.0	31.1	-	2,680
	Adenylic Acid	127.0	72.2	141.0	38.7	-	1,960
	Guanylic Acid	70.8	102.5	91.0	26.6	-	1,565
	Uridylic Acid	86.5	105.5	110.5	26.0	-	1,315

LHI - Laying hen at middle of laying period.
LHII - Laying hen at end of laying period.
C - Cock
TBH - Hen bearing GRCH 15 tumour
T - GRCH 15 tumour
NLH - Non-laying hen.

Figure 10.

L.H.I. Laying hen at middle of laying period.

L.H.II. Laying hen at end of laying period.

C. Cock.

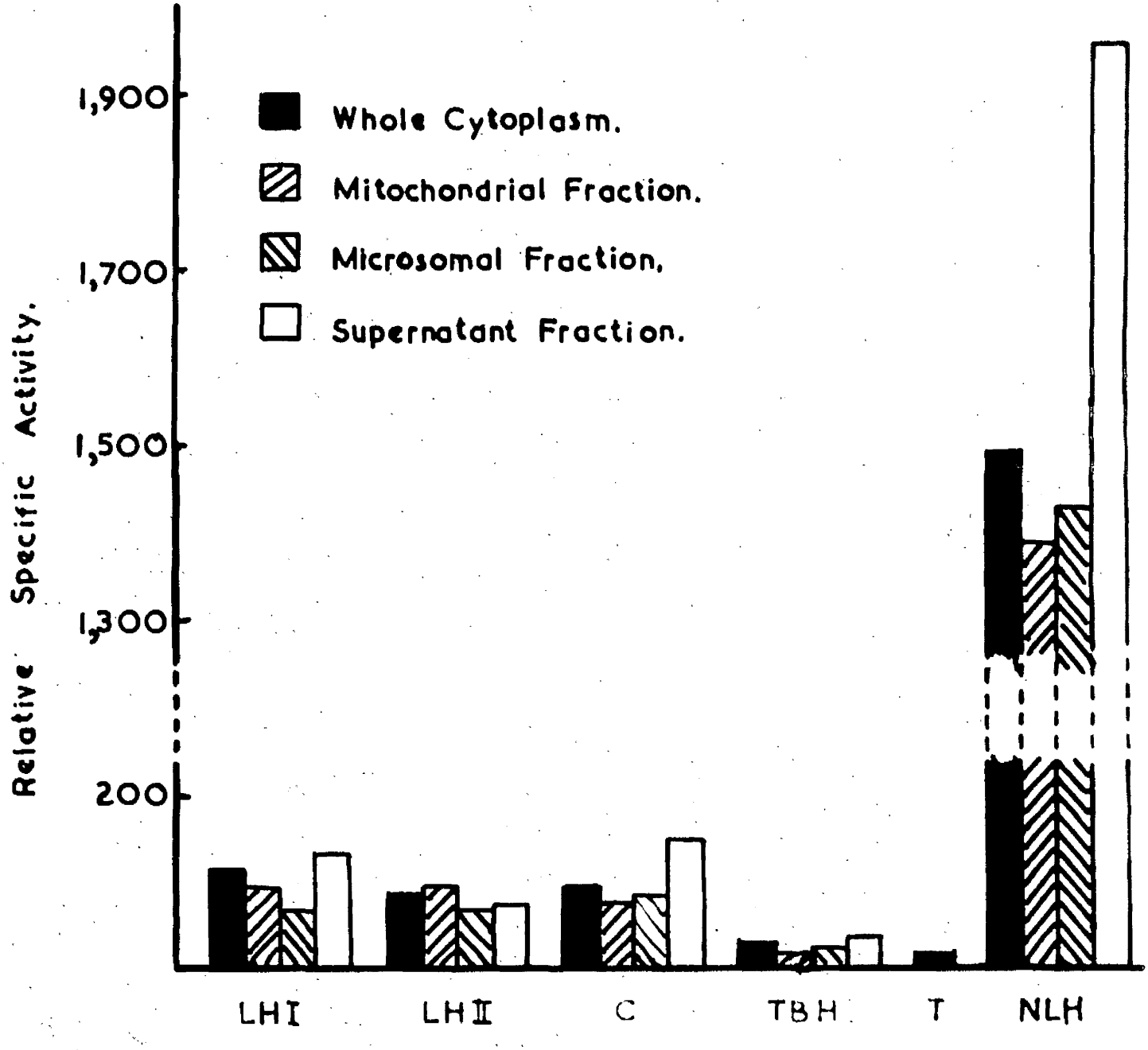
T.B.H. Non-laying hen bearing a GRCH 15 tumour.

T. GRCH 15 tumour

N.L.H. Non-laying hen.

Figure 10.

Specific Activities of the Adenylic Acids from the RNA of the Cytoplasmic Cellular Components, Relative to an Injected Dose of 10^7 Counts/Minute.



there is no evidence, either in the ultraviolet light photograph or in the autoradiograph, of the presence of Components D and E. This is borne out by the fact that the relative specific activities of the uridylic acid approaches more closely that of the other nucleotides.

The activities of the cytoplasmic RNA's obtained are shown in Table 11. The activities of the adenylic acid of the various cytoplasmic fractions of the different experimental birds are shown in Figure 10.

In all instances the activities of the Whole Cytoplasm and the Mitochondrial and Microsomal fractions are of the same order. In all of the livers examined, other than that of the hen at the end of its laying period, the supernatant fraction shows a higher uptake of ^{32}P than do the other cytoplasmic fractions.

The non-laying hen shows a remarkably high incorporation of ^{32}P into the ribonucleotides of the liver cytoplasmic fraction, in comparison to those of the other birds.

The activity of the Whole Cytoplasm of the tumour tissue again shows a value very close to that of the Whole Cytoplasm of the liver from the same bird.

Discussion.

One of the first points, which was considered at the beginning of this work, was the technique of breaking down the tissue cells. The Waring type of blendor produces such a powerful shearing action that, in sucrose medium, the cellular components, particularly the nuclei, are disrupted. In their review of the methods of isolating cell components, Schneider and Hogeboom (1951) advocate the use of a homogeniser devised by Potter and Elvehjem (1936). The main objections to this type of homogeniser are that it is only capable of handling small amounts of material, and in addition a small amount of powdered glass is produced which interferes with the subsequent analysis. It is possible to overcome the second objection by using a plastic pestle, but again only small amounts of material can be handled. It was found, during preliminary experiments, that it was convenient to use a Nelco homogeniser, with a paddle attachment, since it was capable of taking up to 70 ml. of material at one time. By this means it was found possible to obtain good disruption of cells in sucrose without undue destruction of nuclei.

Microscopic examination of these sucrose homogenates showed that there was still a very large number of free and apparently undamaged nuclei present, together with some whole

cells. It is inevitable that there are some whole cells present, for it is impossible to obtain complete breakdown of cells without at the same time causing the disintegration of a large number of nuclei. The results shown in Table 6 confirm that there was very little nuclear breakdown since very little DNA-P was to be found on analysis of the Whole Cytoplasmic fraction, after the removal of the nuclei. Since rabbit liver nuclei contain approximately 10% of the total nucleic acid as RNA (Davidson & McIndoe 1949), it is unlikely that the cytoplasmic fractions were contaminated by nuclear RNA due to nuclear breakdown, although it is possible that some RNA may have been leached out of intact nuclei during the initial stages of fractionation.

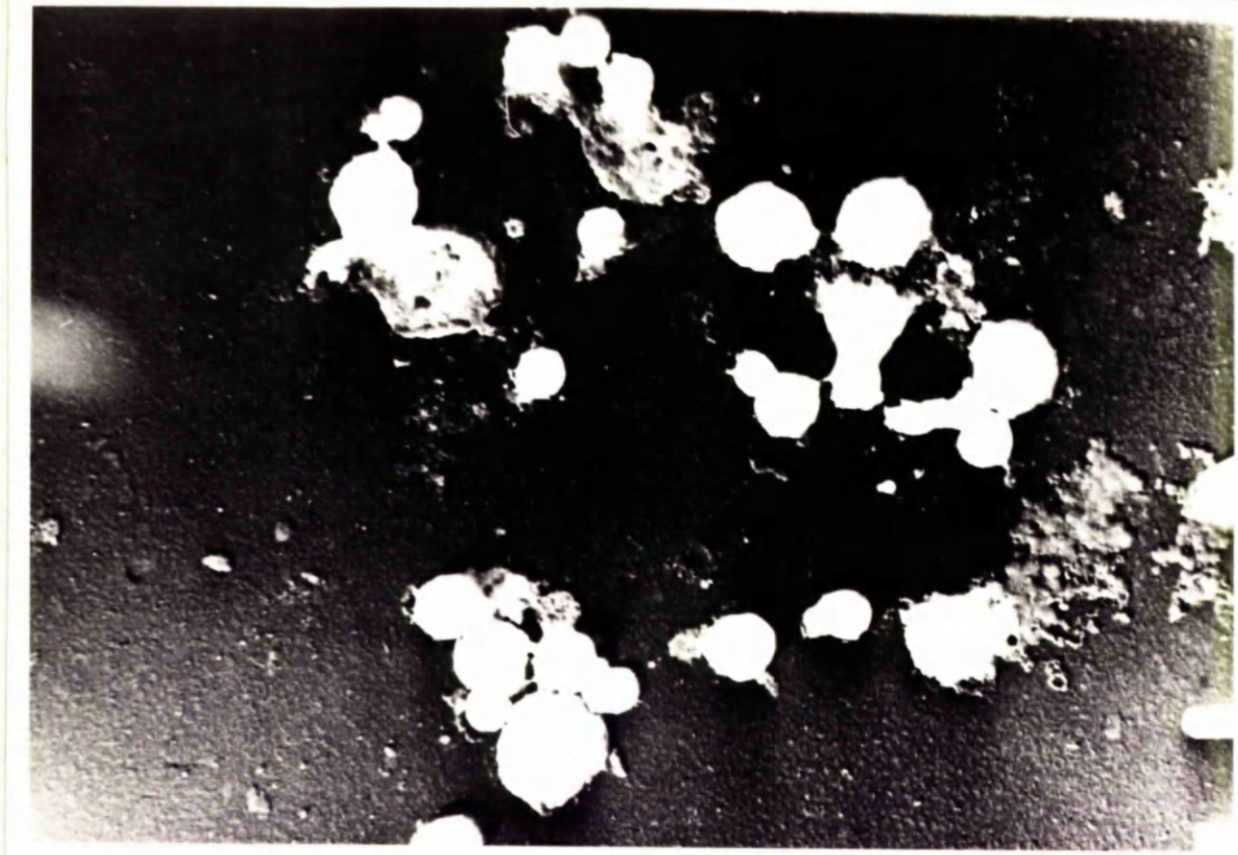
Using the Schneider (1948) procedure of differential centrifugation, it was found possible to obtain discreet mitochondrial and microsomal fractions. Examination of the particulate fractions by means of electron microscopy (Plate 1) indicated that the mitochondrial fraction was free from contamination by microsomes, and consisted of well-defined spherical granules, while the microsomal fraction, although less homogeneous in appearance, was free of contamination by large granules.

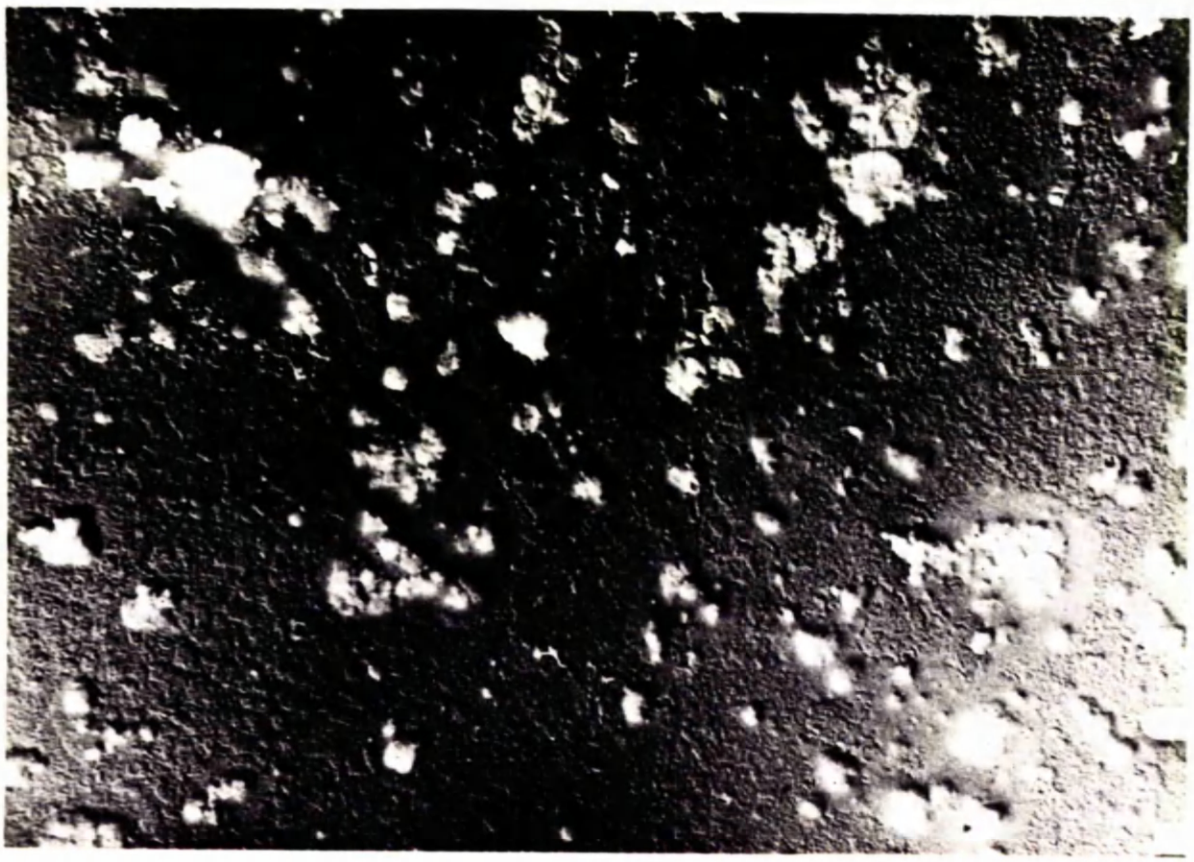
The relative specific activity-time curves obtained from the liver inorganic P of rabbits (Figure 3) agree with

Plate 1.

Electron micrographs of cytoplasmic particles prepared by differential centrifugation from a homogenate of rabbit liver in 0.25 M-sucrose.
Magnification x 18,000. Shadowed at 15° with palladium.

Upper picture, mitochondria; lower picture, microsomes.





those obtained by Barnum and Huseby (1950) and Barnum, Huseby and Vermund (1953) using mice, and Griffiths and Pace (1953) using rats. All of these workers find that there is a rapid fall off in the tissue inorganic P activity until approximately 18 hours after the administration of ^{32}P; thereafter the fall in the activity becomes more gradual.

The relationship between time and the specific activities of the phospholipids has been studied by Ada (1949) using rabbits, and Popjak and Muir (1950) and Griffiths and Pace (1953) using rats. Griffiths and Pace (1953) found that peak of the curves of all of the cytoplasmic particles was at approximately 6 hours after the injection of the isotope. Popjak and Muir (1950) found that the greatest activity of the phospholipids of whole rat liver was reached 12 hours after the administration of ^{32}P. On the other hand, Ada (1949) found that the maxima of the curves of the cytoplasmic particles of rabbit liver was about 24 hours after injection. The order of activities of the cytoplasmic particles, as found by Ada (1949), was microsomes $>$ mitochondria $>$ supernatant, while Griffiths and Pace (1953) found the mitochondria to have the highest activity of all the particles and the microsomes to have the lowest. The results obtained during the present investigation (Figure 4) would appear to suggest that the microsomes have the highest and the mitochondria have the lowest activity, with both of

them reaching their peak of activity at about the same time, namely 24 hours after the injection of the ^{32}P. The supernatant fraction appears to be intermediate in its level of activity and to reach its maximum activity 30 hours after the administration of the isotope. These values, however, are not absolute, since the separation of the different lipid fractions was not absolute, and since small errors in the determination of P or activities give considerable errors in the relative specific activity values.

In the case of the phospholipid activities obtained from the fowls, there would appear to be little uniformity, since each bird appears to have its own particular pattern of the order of activities from the different cytoplasmic particles.

Davidson, Frazer and Hutchison (1951) showed that the determination of the specific activities of protein bound compounds presented many difficulties due to the presence of highly labelled non-nucleotide phosphorus compounds. By using the ionophoresis technique of Davidson and Smellie (1952a) it was possible to remove these interfering phosphorus compounds, as shown by Davidson and Smellie (1952b). The results obtained from rabbit liver, shown in Table 8, and those obtained from fowl livers, shown in Table 11, indicate that the incorporation of ^{32}P into the RNA is of the same order in all four nucleotides from any one of the cellular components. The activity of

adenylic acid, however, appears to be slightly higher, and of guanylic acid slightly lower, than those of the pyrimidine nucleotides. This is in agreement with the findings of Volkin and Carter (1951). The high uridylic acid values, found in the experiments were rabbit tissue was studied, appear to be due to the presence of highly active compounds as found by Davidson and Smellie (1952b). The high specific activity values found in the uridylic acid fraction of chicken liver by Hultin et al. (1951) is probably due to the presence of these highly labelled concomitants. By preliminary extraction of the RNA from the AINLP residue with NaCl before alkaline hydrolysis, as was done in the fowl experiments, these active concomitants appear to be almost completely removed, and the uridylic acid values obtained approach those of the other nucleotides more closely.

