STUDIES IN PLASMA PROTEIN METABOLISM

by

Adam Fleck, B.Sc., M.B., Ch.B.

In previous studies of plasma protein metabolism it had been demonstrated that catabolism is altered by diet and by the administration of hormones. The aim in this thesis was to decide whether the plasma proteins behaved in the same way as the proteins of the liver in response to the stimuli of injury and changes in the protein content of the diet, and also to elucidate the intermediate steps by which the response is mediated.

The effects of injury (fracture of the femur) and the protein content of the diet on the rate of breakdown of plasma albumin in rats was studied using ^{131}I-labelled rat plasma albumin. It was confirmed that the rate of breakdown of albumin was significantly greater when animals were maintained on a high protein diet than when they were deprived of protein. Nevertheless, the catabolic rate of plasma albumin was unaffected by injury in animals fed either a high or a low protein diet.

The total plasma protein concentration and the protein and ribonucleic acid content of the livers of animals fed a diet of high protein content was greater than

in those animals deprived of protein: none of these levels was affected by injury.

It was concluded that although the metabolism of the liver and plasma proteins was affected by diet, they were not involved in the metabolic response to injury, and that the source of the increased nitrogen loss in the urine must have been the carcass.

The effect of the level of protein feeding on protein synthesis in the liver was next studied. Since it had previously been shown that the greater part of the protein produced in the liver was synthesised by the microsomes, this fraction was studied in some detail. It was shown that when animals were deprived of dietary protein, the protein, ribonucleic acid and phospholipid phosphorus content of the microsomes was considerably reduced. The incorporation of ^{14}C-leucine in vitro by the rat microsome fraction was greater two hours after feeding casein than following a fifteen-hour fast. In a study of the ribosome aggregates (polysomes) using a sucrose density gradient technique it was shown that there was a greater amount of free ribosomes in the preparation obtained from fasting animals than in the fraction obtained from animals which

had been fed casein two hours previously. It was concluded that these results were compatible with the hypothesis that feeding protein led to a release of "messenger" ribonucleic acid from the nucleus which in turn led to an increase in the formation of aggregates of ribosomes and subsequently an increased synthesis of protein.

Also in the course of this work, the fractionation of proteins by ion exchange chromatography was investigated, methods of estimating organic nitrogen and protein were reviewed, and an ultraviolet spectrophotometric procedure for the estimation of ribonucleic acid was devised.

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CONTENTS

GENERAL INTRODUCTION

The History of the Plasma Proteins

The ready availability of blood, plasma and serum, together with the medical interest in blood as a basis for the interpretation of changes occurring in pathological states has provided a strong stimulus for the investigation of the plasma proteins.

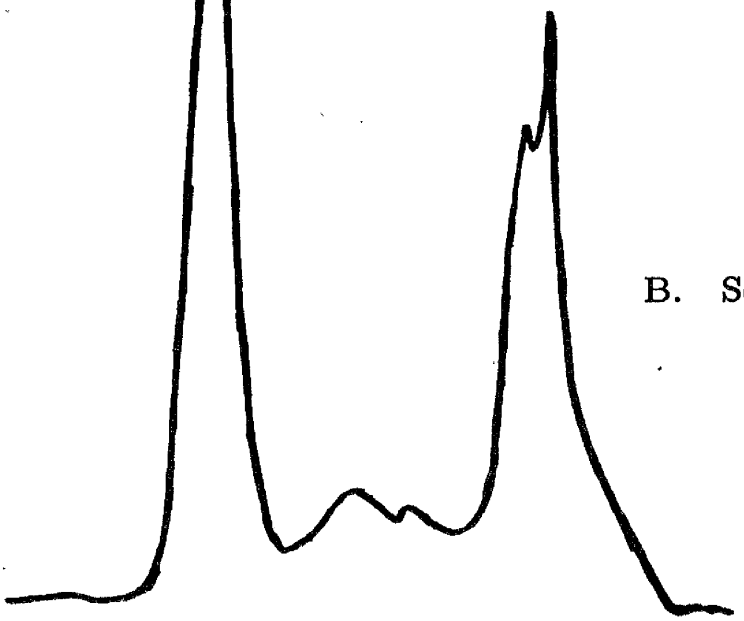
Serum has probably been known since prehistoric time, certainly it was known to Hippocrates (Tullis, 1953). It may have been first observed as the straw-coloured fluid above a retracted blood clot.