In both series of experiments, the specific activity of the supernatant fraction of the cytoplasm would appear on the whole to be higher than those of the cytoplasmic particles, which have approximately the same order of activities, with the microsomal fraction tending to have a slightly lower activity than the mitochondrial fraction. Barnum and Huseby (1950), using saline homogenates of the livers of mice which had received ^{32}P, found that the supernatant fraction had a much higher activity than the microsomal fraction. Hultin et al. (1951), in their experiments with ^{32}P, found that the supernatant

fraction had a higher level of activity than those of the other cytoplasmic fractions. In the present series of investigations, the results obtained for rabbit liver 2 hours after the administration of the isotope are in agreement with these finding, the order of activities at 2 hours being supernatant > mitochondria > microsomes. The results obtained for the fowl livers 4 hours after injection of ^{32}P are not conclusive, since although the supernatant fraction appears to be more active in most instances, in the case of the hen at the end of its laying period, the mitochondria would appear to be the most active fraction; and while in almost every instance the mitochondria have the same or a higher activity than the microsomes, in the case of the non-laying hen the microsomes have an activity appreciably higher than that of the mitochondria.

Jeener and Szafarz (1950) found that in non-multiplying cells the incorporation of ^{32}P at 2 hours into the RNA of the cytoplasmic fractions was in the order supernatant > microsomes > mitochondria. They found, however, that with multiplying cells, such as mice embryos, the order of incorporation of ^{32}P was supernatant > mitochondria > microsomes. It should be emphasised that Jeener and Szafarz (1950) homogenised their tissues in a Waring Blendor with phosphate buffer, and in their centrifugation procedure they used considerably higher

centrifugal fields than were used in the present investigations. Consequently, the mitochondrial fraction as isolated by them is more than likely to be contaminated with agglutinated microsomes, and the microsomal fraction contaminated with ribonucleoprotein from the supernatant fraction. This tendency would be aggravated by their use of phosphate buffer in place of sucrose as a homogenising medium.

On the other hand, Griffiths and Pace (1953) found that the incorporation of ^{32}P into the cytoplasmic fractions of rat liver was in the order mitochondria > microsomes > supernatant, 2 hours after the administration of ^{32}P. It must be pointed out, however, that Griffiths and Pace (1953) carried out their activity determinations on crude phosphorus fractions obtained by the Schneider (1945) procedure. Davidson, Frazer and Hutchison (1951) have shown that the nucleic acid components of the tissue, as isolated by the Schneider (1945) procedure contain other phosphorus compounds, which have a higher activity than the nucleic acids themselves. It may well be that these active compounds differ in their activities according to the cytoplasmic fraction from which they are derived, and that it is these concomitants which are responsible for the order of activities of the cytoplasmic fractions observed.

The very high incorporation of ^{32}P into the nucleotides of nuclear RNA is consistent with the findings of other workers

(Marshak & Calvet 1949; Barnum & Huseby 1950; Jeener & Szafarz 1950; Davidson, McIndoe & Smellie 1951; McIndoe & Davidson 1952) using ^{32}P. Similar results have been obtained using ^{14}C-formate (Smellie, McIndoe & Davidson 1953), ^{14}C-orotic acid (Potter, Recknagel & Hurlbert 1951) and ^{15}N-glycine (Smellie, McIndoe & Davidson 1953). The present studies show that this rapid rate of incorporation of ^{32}P into the nuclear RNA of rabbit liver cells holds for all four nucleotides, which are labelled to almost the same extent. At all of the time intervals after administration of ^{32}P, the nuclear RNA had a specific activity much greater than the RNA derived from any of the cytoplasmic fractions. The peak of activity of the nuclear RNA would appear to fall about 24 hours after the administration of the isotope, while that of the cytoplasmic fractions would appear to fall between 24 and 37 hours after injection. However, Smellie, McIndoe, Logan, Davidson and Dawson (1953) found that the peak of activity of the nuclear RNA was somewhat earlier, namely about 18 hours after the animal had received ^{32}P. The present investigation also shows that the activity of the DNA of the nucleus is at all times very much lower than any of the RNA fractions, with its peak of activity falling about 18 hours after the isotope had been received.

Marshak and Calvet (1949), using rabbits injected with

^{32}P, isolated the nuclei from the liver tissue by the citric acid method of Marshak (1940, 1941), and then used the supernatant to prepare the cytoplasmic fractions. They found that the peak of nuclear RNA activity was between 3 and 13 hours after the injection of ^{32}P. It was also found that the ribonucleotide solution of the microsomal fraction had the highest level of activity throughout the time interval 1 hour to 73 hours after injection, with the peak of activity falling at about 12 hours after injection. On the other hand, the mitochondrial activity was still rising 73 hours after the administration of the isotope. Their method of isolation of the cytoplasmic fractions cannot be regarded as ideal, since Hogeboom et al. (1948) and Schneider and Hogeboom (1951) have shown that electrolytes cause agglutination of the cytoplasmic particles. Another objection to their findings is that they used the whole ribonucleotide fraction (obtained from the Schneider (1946c) modification of the Schmidt & Thannhauser (1945) procedure),which has been shown by Davidson, Frazer and Hutchison (1949) to contain active phosphorus-containing concomitants.

Barnum & Huseby (1950) also obtained a specific activity-time curve for the cytoplasmic fractions between 1 and 24 hours after administration of ^{32}P to mice. They carried out their determinations on isolated RNA from the various

cytoplasmic fractions. Although the specific activities of the cytoplasmic RNA was still increasing up to 24 hours, they did not proceed with the determinations beyond this time. The maximum incorporation into the nuclear RNA they found was about 2 hours after administration of the ^{32}P.

Hurlbert and Potter (1952) found that the maximum incorporation of radioactive orotic acid into nuclear RNA of rats bearing multiple Flexner-Jobling tumours occurred about 4 hours after administration.

Hurlbert and Potter (1954) studied the conversion of labelled orotic acid to uridine compounds and its incorporation into the uridylic acid of the RNA of the nucleus and cytoplasmic fractions of normal rat liver. They found that the orotic acid was incorporated into the uracil of nuclearRNA very rapidly, the maximum incorporation being about 4 hours after administration. At the same time, the orotic acid was also incorporated into the pyrimidines of the cytoplasmic RNA, with the maximum peak of activity falling at about 16 hours after injection. Hurlbert and Potter (1954), from their results, suggest that the orotic acid was first converted to uridine phosphate which acts as an immediate metabolic precursor of the uracil of nuclear RNA and as a major, although possibly indirect, source of the pyrimidines of the cytoplasmic RNA.

It has been suggested by Mauritzen, Roy and Stedman

(1952), that nuclear RNA may be an impurity adsorbed from the cytoplasm during the process of isolation. Every indication, from isotopic studies, would appear to indicate that this is not the case, since the nuclear RNA appears to incorporate ^{32}P much more rapidly than does any of the cytoplasmic particles, and this would appear to be confirmed by the present investigation.

In 1948 Marshak put forward the hypothesis that the nucleus contains a nucleoprotein which behaves as a precursor of the RNA of the cytoplasmic particles of non-proliferating tissues. Marshak and Calvet (1949) claimed that their results were in agreement with this theory and that the specific activity of the microsomal fraction rose until it reached the same level of activity as that of the nuclear RNA. As already stated, the isolation procedure of Marshak and Calvet (1949) is questionable, and also highly active concomitants have been shown to be present in the ribonucleotide fractions which they used.

Jeener and Szafarz (1950) have extended the theory of Marshak (1948) on the basis of their findings at 2 hours after the injection of ^{32}P. They propose that the nucleus synthesises RNA which then passes into the cytoplasmic cell sap. This RNA in the cell sap was then incorporated into the microsomes, which in turn aggregated to form the mitochondria. If this hypothesis were true, the RNA of the supernatant fraction would turn over

more rapidly than that of the microsomes which in turn would turn over more rapidly than the mitochondrial fraction.

The present findings, using rabbit tissue, do not support the hypothesis of Jeener and Szafarz (1950), since all the nucleotides of all the cytoplasmic fractions have the same order of relative specific activities, and their relative specific activity curves all follow the same pattern, with their maximum activity about 24 hours after the administration of the ^{32}P. At the time intervals studied, the specific activities of the nucleotides of the cytoplasmic fractions do not attain the level of activity of the nuclear nucleotides. From the fowl experiments, although only one bird of each type was studied and although there is a very wide scatter of results obtained from birds of different physiological states, a similar picture, as far as the cytoplasmic particles are concerned, is to be found.

PART TWO.

A Study of the Metabolism of the Phosphorus Compounds of Liver, Spleen and Intestinal Mucosa, in vitro, Investigated with the Aid of Radioactive Phosphorus.

1. Introduction.

The study of the metabolism of a tissue in the intact animal, while of great importance, cannot give a complete understanding of the pathways and enzyme systems involved. In order to do this, the metabolism of the tissue under investigation must be studied in vitro. By using in vitro techniques, it is possible to control the experimental conditions much more rigidly. It is also possible to study individually all of the substrates and precursors of the system under investigation, while at the same time using as a control some of the same tissue, to which no addition of substrate or precursor has been made. This is not possible using intact animals, since the control and test experiments must be carried out on different animals. By means of metabolic inhibitors, it is possible to determine in vitro the other systems which may influence the one under investigation. These in vitro studies can be carried out either on tissue slices or on tissue homogenates

Friedkin and Lehninger (1949) reported that they were able to detect the incorporation of ^{32}P into the RNA and phospholipid fractions of rat liver homogenates which had been incubated in the presence of radioactive inorganic

phosphate. They also found that the DNA of this rat liver homogenate did not incorporate ^{32}P under the conditions employed.

Mann and Gruschow (1949) studied the incorporation of ^{32}P into the nucleic acid and phospholipid fractions of tissue slices from rat liver and rat Walker tumours. Their results showed that the incorporation of the isotope into these fractions was dependent on the presence of oxygen.

Goldwasser (1953), using [8-^{14}C] adenine, was able to show that tissue slices of rat liver, rat spleen and pigeon liver and pigeon liver homogenates were capable of incorporating the labelled adenine into their nucleic acid fractions.

In the present investigation, whole tissue homogenates and isolated cellular fractions were incubated with inorganic phosphate labelled with ^{32}P so that a further study might be made to determine whether RNA was indeed synthesised in the nucleus and then passed out into the cytoplasm for inclusion in the cytoplasmic particles, as was postulated by Jeener and Szafarz (1950).

2. Experimental.

I. Preliminary Experiments.

(a) Biological.

In the preliminary experiments the livers from albino rats, from the departmental stocks, were used. The animals were killed by exsanguination and the livers removed, chilled and minced with scissors. The minced livers were then homogenised in four volumes of 0.25 M-sucrose, in an M.S.E. Nelco blendor, with the blades replaced by a paddle. To portions of homogenate, corresponding to 4 g. of tissue, were added 3.2 ml. of buffer pH 7.4, and 50 µc. ^{32}P and the total volume made up to 20 ml.

Two types of buffer solution were used,

(1) 0.5 M-phosphate buffer in 0.25 M-sucrose (89.0 g. $Na_2HPO_4.2H_2O$ 10 ml. 10 N-HCl, diluted to 1 litre with 0.25 M-sucrose).

(2) 0.75 M-glycine buffer in 0.25 M-sucrose (56.25 g. glycine dissolved in 0.25 M-sucrose and adjusted to pH 7.4 with N-NaOH).

All solutions and apparatus were sterilised before use to eliminate complications due to the possible presence of bacteria and fungi in the incubation mixture.

The sucrose solution was gassed with 5% CO_2- 95% O_2 mixture for one hour before use. The homogenates were

incubated at 37°C in 100 ml. Pyrex conical flasks, stoppered with rubber bungs.

A series of preliminary experiments was carried out to find the optimal time of incubation. The reactions in the flasks were stopped, after 0, 1, 2, 3, 4 and 5 hours incubation, by the addition of 10 ml. 30% (w/v) TCA. In general, the incubations were carried out for 2 hours in a thermostat at 37°C, with gentle shaking. A control flask, in which the reaction was stopped immediately, was also set up.

The effect of the addition of various substances was also investigated. Among those studied were adenosine-triphosphate (ATP), diphosphopyridine nucleotide (DPN), cytochrome c (cyt. c), $MgCl_2$, Na_2HPO_4, sodium pyruvate, sodium succinate, sodium fumarate and sodium malate. Each of these compounds was dissolved in sterile distilled water, so that the final solution contained 0.2 mg./ml.. 0.1 ml. of the solution of the substance under investigation, was added to the homogenate immediately before incubation.

The effect of glucose on the incorporation of ^{32}P into the phosphorus-containing components was also studied. 0.05, 0.1, 0.5 and 1.0 ml. of glucose solution(containing 20 mg./ml.) was added to the incubation mixture.

The different cellular fractions were isolated,

from 0.25 M-sucrose homogenates of rat liver, by the procedure of Schneider (1948), described in Part One. The cellular fractions, so isolated, were incubated at 37°C. for 2 hours, as described in the protcols for the individual experiments.

The reaction in the flasks was stopped by the addition of 0.5 volume of 30% TCA. The contents of the flasks were then transferred to a 50 ml. centrifuge tube and the phosphorus containing fractions prepared by the modified Schmidt and Thannhauser (1945) procedure, as described in Part One.

(b) Chemical.

It was invariably found that the nucleic acid fractions, isolated by the modified Schmidt and Thannhauser (1945) procedure, previously described in Part One, contained traces of inorganic phosphate labelled with ^{32}P. Many methods of removing this inorganic ^{32}P were tried, including (1) addition of Na_2HPO_4 to the 10% (w/v) TCA wash solutions (2 g. $Na_2HPO_4.2H_2O$/100 ml. 10% TCA solution).

(2) preparation of the RNA fraction by Procedure 2, as described in Part One. In some instances the RNA, so prepared, was redissolved in 10% (w/v) NaCl and reprecipitated with 2 volumes of ethanol. In one case the RNA was dissolved in NaCl and reprecipitated 4 times.

(3) the RNA fraction, as prepared by Procedure 2, was dissolved in the minimum volume of water and reprecipitated by the addition of 2 volumes of glacial acetic acid, as described by Davidson, Frazer and Hutchison (1951). The precipitated RNA was washed with 70% ethanol, ethanol and ether and finally dried.

The inorganic phosphate, acid soluble organic phosphate and phospholipid fractions were isolated by the methods used in Part One. The RNA, isolated by any one of the above procedures, was hydrolysed overnight with 0.5 N-KOH and the resulting nucleotide solution fractionated into its individual nucleotides by the procedure of ionophoresis (Davidson & Smellie 1952a). The determination of the phosphorus content and of the activities of the solution was carried out as in Part One.

II. The Study of the Transfer of ^{32}P from a Labelled Cellular Fraction into Other Non-radioactive Cellular Fractions.

(a) Biological.

In these experiments, albino rat liver, albino rat spleen, albino rabbit liver and albino rabbit intestinal mucosa were used. Two series of animals were used; in one, the animals received ^{32}P labelled inorganic phosphate 18 hours before sacrifice; rats received 1 mc. ^{32}P each, while rabbits

received 3 mc. each. In the other series, the animals received no treatment prior to being sacrificed. The rats were killed by exsanguination, while the rabbits were killed by cervical dislocation. The organs were removed quickly, and in the case of the liver and spleen, minced finely with scissors and chilled. Rabbit small intestine, after removal from the animal, was washed through with ice-cold 0.25 M-sucrose and slit up. The mucosa was then removed by scraping, collected and chilled.

The tissues, from both series of animals, were homogenised separately with 2 volumes of 0.25 M-sucrose, in an M.S.E. Nelco blendor, with the blades replaced by a paddle. The resulting homogenates were separated into their component cellular fractions by the technique of Schneider (1948), as described in previous sections. The cellular particles, after washing with 0.25 M-sucrose, were suspended in sucrose solution.

A "composite" homogenate was then prepared by mixing in a 100 ml. Pyrex conical flask, the radioactive cellular fraction or fractions from one animal with the non-radioactive cellular fraction or fractions from another animal. At the same time, 6.2 ml. of glycine buffer (pH 7.4) in sucrose were added together with the appropriate additions, such as ATP, DPN, cyt. c, Na_2HPO_4, $MgCl_2$ and sodium malate. The

final volume was made up to 40 ml. with 0.25 M-sucrose solution. In all cases 4 similar flasks were set up, 2 of which were incubated for 2 hours, while the reaction in the other 2 was stopped immediately by the addition of 0.25 M-sucrose - M-citric acid solution. All the solutions and glassware were sterilised before use.

Where dialysed cell sap was used, the cell sap was transferred to a dialysis sac and dialysed against a known volume of sterile distilled water in a rocking dialyser apparatus, for 8 hours in the cold room. At the end of this time the dialysate was removed, collected, and its volume reduced by freeze-drying. The freeze-dried dialysate was then made up to a known volume with 0.25 M-sucrose. Meanwhile, the cell sap was dialysed further against water, with frequent changes, for 16 hours. The dialysed cell sap was collected and made up to a known volume.

Protein fractions were obtained from dialysed cell sap, by fractional precipitation with ammonium sulphate. Three protein fractions were prepared, namely those precipitates at 0-33% saturation and 33-66% saturation of the cell sap with ammonium sulphate, and a third fraction which included all the material not precipitated at 66% ammonium sulphate saturation.

The procedure used in the fractionation of dialysed

cell sap into protein fractions was as follows:-
One half volume of saturated ammonium sulphate solution was added to dialysed cell sap, to give 33% saturation with respect to ammonium sulphate. The resulting precipitate was removed by centrifugation at 600 g. (I.E.C. refrigerated centrifuge, horizontal yoke) for 20 minutes. The precipitate was collected, dissolved in water and the resulting solution dialysed against water to remove the ammonium sulphate present (Fraction I).

The supernatant fluid was then made 66% saturated with respect to ammonium sulphate, by the addition of the calculated amount of solid ammonium sulphate. The resulting precipitate was centrifuged off as before, collected, dissolved in water and this solution dialysed against water (Fraction II).

The supernatant liquid from the second precipitation was dialysed to remove ammonium sulphate, and corresponds to the material which was not precipitated by 66% ammonium sulphate saturation (Fraction III).

All manipulations in the preparation of these protein fractions were carried out aseptically in the cold room.

The test suspensions were incubated in a thermostat, with gentle shaking, for 2 hours at 37°C. Similar

suspensions were set up as controls. The reaction in the controls was stopped immediately, while that of the tests was stopped at the end of the incubation period, by the addition of 4 ml. 0.25 M-sucrose - M-citric acid solution.

Once the reaction had been stopped by the addition of the citric acid solution, the contents of the flasks were transferred to a Potter and Elvehjem (1936) homogeniser, with a plastic pestle. After gentle homogenising, the suspension was centrifuged for 10 minutes at 400 g (I.E.C. refrigerated centrifuge, horizontal yoke). The sediment was washed with 0.01 M-citric acid, until microscopic examination showed that the nuclei were free of extraneous material. The isolated nuclei (N) were then analysed. The supernatant fraction (S) obtained after centrifuging at 400 g was collected and analysed.

10 ml. of 10% (w/v) TCA was added to the nuclear fraction, and 0.5 volume of 30% (w/v) TCA added to the supernatant fraction. The phosphorus containing fractions were then obtained by the modified Schmidt and Thannhauser (1945) procedure, as previously described in Part One.

(b) Chemical.

The inorganic phosphate (P_1), acid soluble organic phosphate (S_1) and phospholipid (LP) fractions were prepared as described in Part One.

The ribonucleotides of the nuclei were prepared by Procedure 1, used in Part One, while the ribonucleotides of the supernatant were prepared by Procedure 2. The ribonucleotides were separated by the technique of Davidson and Smellie (1952a).

The DNA-P of the nuclear fraction was prepared by a modification of the Kay et al. (1952) procedure. The residue obtained on acidification of the alkaline hydrolysis of the AINLP residue, was collected by centrifugation and used to prepare the DNA-P fraction, while the supernatant was used to obtain the nucleotides derived from the nuclear RNA. The residue, containing the DNA, was washed with 0.5 N-perchloric acid, ethanol, ethanol ether (3:1) and finally ether and then dried. The dried powder was suspended in 5 ml. 0.9% (w/v) NaCl and 0.5 ml. sodium dodecyl sulphate solution (5% sodium dodecyl sulphate in 45% ethanol/water) added. After standing 1 hour, with occasional stirring, the solution was made molar with respect to NaCl, by the addition of the calculated amount of solid NaCl. The solution was then centrifuged and the supernatant fluid decanted. Two volumes of ethanol were added and the precipitated DNA centrifuged down. The precipitate was then washed with 70% ethanol, ethanol and ether and finally dried. The total material obtained was then used for the determination of phosphorus

content and of specific activity.

All of the phosphorus fractions derived from the nuclear material were estimated using one-fifth of the amounts of the reagents of the modified Allen (1940) method; otherwise the phosphorus determinations and specific activity determinations were carried out as described in Part One.

In certain instances the purines and pyrimidines of the ribonucleotide solution, obtained after hydrolysis of the RNA by the modified Schmidt and Thannhauser (1945) procedure, were examined chromatographically. The ribonucleotide solution was taken to dryness and hydrolysed for one hour at 100°C with 12 N-perchloric acid (Marshak & Vogel 1951; Wyatt 1951). The pH of the hydrolysate was adjustedto pH 3 with 5 N-KOH and the precipitated potassium perchlorate was removed by centrifugation. A portion of the hydrolysate was then applied to Whatman 3 mm. filter paper. The nucleic acid bases were then submitted to chromatography in iso-propanol - HCl mixture (65 ml. iso-propanol + 16.5 ml. conc. HCl + distilled water to 100 ml.) according to the procedure of Wyatt (1951). After the filter paper had been dried, the bases were detected in ultraviolet light, by the method devised by Holiday and Johnson (1949). The R_F values were determined, and the spots identified. The spots were cut out and eluted with 0.1 N-HCl, except in the case of guanine

which was eluted in 1.6 N-HCl. The concentration of base in the eluate was then determined by measuring the extinction of ultraviolet light, of the appropriate wavelength, in a Beckman Model DU spectrophotometer.

III. Preparation of Compounds labelled with Radioactive Phosphorus.

Various phosphorus-containing fractions of liver tissue, such as total acid soluble phosphorus compounds, acid soluble inorganic phosphate, phospholipids and RNA, were prepared from the livers of animals which had received ^{32}P-labelled inorganic phosphate. These labelled phosphorus compounds were then investigated as possible sources of phosphorus for the synthesis of the nuclear nucleic acids.

(a) Acid Soluble Phosphorus and Acid Soluble Inorganic Phosphate.

Liver tissue from albino rabbits, which had received 2 mc. ^{32}P labelled inorganic phosphate, 18 hours prior to sacrifice, was homogenised in 2 volumes of 0.25 M-sucrose. The resulting homogenate was centrifuged for 75 minutes at 40,000 g (Spinco ultracentrifuge, Rotor No. 21). The supernatant was removed and sufficient 60% perchloric acid to give a final concentration of 0.5 N-perchloric acid. The resulting mixture was centrifuged for 10 minutes at 600 g (I.E.C. refrigerated centrifuge, horizontal yoke). The precipitate was washed with 0.5 N-perchloric acid and the supernatant

and washings combined.

Mathison's (1909) reagent was added to a portion of the extract (1 ml./10 ml. extract) and the resulting solution made alkaline to phenolphthalein with NH_4OH (S.G. 0.88). After standing overnight in the refrigerator, the precipitate was collected by filtering through a Whatman No. 42 paper. The precipitate was washed with 10% (w/v) NH_4OH and dissolved in N-HCl. This acid solution was then passed through a Dowex 50 (H^+ form) column (1 cm. diameter, 8 cm. long) to remove NH^+ and Mg^{++} ions. The eluate was then made up to a known volume (Radioactive P_1).

The remainder of the extract was adjusted to pH 7 with 5 N-KOH and the potassium perchlorate formed was removed by centrifugation. The supernatant was then made up to a known volume (Radioactive Acid Soluble Phosphorus).

(b) Phospholipids.

Liver derived from albino rabbits, which had received 3 mc. ^{32}P labelled inorganic phosphate, 18 hours before sacrifice, was homogenised in 4 volumes 0.9% (w/v) NaCl solution. One half volume of 30% (w/v) TCA was added to the resulting homogenate. The precipitate formed was collected by centrifugation and washed twice with 10% (w/v) TCA. The material precipitated by the TCA was then extracted successively with acetone, ethanol, ethanol ether (3:1)

(twice), and ether. The extracts were collected, combined and taken to dryness on a boiling water bath. The dry residue was extracted with $CHCl_3$ and the extract again taken to dryness. The resulting residue was then emulsified with a known volume of 0.25 M-sucrose, in the presence of a trace of bile salts (Radioactive Lipid P).

(c) Ribonucleic Acid.

RNA was prepared from albino rabbit liver, excised 18 hours after the animal had received 3 mc. ^{32}P as inorganic phosphate. The preparation was carried out using the method of Kay and Dounce (1953).

The tissue was homogenised in 0.9% (w/v) NaCl, containing 0.01 M-sodium citrate. The resulting homogenate was centrifuged at 1,000 g (I.E.C. refrigerated centrifuge, horizontal yoke) for 30 minutes. The supernatant liquid was decanted and recentrifuged to remove any residual precipitate. The final supernatant fluid was decanted and the pH adjusted to 4.5, by the addition of N-HCl. The precipitate formed was removed by centrifugation and the supernatant liquid discarded. The sediment was then suspended in 0.9% (w/v) NaCl solution, so that the final volume was 100 ml. 9 ml. of sodium dodecyl sulphate solution (5% sodium dodecyl sulphate in 45% ethanol) was added with

stirring. The pH of the solution was then raised to 7 by the addition of 2N-NaOH, and the stirring continued for 3 hours. At the end of this time, the solution was made molar with respect to NaCl, by the addition of the calculated amount of solid NaCl. The slightly viscous solution was centrifuged at 20,000 g (Spinco ultracentrifuge, Rotor No. 21) for 30 minutes and the supernatant fluid collected. The RNA present in the supernatant liquid was precipitated by the addition of 2 volumes of ethanol. The RNA, so precipitated, was centrifuged down and washed with 70% ethanol, ethanol and ether and finally dried in air. The dried powder was weighed and dissolved in 0.25 M-sucrose, so that 5 ml. of solution contained 50 mg. RNA (Radio-active RNA).

Table 12.

Summary of the preliminary experiments on the incorporation of inorganic ^{32}P into the RNA of rat liver homogenates, showing the conditions of incubation employed and the technique used to isolate the RNA fraction.

\+ trace of activity detected
\+\+ slight incorporation of ^{32}P
\+\+\+ fair incorporation of ^{32}P
\+\+\+\+ good incorporation of ^{32}P
\+\+\+\+\+ excellent incorporation of ^{32}P

Exp. No.	Tissue Fraction	Time of Incubation	Buffer Solution	Method of Isolating RNA	Conditions of Incubation	Additions to Incubation Medium	Result
1	Whole tissue	2 hrs.	Phosphate pH 7.4	NaCl Extraction	Non-Sterile	None	++
						ATP	++
						DPN	++
						Cyt c	++++
						Succinate	+
				NaCl Extraction and reprecipitation from NaCl solution		None	+
2	Whole Tissue	2 hrs.	Phosphate	NaCl Extraction	Non-Sterile	None	++++
					Sterile	None	++
3	Whole Tissue	2 hrs.	Phosphate	NaCl Extraction and reprecipitation from NaCl solution 6 times	Sterile	None	+++
						ATP, cyt c, succinate	++++
						ATP, cyt c, succinate, $MgCl_2$	+++++

Table 12 (cont'd).

Exp. No.	Tissue Fraction	Time of Incubation	Buffer Solution	Method of Isolating RNA	Conditions of Incubation	Additions to Incubation Medium	Results
3 ct'd.	Whole Tissue	2 hrs.	Phosphate	Carrier Phosphate added to initial NaCl extraction and reprecipitation from NaCl solution 4 times.	Sterile	None ATP, cyt c, succinate ATP, cyt c succinate, $MgCl_2$	+ + ++
4	Whole Tissue	0 hr.	Phosphate	NaCl Extraction Carrier phosphate added to TCA wash solutions. Carrier phosphate added to initial NaCl extraction and reprecipitation from NaCl solution 4 times NaCl extraction and reprecipitation from water with glacial acetic acid.	Non-sterile	None	+++ +++++ ++ +
5	Whole Tissue	0 hr.	Phosphate	NaCl extraction and reprecipitation from water with glacial acetic acid.	Sterile	ATP, cyt c succinate, $MgCl_2$	++

Table 12 (cont'd).

Exp. No.	Tissue Fraction	Time of Incubation	Buffer Solution.	Method of Isolating RNA	Conditions of Incubation	Additions to Incubation Medium.	Results
5 ctd.	Whole Tissue	2 hrs.					++++
	Whole Cytoplasm	0 hr.	Phosphate	NaCl extraction and reprecipitation from water with glacial acetic acid.	Sterile	ATP, cyt c, Succinate, $MgCl_2$	+++
		2 hrs.					++++
	Nuclei	0 hr.					+
		2 hrs.					++
6	Whole Cytoplasm	0 hr.	Phosphate	NaCl extraction and reprecipitation from water with glacial acetic acid	Sterile	ATP, cyt c, succinate, $MgCl_2$	+
		2 hrs.					++++
		0 hr.	Glycine			ATP, cyt c, succinate, $MgCl_2$	—
		2 hrs.				Na_2HPO_4	+++
7	Whole Cytoplasm	2 hrs.	Glycine	NaCl extraction and reprecipitation from water with glacial acetic acid	Sterile	*Succinate	+
						*Succinate DPN	++
						*Pyruvate	++
						*Pyruvate DPN	+
						*Fumarate	++
						*Fumarate DPN	++
						*Malate	+++
						*Malate DPN	+++
8	Whole Cytoplasm	0 hr.	Glycine	NaCl extraction and reprecipitation from water with glacial acetic acid.	Sterile	ATP, DPN, cyt c $MgCl_2$, Na_4HPO_4 and malate	—
	Nuclei						++
	Mitochondria						—
	Microsomes						—
	Cell Sap						++

Table 12 (cont'd)

Exp. No.	Tissue Fraction	Time of Incubation	Buffer Solution	Method of Isolating RNA	Conditions of Incubation	Additions to Incubation Medium	Result
8 ctd.	Whole Cytoplasm Nuclei Mito-chondria Microsomes Cell Sap	2 hrs.	Glycine	NaCl extraction and reprecipitation from water with glacial acetic acid.	Sterile	ATP, DPN, cyt c, $MgCl_2$, Na_2HPO_4 and malate	+++++ +++ + — +

* In all cases ATP, cyt c, $MgCl_2$ and Na_2HPO_4 were also added to the homogenates.

3. Results.

I. Preliminary Experiments.

The main object of the preliminary experiments, listed in Table 12, was to obtain a suitable system for studying the incorporation of ^{32}P into the phosphorus compounds, and in particular into the RNA, of tissue homogenates.

0.25 M-sucrose was used as the homogenising medium in preference to Krebs-Ringer buffer, since it was intended to fractionate the tissue homogenate into its sub-cellular components and to study the incorporation of the isotope into these isolated fractions. Media, containing high concentrations of electrolytes, such Krebs-Ringer buffers, cause agglutination of the cytoplasmic particles and thus prevent the isolation of the intracellular components (Schneider & Hogeboom 1951).

In the first experiment of the series (Experiment No. 1, Table 12,), the effect of adding various substances to rat liver homogenates was studied. The substances, chosen because of their importance in the metabolism of tissues in vivo, were adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), cytochrome c (cyt. c) and sodium succinate. Of these compounds, cytochrome c appears to increase the incorporation of ^{32}P into the RNA of rat

liver homogenates as compared with the controls, while ATP and DPN do not. Succinate, on the other hand, seems to reduce the incorporation of the isotope in comparison with that observed in the control.

A second control was set up at the same time in order to investigate whether traces of inorganic phosphate, labelled with ^{32}P, were being carried over into the RNA fraction during the isolation procedure. In this second control, the RNA, isolated by extraction with hot 10% (w/v) NaCl solution, was redissolved in 10% NaCl solution and reprecipitated with ethanol. It was found that the RNA obtained from this second control had half the activity of that isolated by straight NaCl extraction. This would appear to indicate that labelled inorganic phosphate was still present as a contaminant in the NaCl extracted RNA.

The effect of bacterial growth on the incorporation of ^{32}P into the RNA of tissue homogenates was investigated in Experiment No. 2, Table 12. It was found that when sterile solutions and glassware were used, there was a marked decrease in the amount of activity detectable in the RNA fraction, isolated from the liver homogenate after incubation. At the end of the period of incubation, small amounts of the homogenates were spread over the surface of agar plates. These agar plates were incubated at 37°C for

three days. When the plates were examined, no bacterial colonies were found in the culture from the homogenate in which sterile solutions and glassware had been used. On the other hand, cultures from the homogenate, where no special precautions had been taken, showed the presence of several unidentified bacterial colonies. Although the number of bacterial cells shown to be present was small, it was possible that they could give rise to erroneous results, since these bacterial cells were capable of incorporating the ^{32}P into their own RNA. In consequence, all further experiments were carried out under aseptic conditions.

In Experiment No. 3, Table 12, two points were studied simultaneously, a) the effect of adding ATP, cyt. c and succinate in the presence and absence of Mg^{++} ions, and b) whether the presence of carrier phosphate in the NaCl solution, used to extract the RNA, would remove any contaminating inorganic ^{32}P present in the RNA fraction. It was found that the addition of Mg^{++} ions increases the incorporation of the isotope into the RNA of whole rat liver homogenate, when ATP, cyt. c and succinate are also present. The addition of carrier phosphate to the NaCl solution, used to extract the RNA, was also found to reduce the contamination due to inorganic ^{32}P.