The plasma proteins cannot be said to have been discovered, as knowledge of them has grown slowly since the seventeenth century. Barbato of Padua in 1667 published some observations in which he remarked on the resemblance of serum to the white of an egg (Partington, 1961). Boerhaave (1668-1738) carried out investigations on albumin (Cohn, 1953). Haller in his textbook "Elementa Physiologiae" (1760)mentions the property of heat coagulability of albumin, and in his "Animal Chemistry" Liebig (1842) mentions as the two chief ingredients of blood plasma "fibrine" and "albumen". The latter is the heat coagulable material remaining in plasma after the removal of "fibrine" and so does not correspond exactly to albumin as the term is used at present.

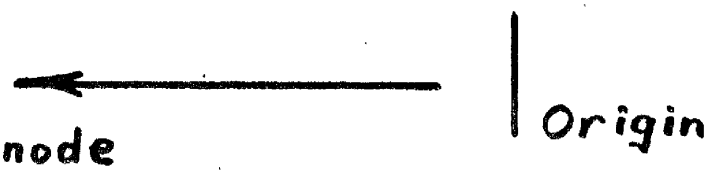
It is of interest to note that the word "Πρωτεῖος" was used by Mulder to denote the acid-insoluble material (containing carbon, hydrogen, nitrogen and oxygen) obtained from what we now refer to as proteins by treatment with hot potassium hydroxide

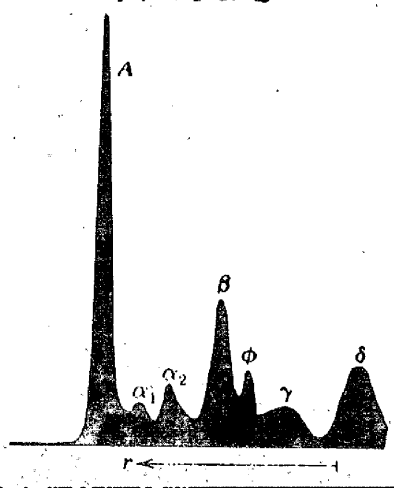
Fig. 1. ELECTROPHORESIS OF PLASMA PROTEINS

A. Paper electrophoresis of rat plasma



B. Scan of A





C. Separation of plasma proteins on Tiselius apparatus

solution (Liebig, 1842). The later rejection by Liebig of the hypothesis formulated by Mulder in connection with this use of "Πρωτεῖος" may account for the use in German of "eiweiss" in referring to protein (Munro, 1963). The term "albumin" is probably derived from Latin meaning "white of egg".

By the later nineteenth century the water-soluble protein of plasma was referred to as albumin and the water-insoluble protein as globulin (Putnam, 1960). Also in the later nineteenth century salt fractionation of serum was introduced (see Putnam, 1960) and subsequently developed by Howe (1925), so that several proteins with different properties were recognised.

Tiselius in 1937 effected a considerable advance in protein biochemistry by the introduction of the technique of electrophoresis of proteins, and with modifications of this original method and the use of paper electrophoresis, this has led to the identification of albumin α_1, α_2, β and γ globulins and fibrinogen in plasma (Cooper, 1960). The technique of electrophoresis is now so well standardised that the plasma proteins are commonly classified by their relative electrophoretic mobility in a barbital buffer pH 8.6 (fig.1).

As the properties of the plasma proteins were investigated the methods for the preparation of proteins developed in parallel so that standard laboratory methods for the preparation of individual plasma proteins are, for example, salt fractionation and electrophoresis. Cohn effected a revolution in the bulk preparation of the plasma proteins by the introduction of the cold ethanol technique (Pennel, 1960).

Table 1

Plasma Proteins

Pre Albumin

Albumin

$\alpha 1$, $\alpha 2$, β, γ globuline

β lipoprotein

Mucoproteins

Fibrinogen

Prothrombin

Antihaemophilic globulin

Christmas factor

Insulin

Parathormone

A.C.T.H.

T.S.H.

F.S.H.

I.C.S.H.

Growth Hormone

Siderophilin

Ceruloplasmin

This was, of course, a rational application of the knowledge of the behaviour of proteins in solutions of different ionic strengths and dielectric constants.

As a result of these developments, the number of proteins believed to be present in plasma has increased from two in 1842 (Liebig, 1842) to more than twenty (table 1). More than half of these can be isolated in fairly pure form from serum or plasma and the remainder, such as plasma enzymes or hormones, can be shown to be present by their biological activity. Many proteins are classified by their biological activity, but as preparations of these active proteins increase in purity other criteria than "specific activity" (equivalent to biological activity measured in appropriate units divided by protein, or protein nitrogen, concentration) become necessary and of great interest in the correlation of structure and function. In recent years in order to characterise a protein, information is required about most of the following:-

1. Biological activity, for example, enzyme or hormone.
2. General chemical characteristics, for example, the presence or absence of a prosthetic group such as haem, flavin or a carbohydrate.
3. Solubility, in water, saline etc.
4. Isoelectric point and relative electrophoretic mobility.
5. Sedimentation coefficient, as determined in the ultracentrifuge.
6. Molecular weight, as is estimated for example, by osmotic pressure, light scattering, sedimentation value and diffusion

Table 2

Albumin Data

Species	N terminal amino acid	C terminal amino acid	S_{20w}	M.W.	Isoelectric point
Man	Asp	Leu	4.6	69,000	
Dog	-	Leu		65,000	
Rabbit	-	Leu			
Horse	Asp	Leu.ala.	4.5	70,000	
Donkey	Asp	Leu.ala.			
Mule	Asp	Leu.ala.			
Pig	Asp	Ala.		72,000	
Cow	Asp	Ala.	4.4	67,000	4.7
Rat	Glut.	Ala.	4.4-4.7	65,000	4.6
Duck	Asp	Ala.			
Chicken	Asp	Ala.			
Turkey	Asp	Val.			

Data obtained from:

Phelps & Putnam, 1960

Charlwood, 1961 and

Petera, 1962

coefficient methods. Also obtainable by these methods are the degree of hydration of the molecule and the molecular diameters.

7. Chemical analysis. This investigation usually begins with the determination of nitrogen, then amino acid content - both qualitative and quantitative - followed by the identification of the terminal amino acids, disulphide bridges, and finally, the amino acid sequence.

8. Molecular configuration and structure. The techniques used are electron microscopy and X-ray diffraction. The potential of the latter in determining molecular structure and in "placing" amino acids in the molecule has recently been demonstrated (Rich and Green 1961).

Obviously it would be impossible to investigate the metabolism of all, or even a few, of the plasma proteins in the course of a few years. Principally for this reason, it was decided to explore the metabolism of plasma albumin. In addition, albumin accounts for a relatively large proportion of the circulating plasma proteins.

The Chemistry of Plasma Albumin

Since albumin is of apparent protean occurrence, although it has been reported to be absent from the blood of certain teleost fish (Sulya, Box and Gunter, 1961), it is probably best to define the serum or plasma albumin of a species as a specific protein of fairly low molecular weight and low iso-electric point (see table 2), which is present in considerable quantities in plasma. A similar protein

Table 3

The Amino Acid Composition of Human and Bovine Plasma Albumin

	Human	Bovine
Glycine	1.6	1.8
Alanine	-	6.3
Valine	7.7	5.9
Leucine	11.9	12.3
Iso-Leucine	1.7	2.6
Proline	5.1	4.8
Phenylalanine	7.8	6.6
Tyrosine	4.7	5.1
Tryptophan	0.2	0.6
Serine	3.7	4.2
Threonine	5.0	5.8
Cystoine / Cystine	6.3	5.9
Methionine	1.3	0.8
Arginine	6.2	5.9
Histidine	3.5	4.0
Lysine	12.3	12.8
Aspartic Acid	10.4	10.9
Glutamic Acid	17.4	16.5
Amido NH_3	1.1	1.0

- Figures are gm. of component per 100 gm. protein
Data obtained from Phelps & Putnam, 1960

occurs in egg-white and milk and albumin has been reported in brain (Robertson, 1957), various other mammalian organs and in tissue culture cells (Pottrell, 1962). The identity of these proteins with plasma albumin has not, however, been established using immunological techniques and the contamination of the specimens with blood has not been excluded in the majority of reports.