In order to investigate further this problem of contamination of the RNA fraction with traces of radioactive inorganic phosphate, Experiment No. 4, Table 12, was carried out. ^{32}P was added to rat liver homogenates which were immediately treated with 30% (w/v) TCA and the fractions isolated. The RNA fraction was isolated and purified by 4 different methods. These methods were:-

(a) NaCl extraction of the AINLP residue and the precipitation of the extracted RNA with ethanol.

(b) After the initial precipitation of the homogenate with 30% TCA, the precipitate was washed twice with 10% NaCl solution which had Na_2HPO_4 added to it. The AINLP residue, so prepared, was extracted with NaCl solution and the RNA precipitated from the extract with ethanol.

(c) Carrier phosphate was added to the initial NaCl solution used to extract the RNA from the AINLP residue. The RNA was then redissolved in NaCl solution and reprecipitated 4 times.

(d) The RNA, extracted with NaCl solution, was precipitated with ethanol. This RNA was then dissolved in a small volume of water and reprecipitated by the addition of 2 volumes of glacial acetic acid, as described by Davidson, Frazer and Hutchison (1951).

The specific activity of the RNA obtained by these different

Figure 11.

Whole rat liver homogenate, from 4 g. tissue was incubated for varying lengths of time.

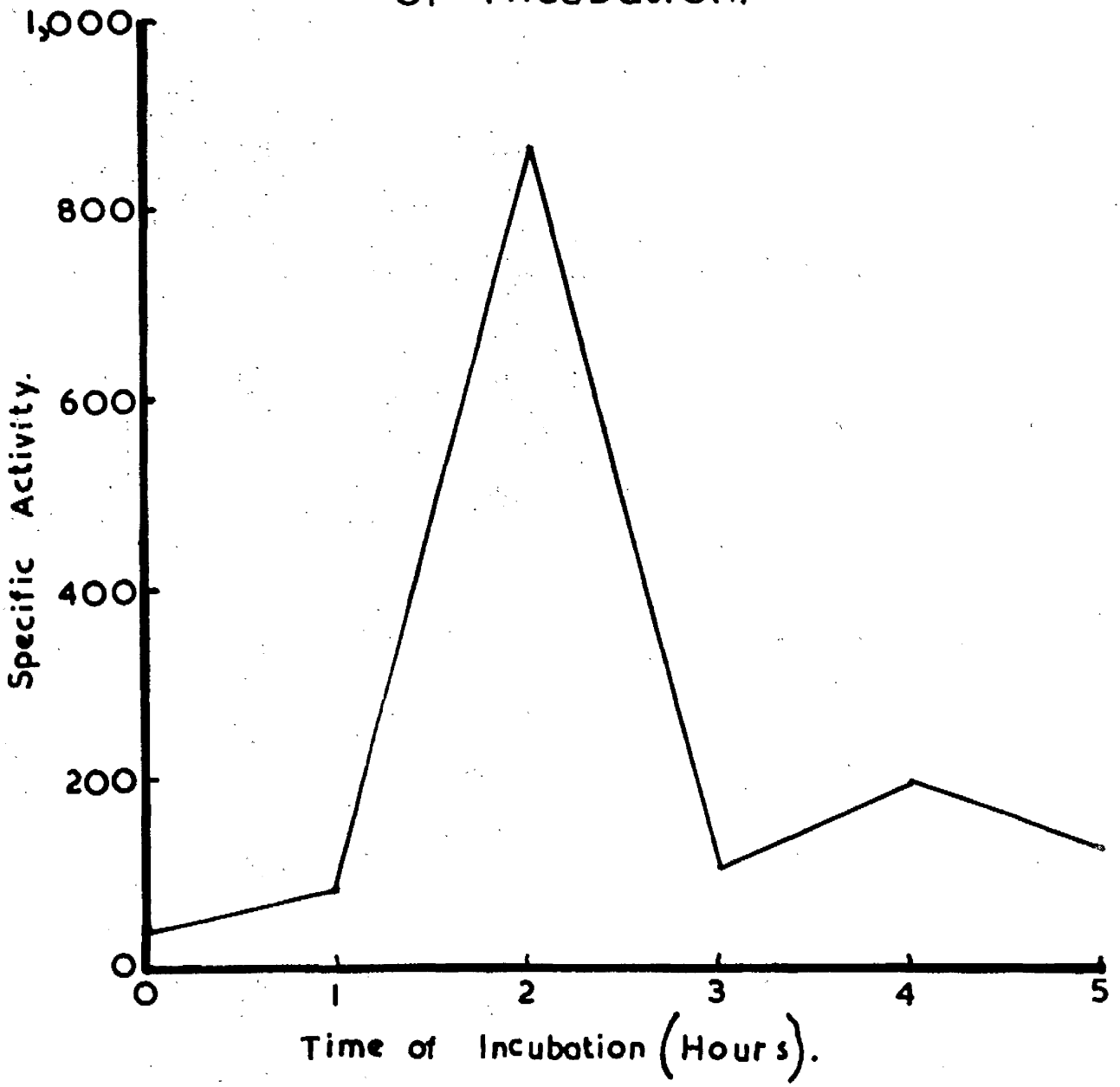
The buffer system used was phosphate buffer pH 7.4, and ATP, DPN, cytochrome c, and $MgCl_2$ solutions were added.

The pH of the homogenate was found to be unchanged throughout the incubation periods studied.

The RNA fraction was isolated by NaCl extraction and purified by reprecipitating from water with glacial acetic acid.

Figure 11.

The Incorporation of Inorganic ^{32}P into the RNA Fraction of Rat Whole Liver Homogenate, with Relation to Time of Incubation.



methods was then determined. Any activity found in these fractions must therefore be due to contamination with inorganic ^{32}P. In this way, it was found that the best method of removing traces of radioactive inorganic phosphate from the RNA fraction, was by reprecipitating the RNA, obtained with NaCl extraction, from water with glacial acetic acid; but even so, there was still an appreciable amount of activity detected, and therefore in all later experiments a control at 0 hours was always carried out.

The effect of the length of incubation on the incorporation of ^{32}P into the RNA of rat whole liver homogenates is shown in Figure 11. The homogenate was incubated for varying lengths of time up to 5 hours, and throughout this period the pH of the medium remained approximately at 7.4. From the graph, it would seem that the optimum incorporation of ^{32}P into the homogenate RNA, under the conditions used, occurs after about 2 hours incubation at 37°C. A likely explanation of the fall in activity of the RNA fraction, after it has reached a maximum level of incorporation in 2 hours, is that the rate of breakdown of the RNA becomes much more rapid than the rate of incorporation of the isotope. In view of these findings, all incubations thereafter were carried out for 2 hours at 37°C.

A preliminary separation of rat liver homogenate

into whole cytoplasm and nuclei was carried out and these two cellular fractions were incubated with inorganic ^{32}P. It was found that both whole cytoplasm and nuclei were capable of incorporating ^{32}P into their RNA fractions independently (Experiment No. 5, Table 12). The uptake of ^{32}P into the RNA of whole cytoplasm, from 4 g. of liver, was noticeably higher than that observed in the RNA of whole liver homogenate, from the same amount of tissue. On the other hand, the incorporation of ^{32}P into the RNA of isolated nuclei, from 4 g. of tissue, was much lower than that observed with either whole liver homogenate or whole cytoplasm.

In Experiment No. 6, Table 12, a comparison is made between the incorporation of isotope observed when phosphate buffer pH 7.4 and glycine buffer pH 7.4 were used in the incubation medium. Although higher incorporation was observed when phosphate buffer was used, it was found that much less contaminating inorganic phosphate was present in the RNA preparations, when glycine buffer was used. It was, therefore, decided to use glycine buffer in all future work.

The effect of various substrates, in the presence and absence of DPN, was studied in Experiment No. 7, Table 12. It was observed that the greatest incorporation occurred

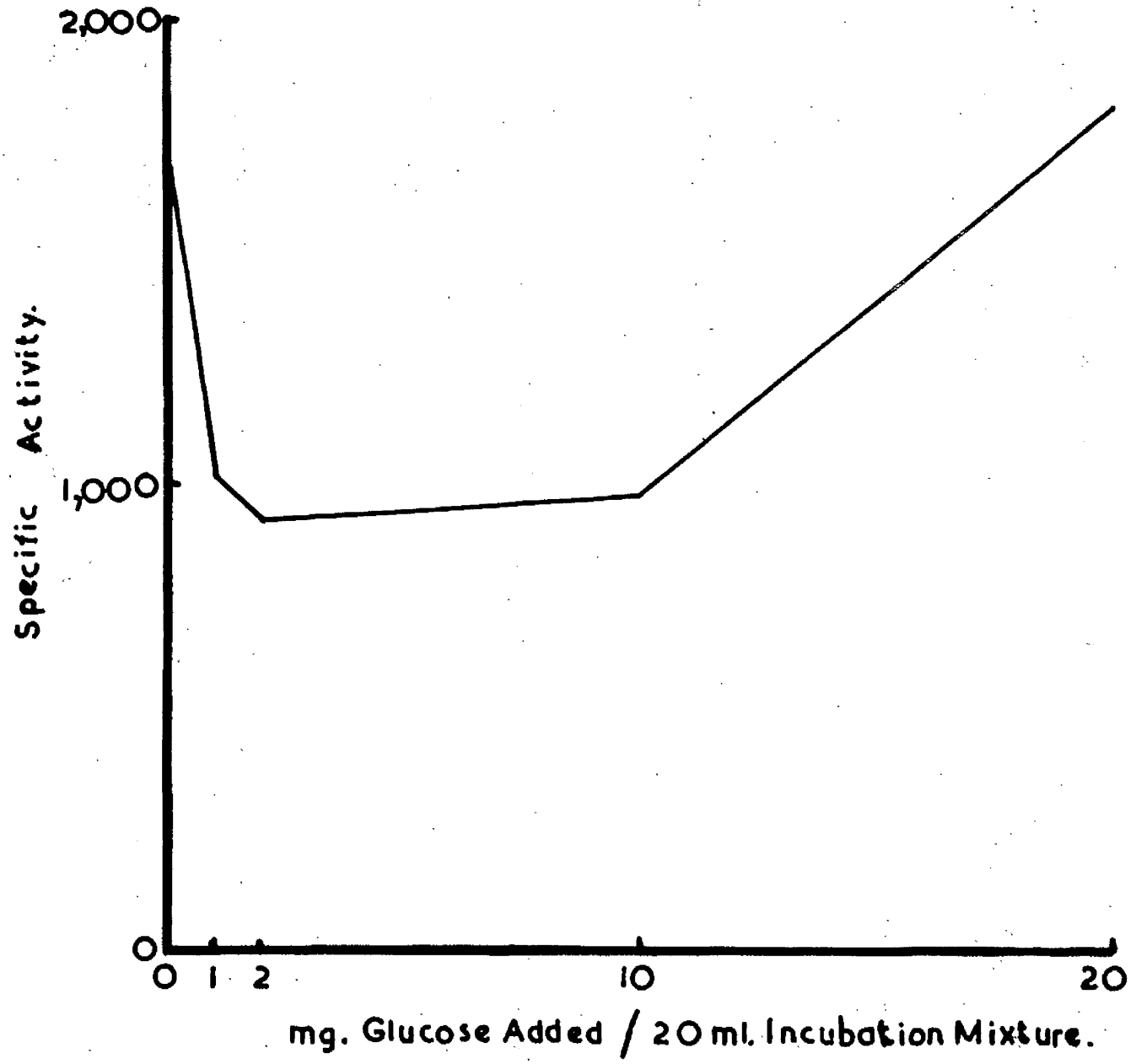
Figure 12.

Rat liver shole cytoplasm, from 4 g. tissue, was incubated with glucose in the presence of ATP, DPN, cytochrome c, $MgCl_2$, Na_2HPO_4, sodium malate and glycine buffer for 2 hours at 37°C.

The RNA fraction was isolated by NaCl extraction and purified by reprecipitating from water with glacial acetic acid.

Figure 12.

The Effect of Glucose on the Incorporation of Inorganic ^{32}P into the RNA of Rat Liver Whole Cytoplasm, in vitro.



whem sodium malate was used, with or without DPN. The uptake of ^{32}P into the RNA in the presence of succinate was enhanced by the addition of DPN, while it was inhibited in the presence of pyruvate on the addition of DPN. The incorporation of the isotope was not affected by DPN when fumarate or malate was present. It was, therefore, decided that in future experiments malate be used as substrate, with added DPN.

Incubation of whole cytoplasm and isolated cellular fractions with radioactive inorganic phosphate (Experiment No. 8, Table 12) gave rise to incorporation of ^{32}P into the RNA fractions of whole cytoplasm, nuclei and mitochondria. When isolated microsomes were incubated with ^{32}P labelled inorganic phosphate, no uptake of the isotope into the microsomal RNA fraction was observed. Cell sap, on incubation with radioactive phosphorus, appeared to show a loss in activity of the RNA fraction. The high activity of the nuclear and cell sap RNA fractions, observed at 0 hours incubation, is probably due to traces of radioactive inorganic phosphate still remaining after rigorous purification of the isolated RNA.

Figure 12 shows the effect on the specific activity of the RNA when whole cytoplasm was incubated with increasing concentrations of glucose. The lower concentrations

of glucose, up to 50 mg./100 ml. have a marked inhibitory effect on the uptake of ^{32}P into the cytoplasmic RNA. The maximum inhibition (40-50%) was obtained at concentrations of 10 mg. glucose/100 ml. Glucose concentrations of 5 and 50 mg. / 100 ml. gave rise to approximately 40% inhibition. The highest concentration of glucose used, namely 100 mg./ 100 ml. appeared to show a very slight increase (6%) in the activity of the RNA fraction, as compared with that of the control.

Throughout these preliminary experiments, the main difficulty encountered was the removal, from the isolated phosphorus compounds, of all traces of radioactive inorganic phosphate. Although it was essential to have sufficient ^{32}P present to give measurable labelling of the phosphorus fraction, such an isotope concentration gave rise to adsorption effects and results which were difficult to interpret. There were also indications that inorganic phosphate did not act as an immediate source of phosphorus for RNA synthesis. In view of this, it was decided to investigate the transfer of ^{32}P from radioactive cellular fractions to non-radioactive cellular fractions on incubation. This would eliminate the necessity of synthesising ^{32}P labelled precursors, in order to study their effect on RNA synthesis. At the same time, by investigating the transfer

Table 13.

The Transfer of ^{32}P from one Labelled Cellular Fraction of Rat Liver to another Unlabelled Cellular Fraction on Incubation. (Counts/min./100µg.P)

(a) Labelled Nuclei and Unlabelled Whole Cytoplasm.

	Nuclei		Whole Cytoplasm	
	0 hr.	2 hr.	0 hr.	2 hr.
Inorganic P	35,400	4,880	15,500	18,025
Acid Soluble Organic P	39,900	6,315	12,100	32,000
Lipid P	38,100	49,350	21,600	27,825
RNA-P	32,350	15,156	8,813	8,033
DNA-P	1,600	1,518	-	-

(b) Unlabelled Nuclei and Labelled Whole Cytoplasm.

Inorganic P	13,250	1,575	50,500	63,700
Acid Soluble Organic P	17,700	5,620	96,400	82,150
Lipid P	137,500	36,750	112,000	81,950
RNA_P	4,973	12,756	22,313	17,133
DNA-P	87	475	-	-

The "composite" homogenates were made up of the nuclear fraction from 6 g. tissue, and the whole cytoplasm from 6 g. tissue. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, $MgCl_2$, Na_2HPO_4, sodium malate and glycine buffer.

of phosphorus from one cellular fraction to another, it would be possible to test the hypothesis of Jeener and Szafarz (1950), that the nucleus synthesises RNA, which then passes out into the cytoplasm, for inclusion into the cytoplasmic particles.

II. The Study of the Transfer of Radioactive Phosphorus from Radioactive Cellular Fraction or Fractions to Non-radioactive Cellular Fraction or Fractions.

Table 13 shows the results obtained when radioactive nuclei from rat liver were incubated with non-radioactive whole cytoplasm from another rat liver. The activities of all the phosphorus fractions of the radioactive nuclei, with the exception of lipid P, were observed to fall after 2 hours incubation with non-radioactive whole cytoplasm; in the whole cytoplasm only the activities of the inorganic P, acid soluble organic P and lipid-P were found to rise, while no change in activity of the whole cytoplasmic RNA fraction was observed.

Also shown in Table 13, are the results obtained when non-radioactive nuclei of rat liver were incubated with radioactive whole cytoplasm. With the exception of inorganic P, all of the phosphorus fractions of radioactive whole cytoplasm showed a fall in activity after 2 hours

Table 14.

The transfer of ^{32}P from one labelled cellular fraction of rat spleen to another unlabelled cellular fraction on incubation. (Counts/min./100 µg.P)

(a) Labelled Nuclei and Unlabelled Whole Cytoplasm

	Nuclei		Whole Cytoplasm	
	0 hr.	2 hr.	0 hr.	2 hr.
Inorganic P	3,440	7,460	917	3,005
Acid Soluble P	7,545	3,980	1,600	3,505
Lipid P	12,750	9,225	1,910	1,285
RNA-P	11,400	5,805	1,096	1,864
DNA-P	7,590	7,185	-	-

(b) Unlabelled Nuclei and Labelled Whole Cytoplasm

Inorganic P	3,890	3,160	4,185	5,415
Acid Soluble P	6,015	4,790	11,200	9,370
Lipid P	904	3,170	7,615	5,470
RNA-P	1,746	3,391	7,480	5,309
DNA-P	163	159	-	-

The "composite" homogenates were made up of the nuclear fraction from 2.5 g. tissue, and the whole cytoplasm from 2.5 g. tissue. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, $MgCl_2$, Na_2HPO_4, sodium malate and glycine buffer.