Although it is well known that crude preparations of albumin may give a positive Molisch test for carbohydrate, it is possible to obtain preparations of albumin free from carbohydrate (Putnam, 1960).

That the plasma albumins of the various mammalian species are closely related is seen from the similarity of: 1. amino acid composition; 2. amino acid end groups; 3. molecular weight; and 4. iso-electric point (tables 2 and 3). The amount of the protein present in the plasma of the various animal species is also remarkably constant; it accounts for about half the total circulating plasma protein (T.A. Douglas, personal communication). Albumin is usually "identified" following electrophoresis as the major fraction with the lowest isoelectric point.

Recent evidence suggests that albumin consists of a single long polypeptide chain coiled upon itself and linked by several S-S "bridges" (Foster, 1960). This structure may account for the observed isomerism of albumin at extremes of pH and for the observed solubility in acid-ethanol (Charlwood, 1961).

Methods of preparation of albumin are discussed in detail later; usually salt or ethanol fractionation is used, the alternatives being electrophoresis or chromatography.

In passing, it is interesting to note that a series of ten lectures entitled "Chemistry of Albumens" by S.E. Schryver was published in 1906 and that it contains little in common with the above brief survey.

Protein Metabolism and Nutrition

As early as 1614 Sanctorius had been attempting dietary "balance" experiments (Lusk, 1928). This field, however, remained sterile until the discovery of nitrogen by Rutherford in 1772 (see Munro, 1963), and the subsequent demonstration of the importance of nitrogen in metabolism by Magendie (1829) and Liebig (1842).

The essential role of dietary nitrogen was demonstrated experimentally by Magendie and from the evidence obtained from his nutritional experiments and observations on disease, he defined nutrition: "There exists, then, in the organs, an insensible motion of the particles.....It is this interior motion, unknown in its nature, that is called nutrition....." (Magendie, 1829). Liebig (1842) later drew the interesting conclusion: "all the nitrogenised constituents of the body, how different soever they may be in composition, are derived from protein".

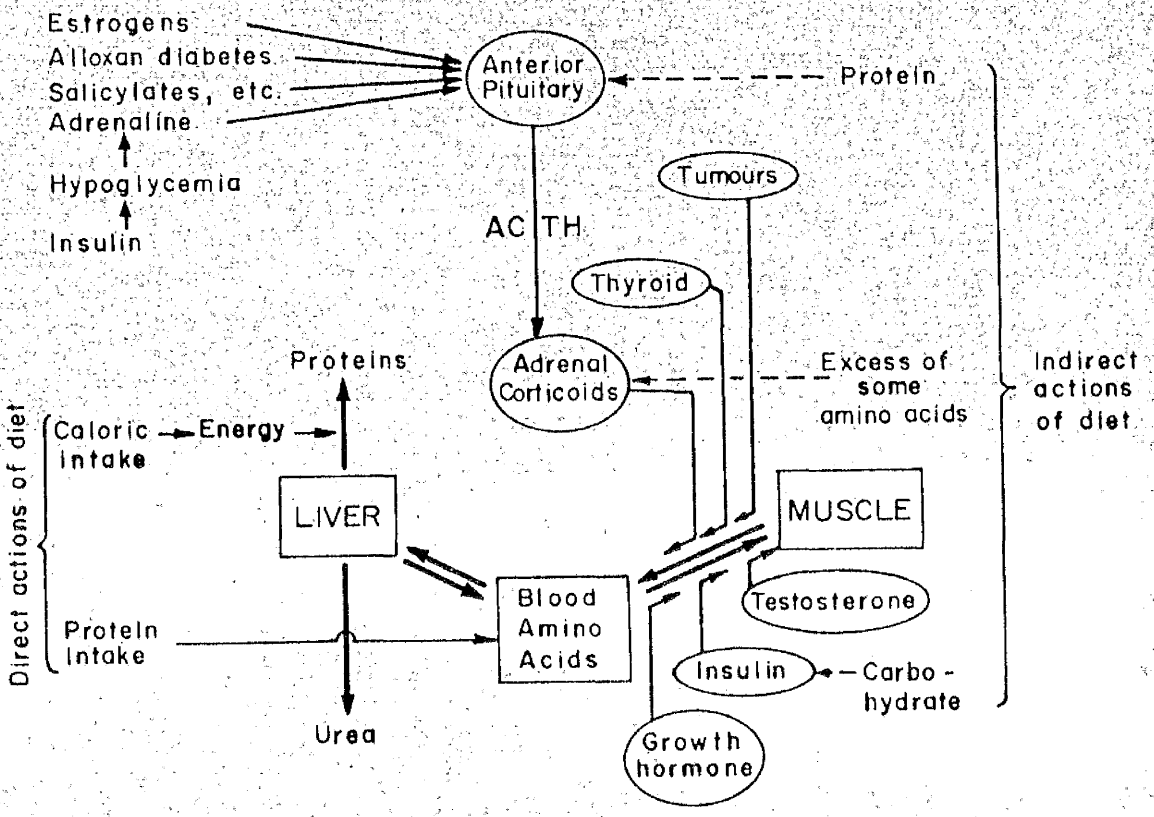
The genesis of scientific nutrition was thus in the first part of the nineteenth century. It has since developed from the establishing of nitrogen balance experiments on a sound quantitative basis by Voit (that is, the experimental demonstration of the fact that the total nitrogen of the urine and faeces is an accurate measure of protein intake (Lusk, 1928)), to the present, when there

is somewhat more emphasis on studies in energy exchange and metabolic pathways.

The fundamental problems in nutrition, the investigation of which was begun by Magendie, are firstly the determination of normal food requirements and secondly, the investigation of the conditions in which food requirements are altered (Magendie, 1829). These problems, in human nutrition, frequently assume great practical importance during wartime or famine, and with regard to protein nutrition have been summarised by Lusk (1928) and Munro (1951). It is now common knowledge that nitrogen balance is influenced by protein intake, fat and carbohydrate, or calorie intake and that carbohydrate has a specific "nitrogen sparing" effect (Munro, 1951). Other factors which affect protein requirements are the energy expenditure of the individual and the temperature of the environment. Lusk (1928) gives evidence that the basal metabolic rate is constant except in undernutrition, when it may fall by up to thirty per cent.

Protein metabolism may be influenced by hormones, the most important of which in this respect are: growth hormone, sex hormones (especially testosterone), cortisone and hydrocortisone, thyroxine and insulin. The administration of growth hormone leads to positive nitrogen balance and an increase in body protein; testosterone has a similar effect (White, 1948). In the normal adult animal, thyroid hormone accelerates protein catabolism (White, 1948). The action of insulin on protein metabolism depends on whether the animal is fasting or fed. The primary action of insulin appears to be to accelerate

Fig. 2. THE ACTION OF HORMONES ON PROTEIN METABOLISM



From Munro (1964)

the accumulation of plasma amino acids in the muscle, and the effect is modified by the availability of a source of energy (Munro, 1956). Cortisone administration produces a protein catabolic effect and negative nitrogen balance, but the effect is complicated by the observation that the liver protein content increases while the carcase or muscle protein decreases. The increase in liver protein is in proportion to the available free amino acids in the blood and thus in proportion to dietary protein. (Fritz 1956; Goodlad and Munro, 1959). Some of the effects of hormones on protein and amino acid metabolism are summarised in fig.2. Other factors which affect protein metabolism and form an important part of nutritional studies are digestion and absorption of proteins and amino acids and the amino acid content, and therefore, the biological value of proteins. The response of the animal to injury also involves changes in protein metabolism: this topic will be dealt with in more detail below.

Plasma Proteins and Nutrition

"The true starting point for all the tissues is, consequently, albumen" (Liebig, 1842).

As is obvious from the above quotation, the belief that the plasma proteins played an important role in the protein metabolism of the organism is of long standing. Whipple and co-workers (Madden and Whipple, 1940) using the technique of plasmapheresis demonstrated that administration of plasma proteins intravenously to a dog on a nitrogen-free diet could maintain the dog in nitrogen balance. They also

demonstrated the presence of body stores of plasma proteins and concluded, from experiments on dogs with Eck fistulae and from a review of clinical evidence, that the liver was the site of plasma protein production. In the course of these experiments, they found that the plasma amino acid level remained constant and this led them to suppose that the plasma proteins could be used directly, that is without prior breakdown to amino acids, to produce tissue proteins.