Table 15.

The transfer of ^{32}P from one labelled cellular fraction of rabbit intestinal mucosa to another unlabelled cellular fraction on incubation (Counts/min./100 µg.P).

(a) Labelled Nuclei and Unlabelled Whole Cytoplasm.

	Nuclei		Whole Cytoplasm.	
	0 hr.	2 hr.	0 hr.	2 hr.
Inorganic P	1,565	1,423	869	1,555
Acid Soluble Organic P	3,110	1,715	1,103	2,415
Lipid P	5,735	3,135	1,428	3,770
RNA-P	5,122	1,120	251	863
DNA-P	2,725	1,475	-	-

(b) Unlabelled Nuclei and Labelled Whole Cytoplasm.

Inorganic P	2,410	4,230	3,355	3,285
Acid Soluble Organic P	5,385	5,340	6,375	3,085
Lipid P	235	2,310	4,695	3,750
RNA_P	370	2,781	6,016	3,525
DNA-P	97	273	-	-

The "composite" homogenates were made up of the nuclear fraction from 6 g. tissue and the whole cytoplasm from 6 g. tissue. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, $MgCl_2$, Na_2HPO_4, sodium malate and glycine buffer.

incubation with non-radioactive nuclei. At the same time there was a fall in activity of the nuclear inorganic P, acid soluble organic P and lipid-P fractions, but the nuclear RNA-P and DNA-P fractions showed a marked increase in activity.

Tables 14 and 15 show the results obtained when similar techniques were applied to rat spleen and rabbit intestinal mucosa homogenates. On incubating non-radioactive nuclei of rat liver or rabbit intestinal mucosa with radioactive whole cytoplasm of corresponding tissues for 2 hours, there was an increase in the activities of the RNA-P and DNA-P fractions of the nuclei, as was found with rat liver. On the other hand, while no increase in activity was noted in the RNA-P of non-radioactive rat liver cytoplasm after incubation with radioactive nuclei from rat liver, the activities of the RNA-P fractions of both non-radioactive rat spleen and rabbit intestinal mucosa were found to increase on incubation with radioactive nuclei.

In order to determine whether the results obtained with radioactive nuclei and non-radioactive cytoplasm of rat spleen and rabbit intestinal mucosa were due to actual transfer of ^{32}P from the radioactive nuclei to the RNA of the cytoplasm, or to contamination of the cytoplasm with nuclear material, the cytoplasmic RNA was hydrolysed to its constituent bases

Table 16.

The molar ratios of the bases present in the A_3S solutions obtained from non-radioactive rat spleen and rabbit intestinal mucosa cytoplasm after incubation with radioactive nuclei.

Molar Ratio of Bases Relative to Adenine Value of 10.0

	0 hr.	2 hr.
Rat Spleen Whole Cytoplasm		
Guanine	19.0	4.07
Adenine	10.0	10.0
Cytosine	5.78	8.92
Uracil	8.68	4.35
Thymine	-	7.48
Rabbit Intestinal Mucosa Whole Cytoplasm.		
Guanine	16.95	17.60
Adenine	10.0	10.0
Cytosine	8.53	15.43
Uracil	7.93	9.88
Thymine	-	-

and these separated by paper chromatography.

The molar proportions of the bases, relative to adenine as 10, obtained for rat spleen and rabbit intestinal mucosa cytoplasmic RNA before and after incubation with nuclei, are shown in Table 16. The results show that the RNA obtained from rat spleen whole cytoplasm, after incubation with nuclei, contained appreciable amounts of thymine. The only possible source of thymine was nuclear DNA which must have been broken down by autolysis during incubation. Consequently, the increase in activity detected in the RNA-P of non-radioactive rat spleen cytoplasm, after incubation with radioactive rat spleen nuclei, can at least in part be attributed to the presence of hydrolysis products of nuclear nucleic acids. On the other hand, chromatographic analysis of the bases present in the RNA isolated from intestinal mucosa cytoplasm, after incubation with nuclei, showed no evidence of the presence of thymine, although there was a considerable change in the molar ratios of cytosine and uracil as compared with those found before incubation. Therefore, the activity detected in the RNA-P of non-radioactive rabbit intestinal mucosa cytoplasm, after incubation with radioactive intestinal mucosa nuclei, was in all probability due to the transfer of ^{32}P from the nucleus into the RNA of the cytoplasm.

Table 17.

The effect of KCN on the transfer of ^{32}P from labelled whole cytoplasm of rabbit intestinal mucosa to unlabelled nuclei of the same tissue (counts/min./100µg.P).

	Addition of KCN (10^{-3}M).							
	No KCN present				KCN present			
	Nuclei		Whole Cytoplasm		Nuclei		Whole Cytoplasm	
	0 hr.	2 hr.	0 hr.	2 hr.	0 hr.	2 hr.	0 hr.	2 hr.
Inorganic P	7,225	8,670	8,560	13,075	6,090	11,250	9,155	14,175
Acid Soluble Organic P	6,325	9,865	17,775	7,200	6,045	10,230	17,355	10,550
RNA-P	565	5,930	12,420	8,070	565	6,406	13,125	7,682
DNA-P	614	826	-	-	613	272	-	-
	Addition of KCN (10^{-1}M).							
Inorganic P	7,090	6,835	6,050	6,840	9,155	7,935	4,660	6,990
Acid Soluble Organic P	5,130	6,040	9,520	5,095	8,020	5,460	10,235	5,435
RNA-P	608	3,020	6,046	4,174	896	3,280	6,220	4,254
DNA-P	315	707	-	-	343	933	-	-

The "composite" homogenate was made up of the unlabelled nuclei from 6 g. tissue and the labelled whole cytoplasm from 6 g. tissue. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, $MgCl_2$, Na_2HPO_4, sodium malate and glycine buffer.

The effect of cyanide on the transfer of ^{32}P from radioactive rabbit intestinal mucosa cytoplasm to non-radioactive nuclei is shown in Table 17. Neither 10^{-3} nor 10^{-1} M-cyanide was found to inhibit the transfer of ^{32}P from the radioactive cytoplasm to non-radioactive nuclei of rabbit intestinal mucosa, and these results show that the process is not dependent on oxidative mechanisms, particularly those involving the cytochrome system, which is extremely sensitive to the presence of cyanide.

Shown in Table 18 are the results obtained for the transfer of ^{32}P from various radioactive cytoplasmic fractions to non-radioactive nuclei, in the presence and absence of other non-radioactive cytoplasmic fractions. Liver was used for this investigation, since it is the tissue which is most readily differentiated into cytoplasmic fractions, and rabbits were used as convenient sources of large amounts of material. In order to compare the results obtained from different mixtures of cytoplasmic fractions, it was desirable to have a common factor. The factor decided upon was that the increment in specific activity of DNA-P after 2 hours incubation would be taken as 100. The change in specific activity of all other phosphorus fractions, was, therefore, calculated relative to an increase in DNA-P specific activity of 100 on incubation (Relative Δ.S.A.).

The "composite" homogenates were made up of the nuclei from 9 g. tissue and the cytoplasmic fraction or fractions from 6 g. tissue. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, $MgCl_2$, Na_2HPO_4, sodium malate and glycine buffer.

Relative Δ S.A. - this expression was derived by calculating the difference between the specific activities (counts/min./100 µg. P)(S.A.) of the phosphorus-containing fractions at 0 hours and 2 hours, relative to a difference between the S.A. of DNA-P, at these times, of 100.

Table 18.

The transfer of ^{32}P into non-radioactive nuclei of rabbit liver from radioactive cellular fractions of the same tissue.

(a) Non-radioactive Nuclei and Radioactive Cell Sap

	Counts/min./100µg.P of nuclear material. 0 hr.	2 hr.	Relative Δ S.A. *
Inorganic P	4,930	6,700	+2,900
Acid Soluble Organic P	10,975	14,085	+5,100
Lipid P	77	3,140	+5,030
RNA-P	109	1,468	+2,230
DNA-P	16	77	+100

(b) Non-radioactive Nuclei and Non-radioactive Cytoplasmic Particles and Radioactive Cell Sap

Inorganic P	6,165	5,060	−794
Acid Soluble Organic P	14,800	9,310	−3,730
Lipid P	208	4,290	+2,780
RNA-P	106	2,641	+1,725
DNA-P	12	159	+100

(c) Non-radioactive Nuclei and Radioactive Cytoplasmic Particles.

Inorganic P	1,833	1,721	−99
Acid Soluble Organic P	6,275	4,040	−1,975
Lipid P	373	1,695	+1,170
RNA-P	243	501	+227
DNA-P	15	128	+100

(d) Non-radioactive Nuclei and Non-radioactive Cell Sap and Radioactive Cytoplasmic Particles.

Inorganic P	2,460	1,149	−728
Acid Soluble Organic P	1,223	1,098	−70
Lipid P	107	760	+353
RNA-P	46	184	+77
DNA-P	82	262	+100

From the results, shown in Table 18, it would appear that when non-radioactive rabbit liver nuclei are incubated with radioactive rabbit liver cell sap there is a considerable transfer of ^{32}P to the nucleic acids of the nucleus. However, when non-radioactive cytoplasmic particles are also incubated with non-radioactive nuclei in the presence of radioactive cell sap, there is a considerable reduction in the transfer of ^{32}P from the radioactive cell sapto the nucleic acid fractions of the nucleus. The effect of the cytoplasmic particles is possibly due either to a dilution of the level of ^{32}P of the radioactive cytoplasm, or to the transfer of ^{32}P from the radioactive cytoplasmto the non-radioactive cytoplasmic particles as well as to the non-radioactive nuclei.

When radioactive cytoplasmic particles are incubated with non-radioactive nuclei, there is very little transfer of ^{32}P to the nucleic acids of the nucleus. The addition of non-radioactive cell sap to an incubation mixture, containing radioactive cytoplasmic particles and non-radioactive nuclei, reduces the amount of ^{32}P transferred to the RNA-P of the nucleus, without decreasing the transfer of ^{32}P into the DNA-P of the nucleus.

These results would suggest that the cell sap provides a source of phosphorus for transfer into the

Table 19.

The effect of dialysis on the transfer of ^{32}P from radioactive cell sap into non-radioactive nuclei of rabbit liver.

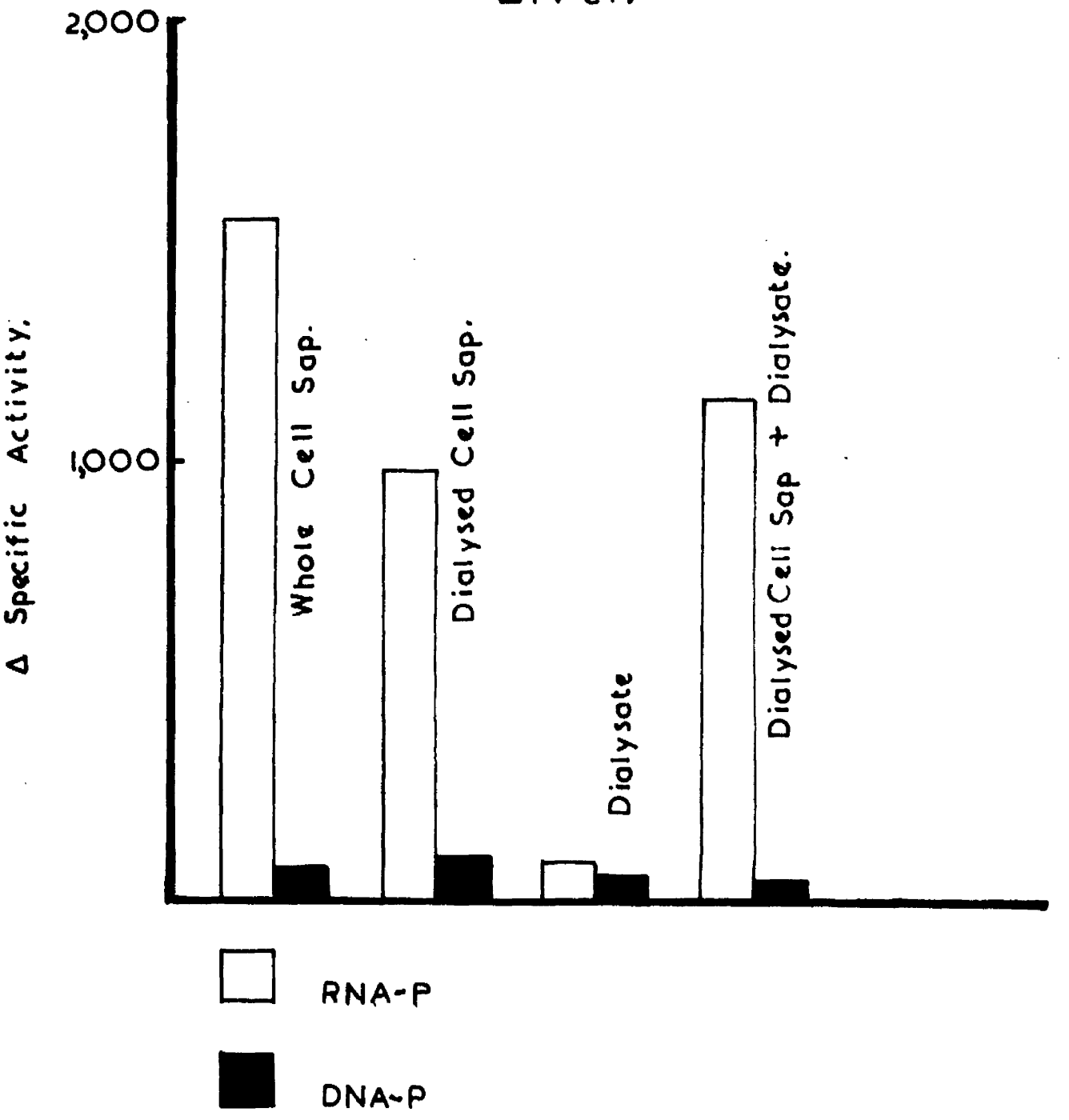
	Specific activity of Nuclear Components (counts/min./100 µg. P)		Δ S.A.
	0 hr.	2 hr.	
(a) Whole Cell Sap			
Inorganic P	2,410	3,915	+1,305
Acid Soluble Organic P	9,165	10,420	+1,265
RNA-P	68	1,605	+1,537
DNA-P	29	103	+ 74
(b) Dialysed Cell Sap			
Inorganic P	3,500	2,640	- 860
Acid Soluble Organic P	10,900	7,140	-3,760
RNA-P	70	1,038	+968
DNA-P	9	98	+ 89
(c) Dialysate			
Inorganic P	2,605	1,420	-1,185
Acid Soluble Organic P	3,860	3,010	- 850
RNA-P	13	95	+82
DNA-P	5	60	+55
(d) Dialysed Cell Sap and Dialysate			
Inorganic P	2,945	2,213	- 732
Acid Soluble Organic P	11,125	8,730	-2,395
RNA-P	49	1,174	+1,125
DNA-P	21	46	+ 25

Δ S.A. is the difference in specific activity between 0 and 2 hr. values.

The "composite" homogenate was made up of the non-radioactive nuclei from 9 g. tissue and the radioactive cell sap (or dialysed cell sap and/or dialysate) from 6 g. tissue. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, Na_2HPO_4 , sodium malate and glycine buffer.

Figure 13.

The Effect of Dialysis on the Transfer of ^{32}P from Radioactive Cell Sap to Non-radioactive Nuclei of Rabbit Liver.



nucleic acids of the nucleus, particularly the RNA.

The effect of dialysis of radioactive rabbit liver cell sap, on the transfer of ^{32}P to non-radioactive rabbit liver nuclei, is shown in Table 19 and Figure 13. When dialysed radioactive cell sap was incubated with non-radioactive nuclei, there was a reduction in the transfer of ^{32}P into the RNA-P of the nucleus, as compared with whole radioactive cell sap. At the same time, transfer of ^{32}P from dialysed cell sap into the DNA-P of the nucleus would appear to have been slightly enhanced, compared with that observed with whole cell sap.

When the dialysate from radioactive cell sap alone was incubated with non-radioactive nuclei, there was very little transfer of ^{32}P into the nuclear RNA. At the same time, there was an decrease in the activity of the DNA-P fraction compared with that observed with whole cell sap.

However, when dialysed cell sap and dialysate were both present, an increase in the transfer of ^{32}P into the nuclear RNA was observed, although the transfer of phosphorus into the DNA-P appeared to fall off.

It would appear from these results that both the non-dialysable material and dialysable components of the cell sap are necessary for the transfer of phosphorus from the cell sap into the RNA of the nucleus. However, it is

Table 20.

Incorporation of ^{32}P derived from Total acid soluble P or Inorganic P into non-radioactive rabbit liver nuclei in the presence and absence of non-radioactive cell sap. (Counts/min./µg.P).