This type of approach, that of classical nutrition, has been extended by Chow and co-workers (Barrows and Chow, 1959). Following experiments in which plasmapheresis was combined with variation of protein intake, it was concluded that after plasmapheresis the reserve protein stores of the experimental animals were not totally depleted although the plasma proteins were considerably reduced, and conversely, animals receiving a protein-free diet lost the "reserve protein stores" before the plasma proteins became depleted.

With the advent of isotopes and turnover studies (see later), some of these conclusions, particularly those of Whipple et al. regarding the utilisation of the plasma proteins by the tissues, must be re-examined. It is reasonable to assume that the rates of synthesis and breakdown of the many different plasma proteins are different, and this has been established experimentally by many workers (Anker, 1960). This may explain in part the changes of albumin:globulin ratio which occur in disease and states of altered nutrition. Because the turnover of albumin is more rapid than globulins, any conditions which produces decreased synthesis of plasma proteins will

produce initially a low albumin:globulin ratio. However, were the condition to result in a new "steady state" lasting for up to two to three times the half life of the globulins, then the albumin:globulin ratio would increase slightly to a new equilibrium level. Thus, changes in the albumin:globulin ratio cannot be used as a "therapeutic index". It has been shown that the uptake by the tissues of the free amino acids of the plasma is extremely rapid; 90% of the free amino acids of plasma is taken up by the tissues in 5 minutes (McFarlane, 1957). It is scarcely surprising that Whipple et al. did not detect alterations in the concentrations of the free amino acids of the plasma, and obviously, their conclusion that the plasma proteins are utilised directly by the tissues for the production of tissue proteins is unjustified. Further experimental evidence in this connection is quoted by McFarlane (1957) and it is at present agreed that proteins are synthesised from free amino acids, not from peptides (Hoagland, 1960).

The introduction of isotopes also allowed for more "physiological" experiments instead of the rather extreme nutritional conditions utilised by Whipple et al., for example, and in addition the rates of breakdown of molecules could be estimated by tracer techniques. Since albumin is so readily available (especially human serum albumin) it has been used in preference to other serum proteins in metabolic studies such as the effects of low and high protein diets and thyroid and adrenal hormones on serum protein metabolism. It has been shown using rats (Jeffay and Winzler, 1958) and humans

(Iber, Nassau, Plough, Berger, Meroney and Fremont-Smith, 1958) that the rate of turnover of albumin increases following the administration of a high protein diet and conversely decreases during the administration of a low protein diet. The reduction in concentration of various tissue enzymes which occurs in hyponutrition may be related to this phenomenon and it is of interest to note that the response of the enzymes of various tissues is variable; intestinal mucosa and liver showing the largest and most rapid response, and heart muscle and brain minimal changes (Fisher, 1954; Munro, 1964). Iber et al. also studied the effects of tri-iodothyronine and demonstrated that it caused an increase in the turnover of albumin. Hydrocortisone is also reported as causing an increase in the turnover of albumin (Grossman, Yalow and Weston, 1960). In all these cases, the concentration of albumin in serum did not change markedly, certainly not in proportion to the change in metabolic rate during the course of the experiment.

All the evidence adduced so far is in favour of the hypothesis that the plasma proteins respond to hormones and changes in nutrition in a similar fashion to the other proteins of the body.

In the course of this work, two main groups of experiments are described. The first group was carried out in order to decide whether the plasma proteins behave metabolically as liver proteins, as might be expected since they are in the main synthesised there, or whether they behave as transporters of nitrogen to the tissues. The second group of experiments was concerned with the effects of diet on the synthesis of protein by the liver.