(a) Radioactive Acid Soluble Phosphorus

	In absence of Cell Sap*			In presence of Cell Sap*		
	0 hr.	2 hr.	ΔS.A.	0 hr.	2 hr.	ΔS.A.
Inorganic P	12,250	8,350	-3,900	1,226	550	-676
Acid Soluble Organic P	10,925	7,105	-3,820	2,370	1,295	-1,075
LP	6	34	+28	217	9	-208
RNA-P	11	30	+19	0	62	+62
DNA-P	0	4	+4	51	607	+546

(b) Radioactive Inorganic P

Inorganic P	2,695	667	-2,028	4,280	2,405	-1,875
Acid Soluble Organic P	3,155	2,520	-635	3,840	3,445	-395
LP	0	8	+8	108	8	-100
RNA-P	23	55	+32	0	73	+73
DNA-P	16	23	+7	0	138	+138

* In the absence of added cell sap, the total acid soluble P and inorganic P added to the incubation mixtures was derived from cell sap from 6 g. rabbit liver.

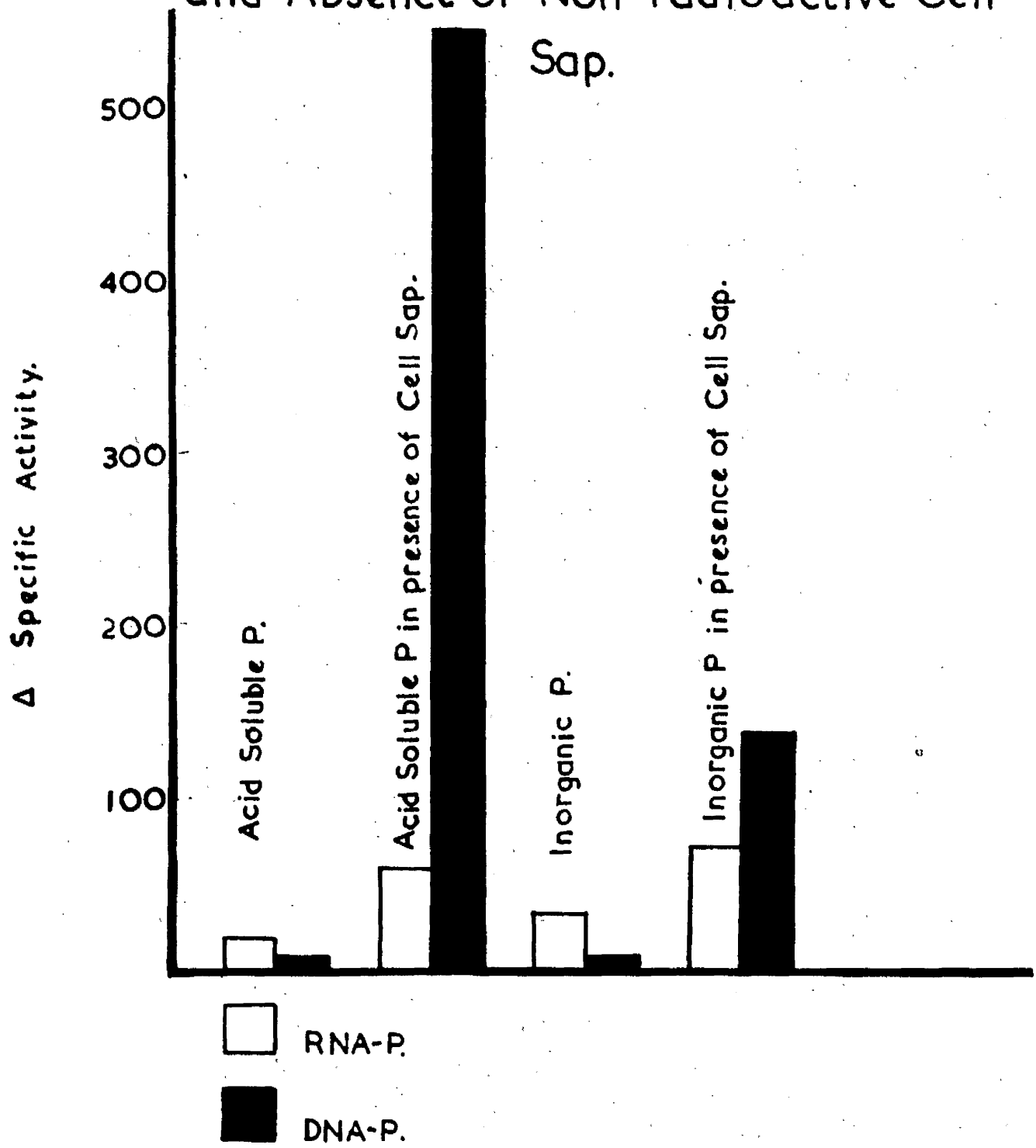
In the presence of added cell sap, the total acid soluble P added was derived from cell sap from 1.2 g. tissue, while the inorganic P was derived from 12 g. tissue.

The amounts of radioactive P added was calculated so that the final concentration of P in the "composite" homogenate was similar to that found in whole tissue.

The "composite" homogenates consisted of the non-radioactive nuclei from 9 g. tissue, and where non-radioactive cell sap was added, it was derived from 6 g. tissue. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, $MgCl_2$, sodium malate and glycine buffer.

Figure 14.

The Incorporation of ^{32}P, Derived from Acid Soluble P and Inorganic P, into Non-radioactive Nuclei in the Presence and Absence of Non-radioactive Cell Sap.



difficult to interpret the results obtained for the transfer of phosphorus from the cell sap to the nuclear DNA-P. The dialysed cell sap would appear to be able to transfer ^{32}P much more readily to the DNA fraction than does the whole cell sap or dialysed cell sap and dialysate. The dialysate alone apparently is more capable of transferring phosphorus to the DNA-P than is the reconstituted cell sap. It seems, therefore, that the purity of the DNA-P fractions must be questioned.

Table 20 and Figure 14 show the results obtained when the radioactive acid soluble phosphorus components of rabbit liver cell sap were incubated with non-radioactive rabbit liver nuclei, in the presence and absence of non-radioactive cell sap. These acid soluble phosphorus fractions of cell sap were studied because the effect of cell sap in transferring phosphorus to the nucleus may be due to its inorganic phosphate content or to the presence of soluble organic phosphorus-containing compounds in the cell sap. In the process of cellular fractionation, these soluble phosphorus compounds are almost exclusively isolated in the cell sap fraction. The results obtained would suggest that, in the absence of cell sap, inorganic P and total acid soluble P are not capable of acting as sources of phosphorus for transference to the nucleic acids of the nucleus. Even in the presence of cell sap, these phosphorus fractions do not

Table 21.

Incorporation of ^{32}P derived from radioactive Lipid P and RNA-P, into non-radioactive rabbit liver nuclei in the presence and absence of dialysed non-radioactive cell sap(Counts/min./100µg.P).

(a) Radioactive Lipid P (corresponding to 7.84mg. Lipid P)

	Absence of Dialysed Cell Sap			Presence of Dialysed Cell Sap		
	0 hr.	2 hr.	ΔS.A.	0 hr.	2 hr.	ΔS.A.
Inorganic P	1,170	1,258	+88	21,650	2,355	-19,295
Acid Soluble Organic P	6,140	5,020	-1,120	6,524	3,195	-3,329
RNA-P	1,084	995	-89	4,345	6,890	+2,545
DNA-P	115	126	+11	173	185	+13

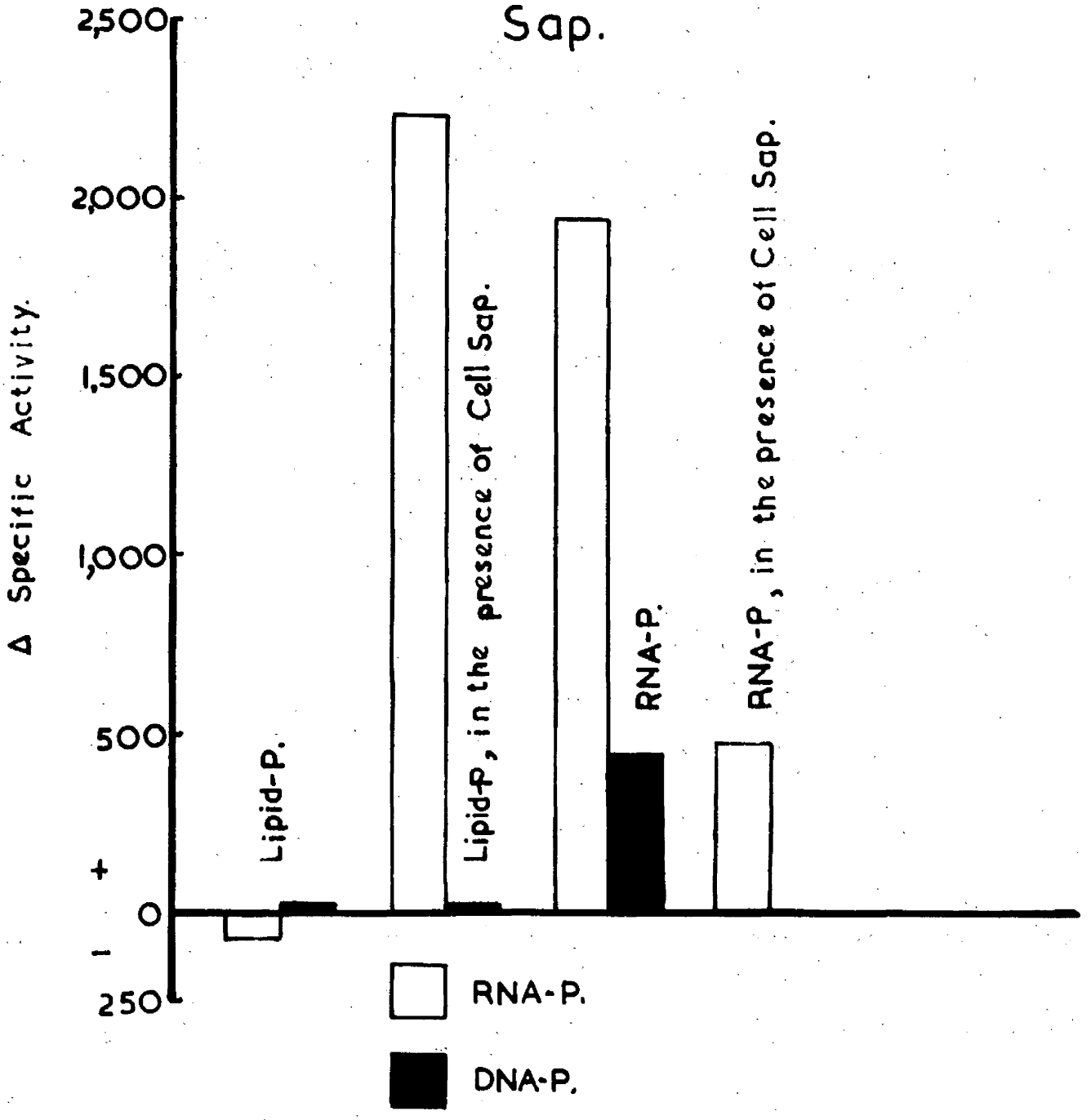
(b) Radioactive RNA-P (corresponding to 1.54 mg. RNA-P)

Inorganic P	4,835	8,400	+3,665	987	1,340	+353
Acid Soluble Organic P	3,340	3,560	+220	2,070	2,060	-10
RNA-P	578	2,520	+1,942	699	1,180	+481
DNA-P	7	452	+445	Fraction lost	Fraction lost	-

The "composite" homogenates consisted of non-radioactive nuclei from 9 g. rabbit liver and dialysate from cell sap from 6 g. non-radioactive liver. Where cell sap was present, it was derived from 6 g. non-radioactive liver. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, Na_2HPO_4, sodium malate and glycine buffer.

Figure 15.

The Incorporation of ^{32}P, Derived from Radioactive Lipid-P and RNA-P, into Non-radioactive Nuclei, in the Presence and Absence of Non radioactive Cell Sap.



appear to participate in the transfer of phosphorus to the nuclear RNA. Both of these phosphorus fractions, however, transfer phosphorus to the DNA of the nucleus when cell sap is present. Total acid soluble P, which contains organic phosphorus compounds as well as inorganic phosphate, seems to be a better donor of phorphorus for inclusion into the DNA-P fraction than inorganic phosphate alone. Hence the action of cell sap is not due merelyto its inorganic phosphate content.

In Table 21 and Figure 15, the results, obtained when radioactive lipid-P and RNA-P were incubated with non-radioactive rabbit liver nuclei, in the presence and absence of non-radioactive cell sap, are shown. Lipid-P would appear to be inactive in the absence of cell sap, but when cell sap was also present a marked transfer of ^{32}P into the the nuclear RNA fraction was observed, without any apparent transfer of ^{32}P into the DNA-P fraction of the nucleus. It was noted, however, during the isolation of the nuclei, after incubation with added lipid, that there were large amounts of lipid material adsorbed on to the isolated nuclei. The observed transfer of phosphorus into the nuclear RNA may, therefore, be due to the contamination of the nuclear RNA fraction with lipid material or its degradation products. Incubation of non-radioactive nuclei with radioactive RNA-P in the absence of any cell sap would appear to give rise to

Table 22.

The effectiveness of different fractions of dialysed radioactive cell sap in transferring ^{32}P to non-radioactive nuclei on incubation (counts/min./100 µg. P).

(a) Dialysed Cell Sap and Dialysate

	0 hr.	2 hr.	Δ S.A.
Inorganic P	3,310	7,615	+3,805
Acid Soluble Organic P	1,455	15,650	+14,195
RNA-P	104	561	+457
DNA-P	228	304	+76

(b) Fraction I of Dialysed Cell Sap (precipitated with 0-33%$(NH_4)_2SO_4$ Saturation) and dialysate

Inorganic P	726	4,765	+4,039
Acid Soluble Organic P	10,975	12,275	+1,300
RNA-P	80	265	+185
DNA-P	41	227	+186

(c) Fraction II of Dialysed Cell Sap (precipitated with 33-66%$(NH_4)_2SO_4$ Saturation) and dialysate

Inorganic P	2,060	4,050	+1,990
Acid Soluble Organic P	13,750	12,175	−575
RNA-P	179	677	+498
DNA-P	32	90	+58

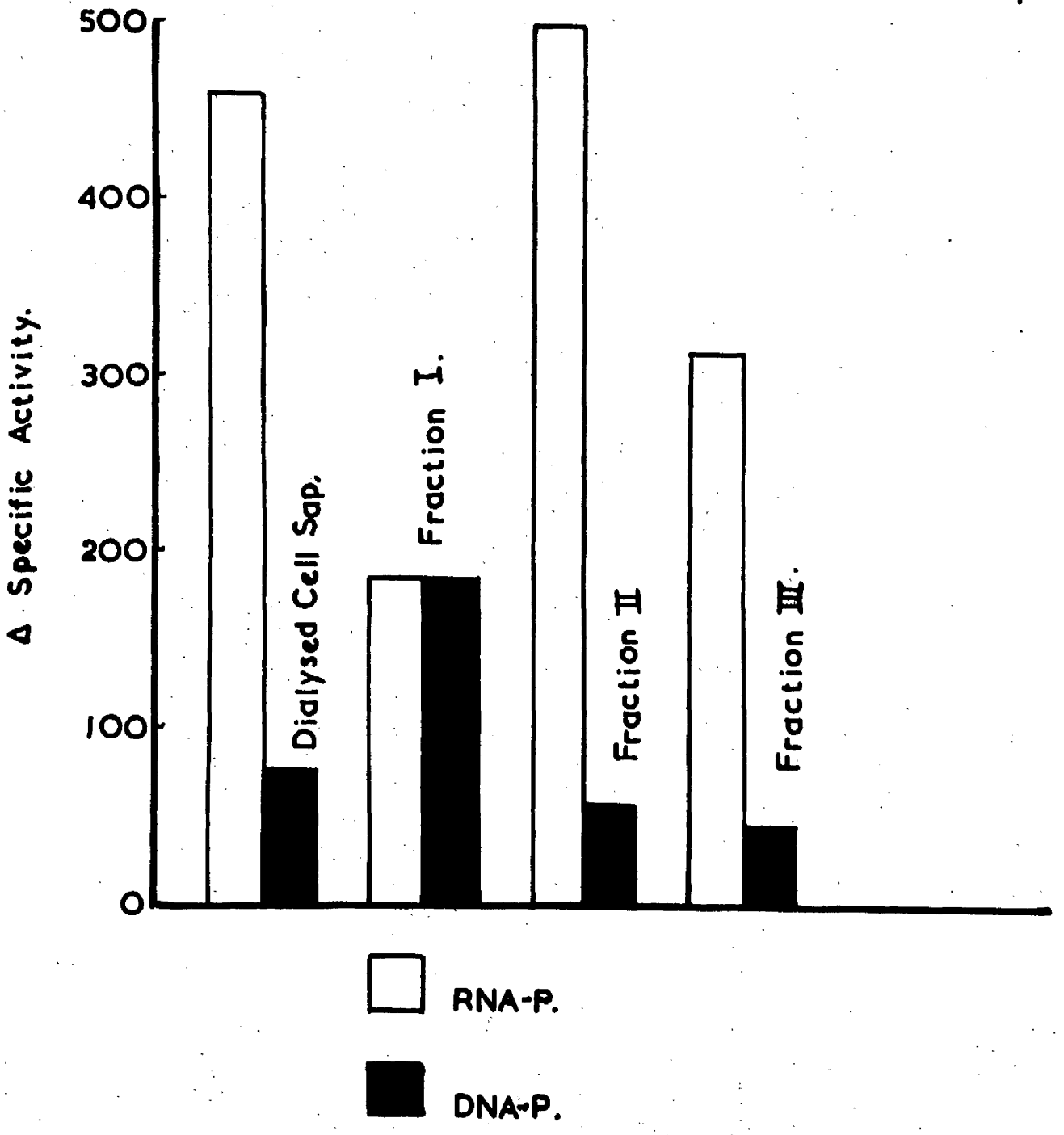
(d) Fraction III of Dialysed Cell Sap (not precipitated with 66%$(NH_4)_2SO_4$ Saturation) and dialysate

Inorganic P	5,995	2,595	−3,400
Acid Soluble Organic P	10,340	8,765	−1,675
RNA-P	120	433	+313
DNA-P	11	56	+45

The "composite" homogenate was made up of the non-radioactive nuclei from 9 g. rabbit liver and dialysed cell sap (or fraction) and dialysate from 6 g. radioactive liver. The "composite" homogenate was incubated in the presence of ATP, cyt c, DPN, Na_2HPO_4, sodium malate and glycine buffer.

Figure 16.

The Effectiveness of the Different Fractions of Dialysed Radioactive Cell Sap in Transferring ^{32}P to Non-radioactive Nuclei, in the Presence of Radioactive Dialysate.



the transfer of phosphorus to both of the nuclear nucleic acid fractions. Addition of non-radioactive cell sap to the incubation mixture gives rise to a marked reduction in the amount of phosphorus transferred from the labelled RNA-P to the nuclear RNA. The DNA fractions in this experiment were both lost and consequently, no observations can be made as to whether RNA can act as a donor of transferable phosphorus for DNA synthesis. The results obtained in these experiments on the transfer of ^{32}P from labelled RNA to nuclear RNA, could be explained by the adsorption of the labelled RNA on to the nuclei. Such adsorbed RNA would therefore contaminate the nuclear RNA.

Fractionation of radioactive cell sap, with saturated ammonium sulphate, into three protein fractions, which were then incubated with non-radioactive nuclei gave the results shown in Table 22 and Figure 16. From these results, it is clear that the various protein fractions differed in their abilities to transfer ^{32}P to nuclear RNA and DNA.

Fraction I of dialysed cell sap, i.e. that fraction precipitated by 0-33% ammonium sulphate saturation, seems to be very active in transferring phosphorus to the DNA-P fraction of the nucleus. It is much less active in the transfer of phosphorus to the nuclear RNA in comparison with whole cell sap or the other protein fractions of cell sap.

Fraction II of dialysed cell sap, i.e. that fraction precipitated by 33-66% ammonium sulphate saturation, is very active in the transfer of ^{32}P to nuclear RNA. It is more active than whole cell sap as a source of transferable phosphorus for inclusion into the nuclear RNA. On the other hand, it is not as active as whole cell sap or Fraction I in transferring phosphorus to the DNA-P of the nucleus.

Fraction III of dialysed cell sap, i.e. that fraction not precipitated by 66% ammonium sulphate saturation, although more active in the transfer of phosphorus into nuclear RNA than Fraction I, is not as active as whole cell sap or Fraction II. It would also appear that Fraction III is less capable of supplying phosphorus for transfer into the DNA-P of the nucleus.

4. Discussion.

One of the main difficulties encountered, in these studies of the incorporation and transfer of ^{32}P in homogenates, has been to differentiate between true incorporation and transfer of the radioactive phosphorus, on the one hand, and adsorption or exchange of the labelled inorganic phosphorus in the nucleic acid fractions, on the other. True incorporation of ^{32}P into the nucleic acids occurs when the nucleic acid is synthesised from its precursors. During this process, the radioactive phosphorus is transferred from the precursors to the newly formed nucleic acid molecule. This process differs from exchange, since during exchange there is a transient break in the nucleic acid molecule at the phosphate group with the liberation of phosphate. The liberated phosphate group then exchanges with the phosphate of the medium before the nucleic acid chain is reconstituted. If this occurs in the presence of inorganic ^{32}P, the ^{32}P atoms of the medium can exchange with the ^{31}P atoms of the nucleic acid molecule. On the other hand, when adsorption occurs, the radioactive inorganic phosphate is bound to the nucleic acid by purely physical means, rather than by entering into chemical combination with it.

In the preliminary experiments, when zero time control

flasks were set up, it was invariably found that the isolated nucleic acid fractions contained traces of adsorbed radioactive phosphorus which could be partially removed by drastic purification of the isolated compound. However, even the ribonucleotides, isolated by ionophoresis, contained ^{32}P; all had approximately the same specific activity. Since the inorganic phosphate is not found in the same regions of the ionophoretic paper as any of the nucleotide spots, this activity could not, therefore, be due to contamination by radioactive inorganic phosphate. This activity must, therefore, be due to exchange of the ^{32}P atoms of the labelled inorganic phosphate with the ^{31}P atoms of the nucleic acid molecules. Because of this exchange reaction, it was imperative to carry out control experiments in order that allowance could be made for these exchange and adsorption effects. Thus, when the effect of adding known compounds was being studied, a control experiment, where no addition was made, was also carried out. Zero time controls were determined in almost all cases, and any activity detected was due entirely to exchange and adsorption of the radioactive phosphorus.

Friedkin and Lehninger (1949) found the same difficulty, in their investigations of the incorporation of ^{32}P into the nucleic acids of cell-free rat liver homogenates. Even after treatment of the nucleic acid fractions of the

unincubated rat liver homogenates with carrier phosphate and subsequent precipitation with magnesia mixture, they were still able to detect ^{32}P in the RNA fraction. In the present studies, the zero time controls were found to contain traces of ^{32}P in the RNA after purification by reprecipitation from water with glacial acetic acid, and separation of the nucleotides by ionophoresis.

In most of the in vitro studies, in which either tissue slices of tissue homogenates have been used, the medium has invariably been of the Krebs-Ringer type. In the initial experiments of this series, the ultimate aim was the study of the incorporation of ^{32}P into the cellular fractions. It was, therefore, decided to use a system whereby these cellular fractions could be isolated and studied. Since it has been shown that the presence of electrolytes prevent the preparation of clean cytoplasmic particles (Schneider & Hogeboom 1951), and the best known method of preparing cellular fractions utilises 0.25 M-sucrose solution (Schneider 1948), it was decided to devise an incubation medium which had sucrose as its basis.

The final incubation medium suffered from certain disadvantages, but these were, on the whole, outnumbered by the advantages. The main disadvantage of sucrose solution as an incubation medium is that it does not test the metabolism of the tissue under physiological conditions, since it is not

a normal constituent of the tissue fluids. 0.25 M-sucrose is, however, isotonic with respect to the cellular components, and consequently does not injure the intracellular fractions to the same extent as would hypertonic or hypotonic solutions. Schneider (1948) claimed that 0.25 M-sucrose solution did not inhibit the activity of the enzyme systems of the cellular fractions to the same extent as did 0.88 M-sucrose. Even so, it is possible that 0.25 M-sucrose does inhibit some of the enzymes present in the intracellular components. Another defect, in the use of 0.25 M-sucrose solution, is that the nuclei prepared in this medium are much larger and more fragile in comparison with those prepared in citric acid (Dounce 1943a, 1943b, 1950). However, it is impractical to use citric acid nuclei, since the cytoplasmic components are completely agglutinated by the low pH and may not subsequently be isolated and moreover most of the enzymes will be inactivated. Peterman and Schneider (1950, 1951) have advocated the addition of calcium chloride to the sucrose solution used in the preparation of nuclei, to reduce their fragility. Since the effect of calcium chloride on the cytoplasmic particles, particularly during incubation, was not known, this technique was rejected.

0.25 M-sucrose - 0.03 M-phosphate buffer solution was used as incubation medium by Siekevitz (1952) in his investigation of the metabolism of rat liver homogenates and of

the isolated cellular fractions. Siekevitz (1952) found that [1-^{14}C]-alanine was incorporated into the proteins of the microsomal fraction of sucrose homogenates of rat liver much more rapidly than into the nuclear, mitochondrial or cell sap fractions. No incorporation of labelled alanine into the proteins of either isolated microsomes or mitochondria occurred on incubation, but when the mitochondrial and microsomal fractions were incubated together, marked incorporation of radioactive alanine was observed. Using the same incubation medium, Allfrey (1954) found that isolated calf thymus nuclei were capable of incorporating [1-^{14}C]-alanine into their proteins, in the presence of added α-ketoglutarate, as long as the nuclear DNA was still intact. These findings indicate that the sucrose medium does not affect the metabolism of isolated cellular fractions to any marked extent.

The Schneider (1948) procedure for the isolation of intracellular components has been found by many workers to be a very useful method of preparing clean and homogeneous cellular fractions. It has the added advantage that it does not require prolonged centrifugation at high centrifugal speeds, which in themselves may have an adverse effect on the enzymes present in the intracellular particles. In addition, the pH of the sucrose solution is readily adjusted by means of relatively weak buffer solutions. Moreover, as an incubation medium it

offered the opportunity of reisolating the cytoplasmic particles after incubation.

In the study of the transfer of ^{32}P from radioactive cell fractions to non-radioactive cell fractions, it was found advantageous to isolate the nuclei initially in 0.25 M-sucrose (Schneider 1948) and subsequently to re-isolate the nuclei, after incubation, using the modified citric acid method already described. By using both methods of obtaining nuclei from liver tissue, it was possible to obtain the initial nuclei free from electrolytes and with their enzymes relatively intact. After incubation, it was no longer necessary to exclude electrolytes or to preserve the nuclear enzyme systems, and so the much more drastic citric acid method was employed to re-isolate nuclei free from cytoplasmic contamination.

It must be pointed out, however, that the nuclei re-isolated after incubation were changed in appearance, when examined microscopically, and also in their ability to stain with methylene blue. The nuclei, before incubation, were large round discrete bodies, which stained readily with methylene blue, but after incubation, the nuclei obtained were small irregular bodies, which did not stain readily.

Glycihe buffer was used in preference to phosphate buffer in the incubation medium, since, although it did not

give rise to such a high uptake of ^{32}P into the RNA fractions of the rat liver homogenates, it avoided the presence of a large excess of inorganic phosphate ions in the medium (Experiment No. 6, Table 12). Although the phosphate buffer did not appear to reduce the incorporation of the labelled phosphate by dilution effects, it did increase the difficulties of isolating the phosphorus containing fractions, particularly the RNA-P and DNA-P fractions, due to adsorption of phosphate ions on the precipitated material.

Throughout these in vitro experiments, the time of incubation used was 2 hours. In the experiment, in which the optimum time of incubation was investigated (Figure 11), it was found that the uptake of ^{32}P into the RNA of whole rat liver homogenate increased with time up to 2 hours, and thereafter decreased rapidly. This may be due to the uptake of the isotope being greater than the autolysis of the RNA fraction with periods of incubation up to 2 hours, but during longer periods of incubation, autolysis may increase to a marked extent and outstrip the incorporation of the ^{32}P. The length of incubation was restricted, therefore, to 2 hours at 37°C.

Friedkin and Lehninger (1949) found that there was incorporation of ^{32}P into the RNA of suspensions of rat liver nuclei and mitochondria, on incubation with inorganic ^{32}P. This incorporation was observed even after rigorous purification of

the RNA fraction. They obtained, however, no evidence of incorporation of the isotope into the nuclear DNA. Their incubation mixture contained ATP, KCl, $MgSO_4$ and they found that the presence of malate greatly increased the incorporation. During the present investigations, Experiment No. 7, Table 12, the uptake of ^{32}P into the RNA fraction of rat liver whole cytoplasm suspensions, in the presence of ATP, DPN, cytochrome c, $MgCl_2$ and Na_2HPO_4, was also found to be enhanced by the addition of sodium malate. It was also observed that malate was superior to succinate, fumarate or pyruvate in its effect on the incorporation of the isotope.

Mann and Gruschow (1949) were able to demonstrate the incorporation of inorganic ^{32}P into the nucleic acids of rat liver, rat kidney and rat Walker tumour slices. They showed at the same time that oxygen was necessary for this to occur. The addition of glucose to the tissue slices was capable of partially reversing the inhibition brought about by anaerobic conditions. Although it is very difficult to compare results obtained by tissue slice and homogenate techniques, it was found during the present work that the addition of glucose, to the incubation medium in the presence of oxygen, did not increase the incorporation of ^{32}P, but rather inhibited this process, particularly at low concentrations of glucose (Figure 12).

Rossiter and his co-workers (Deluca, Rossiter & Strickland 1953; Findlay, Rossiter & Strickland 1953; Strickland & Rossiter 1953) showed that brain slices were capable of incorporating ^{32}P into the RNA, although when brain homogenates were used no uptake was observed. The incorporation of ^{32}P into the RNA of cat brain slices was greatly increased by the addition of glucose to the incubation medium. Succinate and malate both had inhibitory effects, while pyruvate and lactate stimulated the incorporation of the isotope. It is difficult to draw comparisons between their findings and those found during the present investigation, since two different techniques were used, namely tissue slices and tissue homogenates, as well as two different tissues, brain and liver. It is possible, however, to point out the main differences between the two sets of results. The present work showed partial inhibition of incorporation of ^{32}P into the RNA of liver tissue in the presence of glucose (Figure 12). It was also found that pyruvate and succinate did not enhance the incorporation of the isotope into the RNA of liver whole cytoplasm, while malate did increase the uptake of ^{32}P into the nucleic acids of liver whole cytoplasm suspensions (Experiment No. 7, Table 12).

Khouvine and Montreuil (1954), following up their in vivo studies on the incorporation of ^{32}P into the RNA of the

livers of normal and Guerin epithelioma-bearing rats and the Guerin epithelioma, investigated the uptake of ^{32}P by the RNA of the same tissues in vitro, using the tissue slice technique. They found that ^{32}P was incorporated into the nucleic acids of these tissues in the presence of oxygen, but under anaerobic conditions there was partial inhibition of the uptake of the isotope. They also studied the effects of such inhibitors as 2,4-dinitrophenol, malonate and mono-iodoacetate. With the exception of malonate, all of these compounds gave rise to the inhibition of the incorporation of the ^{32}P. These findings are in agreement with those obtained in the present work, in that it is possible to show that ^{32}P is incorporated into the nucleic acids of liver tissue after its removal from the body. However, since no work was carried out on the effect of inhibitors, it is not possible to compare further the present findings with those obtained by Khouvine and Montreuil (1954).

Hokin (1952), and Hokin and Hokin (1953, 1954), in their studies on protein synthesis, have investigated the secretion of amylase by pigeon pancreas slices and the simultaneous uptake of ^{32}P into the RNA of the tissue. It is interesting to note that while they found the addition of a mixture of all known amino acids stimulated the incorporation of ^{32}P into the RNA of the pancreas slices, omission of tryptophan, from the amino acid mixture, inhibited the

secretion of amylase without affecting the uptake of ^{32}P into the RNA. Hokin and Hokin (1953, 1954), therefore, came to the conclusion that RNA did not act directly in the synthesis of protein in pigeon pancreas slices. While of no direct bearing on the present work, these findings do show that the uptake of ^{32}P can be observed in widely different types of tissues, and that different tissues may be utilised to study different aspects of nucleic acid metabolism in vitro.

Other workers have demonstrated the incorporation of labelled purines and pyrimidines into the nucleic acids of tissues in vitro. Goldwasser (1953), using [8-^{14}C]-adenine, was able to show that it was incorporated in vitro into the RNA of rat liver, rat spleen and pigeon liver slices and of pigeon liver homogenates. This incorporation of labelled adenine required the presence of oxygen and was inhibited by the presence of cyanide. From the results obtained by Goldwasser (1953), it would appear that the incorporation of adenine was a true incorporation and not adsorption of the labelled adenine.

Grossman and Visser (1954) found that [4-^{14}C]-cytidine was incorporated into the RNA of rat liver slices. They were also able to show that the uptake of the labelled cytidine into the nuclear RNA was much more rapid than that observed in the cytoplasmic RNA. These in vitro findings of Goldwasser(1953)

and Grossman and Visser (1954) are in agreement with those obtained in intact animals and support the conclusion that the results obtained by in vitro studies are due to true incorporation and not to contamination or exchange reactions during incubation.

Jeener and Szafarz (1950) have put forward the hypothesis that the nucleus is the site of RNA synthesis in the cell. The RNA so formed, they postulated, was passed out of the nucleus into the sap of the cytoplasm where it was built up into the cytoplasmic particles. In the present work with rat liver homogenates (Experiment No. 8, Table 12), it was possible to demonstrate that the RNA of the whole cytoplasm was capable of incorporating ^{32}P. At the same time it was found that the isolated nuclei also incorporated the isotope into the nuclear RNA. This would indicate that the cytoplasm was able to synthesis its own RNA.

The findings of Brachet and Szafarz (1953), who showed that both the nucleated and enucleated fragments of the algae, Acetabularia mediterranea, were capable of assimilating [2-^{14}C]-orotic acid into their nucleic acids up to 71 days after section in the presence of oxygen, lends further support to this conclusion.

Reichard (1952), using regenerating rat liver slices, has demonstrated that the nitrogen of $^{15}NH_4Cl$ was incorporated into the pyrimidines of RNA. The first step in the

incorporation of the labelled nitrogen is apparently the formation of labelled orotic acid, which in turn is converted to labelled pyrimidine bases.

It is, therefore, reasonable to suppose that under the conditions used by Brachet and Szafarz (1953) the labelled orotic acid was incorporated directly into the nucleic acid pyrimidines, without previously being degraded to some compound of lower molecular weight.

Lang and his co-workers (Siebert, Lang, Lucius and Rossmüller 1953; Lang, Lang, Siebert and Lucius 1953; Lang, Siebert & Rossmüller 1953) have studied the metabolism of isolated pig kidney nuclei, in relation to the source of phosphorus and carbon compounds required for the synthetic processes of the nucleus. Siebert et al. (1953) were able to show that inorganic phosphate labelled with ^{32}P was incorporated into the RNA and DNA of isolated pig kidney nuclei. They concluded from their results that the incorporation of the inorganic phosphate into the nucleic acids of the nucleus did not require the formation of high energy bonds. Lang, Siebert & Rossmüller (1953) investigated the possibility that phospholipid phosphorus was capable of acting as a source of phosphorus for the synthesis of nuclear RNA and DNA. They found that ^{32}P labelled phospholipid did not act as a source of phosphorus for the synthesis of nuclear RNA and DNA. Lang,

PROFESSOR C. J. FORDYCE, M.A.
Clerk of Senate.

THE UNIVERSITY,
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11th July, 1955

Professor Davidson,
Biochemistry.

Dear Professor Davidson,

I send to you, as Convener of the Sub-Committee, the duplicate copy of Mr. Logan's thesis for the degree of Ph.D. The other members of your Committee are Dr. Munro, Dr. Hutchinson and Dr. Smellie.

Yours sincerely,

C. J. Fordyce

CLERK OF SENATE

Lang, Siebert and Lucius (1953), using ^{14}C-labelled glycine, were able to show that the glycine was incorporated into the purine of the nucleus, as well as into the nuclear proteins. They were, therefore, able to show that total synthesis of the purine ring can occur in the nucleus in vitro.

The observations made by Siebert et al. (1953), using inorganic ^{32}P incorporation into isolated pig kidney nuclei, are in agreement with those obtained, in Experiment No. 9, Table 12, on the uptake of phosphorus into isolated rat liver nuclei.

Stich and Hämmerling (1953) have studied the uptake of radioactive phosphorus into isolated nucleoli, obtained from the algae Acetabularia mediterranea. They found that there was incorporation of the ^{32}P into the RNA and protein of the nucleolus, which would appear to indicate that the nucleolus, as well as the nucleus as a whole, is capable of synthesising RNA

The evidence put forward by Lang, Lucius and Rossmüller (1953), Lang, Lang, Siebert and Lucius (1953) and Siebert et al. (1953) suggeststhat the nucleus is capable of synthesising its own nucleic acids, as well as precursors such as the purine bases. The incorporation of radioactive phosphorus observed in the present work, using isolated rat liver nuclei, is in keeping with this view. The findings of Stich and Hämmerling (1953) indicate that one part of the nucleus, namely the

nucleolus, is particularly concerned with the synthesis of RNA.

Brachet and Szafarz (1953), using nucleated and enucleated sections of Acetabularia mediterranea, have confirmed our findings with rat liver homogenates that the cytoplasmic constituents of the cell sap are able to incorporate labelled precursors into the cytoplasmic RNA and so act independently of the cell nucleus. These observations invalidate the earlier hypothesis of Jeener and Szafarz (1950) that the nucleus synthesises all the nucleic acid of the cell and that the RNA formed in the nucleus passes out into the cell sap where it is built up into the cytoplasmic particles.

The evidence against the Jeener and Szafarz (1950) theory would seem to be further substantiated by the results obtained when the transfer of ^{32}P from radioactive rat liver nuclei to non-radioactive rat liver whole cytoplasm was investigated (Table 13). If the Jeener and Szafarz (1950) theory were correct, it would be expected that transfer of ^{32}P from the radioactive nuclei into the RNA of the non-radioactive cytoplasm would be observed. However, there was no evidence of such a transfer occurring; on the contrary, when non-radioactive rat liver nuclei were incubated with radioactive rat liver cytoplasm, a very marked transfer of ^{32}P from the radioactive cytoplasm to the nucleic acids of the non-radioactive nuclei was obtained (Table 13). This suggests that the cytoplasm

contains some active precursor of nuclear nucleic acids, and that once the nucleic acids have been synthesised in the nucleus, they cannot diffuse out through the nuclear membrane.

The results, obtained when rat spleen (Table 14) and rabbit intestinal mucosa (Table 15) were used, confirm that there is a transfer of radioactive compounds from the cytoplasm into the nucleus where they are used in the synthesis of the nuclear nucleic acids. However, when the transfer of ^{32}P from the nucleus to the cytoplasm of these tissues was examined, the results at first appeared to indicate that the nucleus was acting as a source of cytoplasmic RNA. Chromatography of the bases obtained from the cytoplasmic nucleic acid of rat spleen tissue, however, showed the presence of the base thymine (Table 16). Thymine can be derived only from DNA, which in turn could be present in the cytoplasmic fraction only if autolysis of the nuclei occurred during incubation. Consequently, if nuclear DNA was detected in the cytoplasmic nucleic acid fraction of spleen homogenates, there must be some nuclear RNA also present, since it would be released from the nuclei by autolysis at the same time. The activity detected in the cytoplasmic RNA fraction of spleen, when non-radioactive cytoplasm was incubated in the presence of radioactive nuclei, can therefore be accounted for by the release of active nuclear nucleic acids.

When non-radioactive cytoplasm from rabbit intestinal

mucosa was incubated in the presence of radioactive nuclei, the results cannot be explained on this basis, since no thymine was detected when the bases derived from the cytoplasmic nucleic acids were separated by chromatography. The ratios of the bases obtained on chromatography, however, suggest that there has been some enzymic reaction taking place, other than straightforward transfer of phosphorus, since there is a rise in the relative amount of cytosine and uracil present although there is no change in the relative amounts of adenine and guanine found (Table 16). Other than this redistribution of the relative amounts of bases in the cytoplasmic RNA, there is no evidence for autolysis of the nuclei during incubation of the intracellular components of rabbit intestinal mucosa. It would seem, therefore, that in the case of intestinal mucosa there is some evidence of a transfer of phosphorus from the nucleus to the cytoplasmic RNA.

The effect of cyanide on the transfer of ^{32}P from the radioactive rabbit intestinal mucosa cytoplasm to non-radioactive rabbit intestinal mucosa nuclei (Table 17) indicates that the transfer mechanisms involved are independent of the cytochrome systems. Findlay et al. (1953) showed that the incorporation of ^{32}P into the RNA of cat brain slices is almost completely abolished by addition of cyanide to the medium. Goldwasser (1953) found that cyanide had the same effect on the incorporation of labelled adenine into rat liver, rat spleen and

pigeon liver slices and into pigeon liver homogenates. It seems, therefore, that the transfer of phosphorus from the cytoplasm into the nucleic acids of the nucleus is controlled by different mechanisms from those involved in the incorporation of precursors into the nucleic acids of the cell. It also suggests that cyanide inhibition affects preferentially the mechanism of incorporation of the labelled compounds into precursors of the nucleic acids.

When the transfer of radioactive phosphorus from different radioactive cytoplasmic fractions to non-radioactive nuclei of rabbit liver was investigated (Table 18), it was found that, of all the possible combinations, radioactive cell sap was the best source of transferable phosphorus for the synthesis of nuclear nucleic acids. The presence of non-radioactive cytoplasmic particles reduced the amount of phosphorus transferred from the cell sap to the nucleus. This effect was possibly due to the dilution of the ^{32}P present, or more probably to the transfer of the ^{32}P from the radioactive cell sap to the non-radioactive cytoplasmic particles themselves, since the cell sap is the most likely source of phosphorus for the synthesis of the components of the cytoplasmic particles.

Radioactive cytoplasmic particles would not appear to be a good source of transferable ^{32}P for the synthesis of nuclear

RNA, either in the presence or absence of non-radioactive cell sap. However, they appear to act as a source of transferable phosphorus for the synthesis of nuclear DNA. It may be concluded from these observations that the phosphorus required for the synthesis of nuclear RNA and DNA is derived from different sources.

The results, shown in Table 18, indicated that the soluble phosphorus compounds of the cell sap were the most likely sources of phosphorus for transfer from the cell sap into the nuclear nucleic acids. In order to test this hypothesis, the cell sap was dialysed against water and the dialysed cell sap and dialysate from radioactive rabbit liver tested for their abilities to transfer phosphorus to the nucleus. The results shown in Table 19 and Figure 13, show that the transfer of the phosphorus from the cell sap to the nucleus required the presence of both the dialysate and the dialysed material. It may be, therefore, that this transfer of phosphorus is not simply a diffusion effect, but is under the control of one or more enzymes.

The sources of transferable phosphorus was further investigated by studying the transfer of phosphorus from the different phosphorus fractions prepared from radioactive cell sap. From Table 20 and Figure 14, it was concluded that the acid soluble phosphorus compounds, particularly the acid soluble organic phosphorus compounds, could act as a

source of phosphorus for transfer from the cell sap into the nuclear DNA. These organic phosphorus-containing compounds include many mononucleotides. The results also suggest that these acid soluble compounds were not a good source of phosphorus for transfer from the cell sap into the nuclear RNA.

Siebert et al. (1953) showed that isolated pig kidney nuclei, in the absence of any other intracellular fraction, were capable of incorporating inorganic ^{32}P into the RNA and DNA of the nucleus. This was also demonstrated in the preliminary experiments of the present series (Experiment No. 8, Table 12). The addition of non-radioactive cell sap greatly enhanced the incorporation of the inorganic ^{32}P, but even so, the incorporation into the DNA was not as great as when radioactive acid soluble phosphorus compounds were used as a source of phosphorus. This suggests that the organic phosphorus compounds, probably the soluble nucleotides, act as precursors.

It is suggested by the results, shown in Table 21 and Figure 15, that phospholipids may act as a source of phosphorus for transfer into the nuclear RNA. This would be in keeping with the views of Brachet (1950) who postulated that lipid phosphorus was a precursor of RNA. However, the nuclei isolated after incubation, in the presence of phospholipids, contained large amounts of adsorbed lipid material, and as has previously been pointed out, it is possible that this adsorbed lipid or

its degradation products could give rise to the activities noted in the nuclear RNA fraction.

Lang, Siebert and Rossmüller (1953) have also investigated phospholipid as a precursor of nuclear nucleic acids, but they were unable to detect any incorporation of ^{32}P into the nuclear nucleic acids. However, in their experiments isolated pig kidney nuclei were incubated with radioactive phospholipid material, without any added cell sap. It may be the case that the cell sap contains enzymes which hydrolyse the phospholipid to some more readily acceptable phosphorus donor, or precursor for nuclear RNA synthesis.

When radioactive RNA was incubated with non-radioactive nuclei, the results obtained (Table 21 and Figure 15) show that there is apparent uptake of ^{32}P into the nuclear RNA and DNA fractions, particularly in the absence of added cell sap. Although these results seem to indicate that the cell sap RNA was acting as a donor of phosphorus for incorporation of nuclear nucleic acids, it is more probable that they are due entirely to the adsorption of the radioactive RNA on to the nucleus, and subsequent estimation of the adsorbed material as being derived from the nucleus. It is possible, however, that the uptake noted in the DNA fraction of the nucleus in absence of added cell sap, is true incorporation and not due to adsorption effects. Again, this would be in keeping with the

hypothesis put forward by Brachet (1950) who postulated that RNA was converted to DNA in the nucleus.

Fractionation of radioactive cell sap into protein fractions, by means of ammonium sulphate, shows that the three protein fractions obtained differ from each other and from whole cell sap in their abilities to transfer phosphorus to the nuclear RNA and DNA (Table 22 and Figure 16). These observations definitely indicate that the transfer of phosphorus from the cell sap to the nuclear nucleic acids is an enzymic process, and that different mechanisms are involved in the transfer to RNA and DNA of the nucleus. Fraction I of the cell sap would appear to be involved in the transfer of phosphorus to the nuclear DNA, while Fractions II and III apparently control the transfer of phosphorus to the nuclear RNA.

Khouvine and Mortreuil (1954), in their work on the incorporation of ^{32}P into the nucleic acids of liver slices from normal and Guerin tumour-bearing rats, also came to the conclusion that the mechanism of supplying phosphorus for the synthesis of the two types of nuclear nucleic acids was an enzymic process and that two different pathways were involved. It is a reasonable supposition that two such mechanisms do exist, since in vivo studies have shown that nuclear RNA and DNA incorporate labelled precursors at entirely different rates. Throughout the present series of experiments, it has also been

apparent that nuclear RNA and DNA require different sources of phosphorus for incorporation into their molecular structure.

In a recent paper, Brawerman and Chargaff (1954) reported that they were able to obtain from rat liver extracts enzymes which were capable of transferring phosphorus from sodium mono-phenylphosphate to certain nucleosides. These nucleoside phosphotransferases, they claimed, were important in the synthesis of the nucleic acids of the cell. The effects which have been observed in the present study of the transfer of phosphorus from the cell sap of rabbit liver to the nuclei of the same tissue, may well be due to the action of these nucleoside phosphotransferases. It would be possible to explain the incorporation of ^{32}P into the nuclear nucleic acids obtained when isolated nuclei were incubated in the presence of the different protein fractions of cell sap. The results obtained when nuclei were incubated with different labelled compounds in the presence of cell sap could easily be explained by the action of such phosphotransferases, however, it would not account for some of the results observed when no cell sap was added. The dialysis experiments carried out in the present work do indicate that the transfer of phosphorus into the nucleus is an enzymic process, and this is further supported by the evidence obtained when dialysed cell sap was separated into protein fractions by ammonium sulphate fractionation.

Summary.

1. The incorporation of radioactive phosphorus (^{32}P) into the phospholipids and ribonucleic acid (RNA) of the isolated nuclei, mitochondria, microsomes and cell sap and into the deoxyribonucleic acid (DNA) of the isolated nuclei of rabbit liver has been investigated at various time intervals after administration of the isotope. The nuclear RNA showed a greater uptake of ^{32}P than was found in the RNA of the cytoplasmic fractions. The uptake of ^{32}P into the RNA was approximately the same in the different cytoplasmic fractions. Maximum incorporation into the RNA of all fractions occurred about 24 hours after the administration of the isotope. The incorporation of ^{32}P into the DNA was very low and the maximum was reached about 18 hours after administration.

2. The incorporation of ^{32}P into the phospholipids and RNA of the liver cytoplasmic fractions of fowls, in different physiological states, was studied. No correlation was found between the physiological state of the birds and the incorporation of the isotope.

3. An ionophoretic separation of the nucleotides of the RNA fractions of rabbit and fowl livers was carried out. It was found that the specific activities of the nucleotides of any one RNA fraction were all of the same order.

4. The in vitro incorporation of inorganic ^{32}P into the RNA of sucrose homogenates of rat liver was investigated. Such rat liver homogenates were found to be capable of incorporating ^{32}P into RNA in the presence of ATP, DPN, cytochrome c, Na_2HPO_4 and $MgCl_2$.

5. It was found that isolated nuclei and whole cytoplasm of rat liver were each able to incorporate ^{32}P into their RNA fractions on incubation. Incorporation of inorganic ^{32}P into the RNA of isolated mitochondria was also demonstrated, but no uptake of ^{32}P into the RNA of isolated microsomes or cell sap was observed.

6. The transfer of ^{32}P from radioactive cellular fractions into non-radioactive cellular fractions of rat liver, rat spleen, rabbit liver and rabbit intestinal mucosa was investigated.

7. Using rat liver, rat spleen and rabbit intestinal mucosa, it was found that there was a transfer of ^{32}P from radioactive whole cytoplasm into the nucleic acids of non-radioactive nuclei. No evidence was obtained for the transfer of phosphorus from radioactive rat liver nuclei into the RNA of non-radioactive rat liver cytoplasm. However, in rat spleen and rabbit intestinal mucosa, it was observed that some transfer of ^{32}P from the radioactive nuclei to non-radioactive cytoplasm occurred.

8. The transfer of ^{32}P from radioactive cytoplasm to non-radioactive nuclei of rabbit intestinal mucosa was not inhibited by cyanide.

9. Investigation of the transfer of phosphorus from radioactive cytoplasmic components to non-radioactive nuclei of rabbit liver showed that cell sap was the best donor of phosphorus for incorporation into nuclear nucleic acids.

10. Dialysis of radioactive cell sap, prior to incubation with non-radioactive nuclei of rabbit liver, partially inhibited the transfer of ^{32}P into the nuclear nucleic acids.

11. Various phosphorus compounds of the radioactive cell sap were investigated as possible donors of phosphorus for inclusion into the RNA and DNA of the nucleus.

12. Three protein fractions were obtained from radioactive cell sap, by ammonium sulphate fractionation. It was demonstrated that they differed in their abilities to promote the transfer of ^{32}P to the RNA and DNA of the nucleus.

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