PART ONE

PLASMA PROTEIN METABOLISM AND ITS RELATION TO DIET AND INJURY

Protein Metabolism and Injury

The response of the animal organism to injury includes some interesting effects on protein metabolism. An early observation was that of Cuthbertson (1930) who noted an increase in urinary nitrogen excretion following injury. That this increase in nitrogen output was not simply due to the traumatic local destruction of tissue was shown by Munro and Chalmers (1945) and Munro and Cumming (1948). Somewhat earlier, however, Cuthbertson (1932, 1964) had investigated the changes in body temperature, pulse rate, basal oxygen consumption, and urinary nitrogen, sulphur, and phosphorus output following trauma, with the results shown in figs.3 and 4. It is apparent that injury produces a response in the animal body which results in increased catabolism of protein.

The endocrine changes which occur in response to trauma have been summarised by Born (1962):-

1. adrenaline production (which leads to symptoms of shock and transient hyperglycaemia)
2. stimulation of the hypothalamus and in turn the anterior pituitary
3. increased production of ACTH
4. stimulation of the adrenal cortex

In adrenalectomised animals the general response to trauma described above is absent, but if adrenalectomised animals are given maintenance doses of cortisone, the usual increase in urinary nitrogen output following injury occurs (Campbell, Sharp, Boyne and Cuthbertson, 1954). It is believed that in these circumstances, cortisone is

Fig. 3. METABOLIC CHANGES IN INJURY - I

Changes in the urine

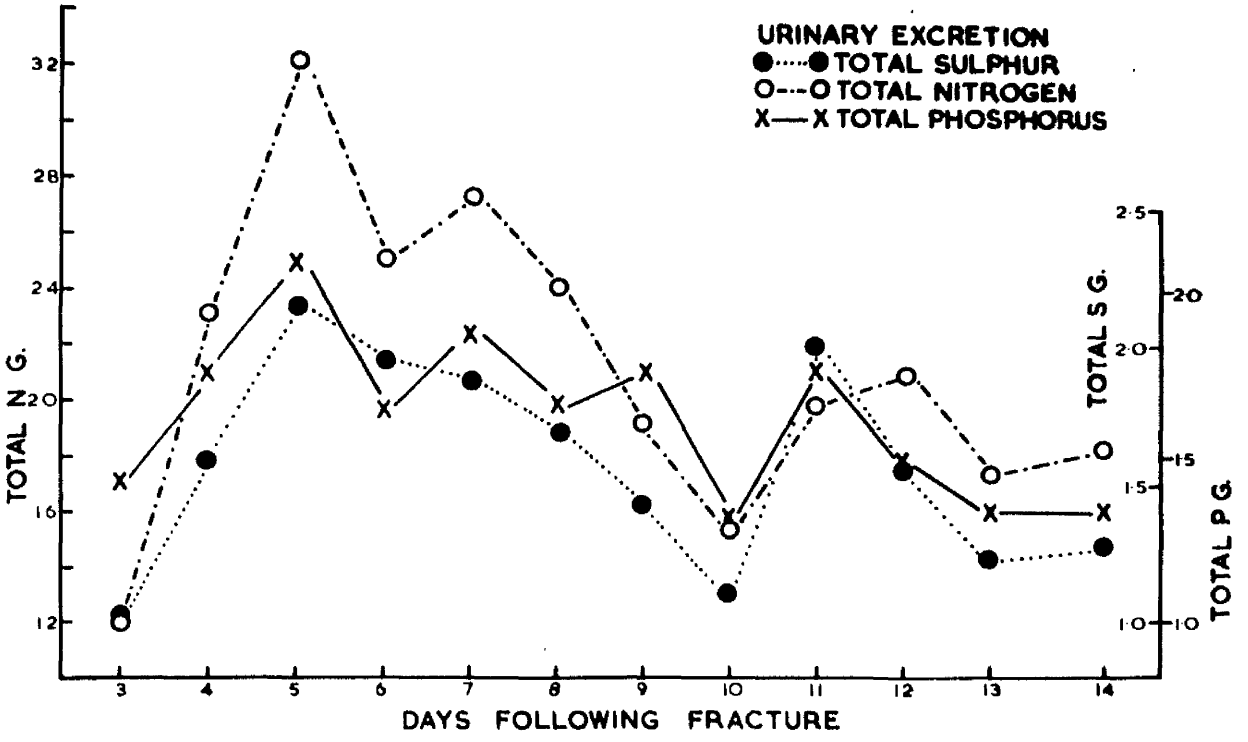
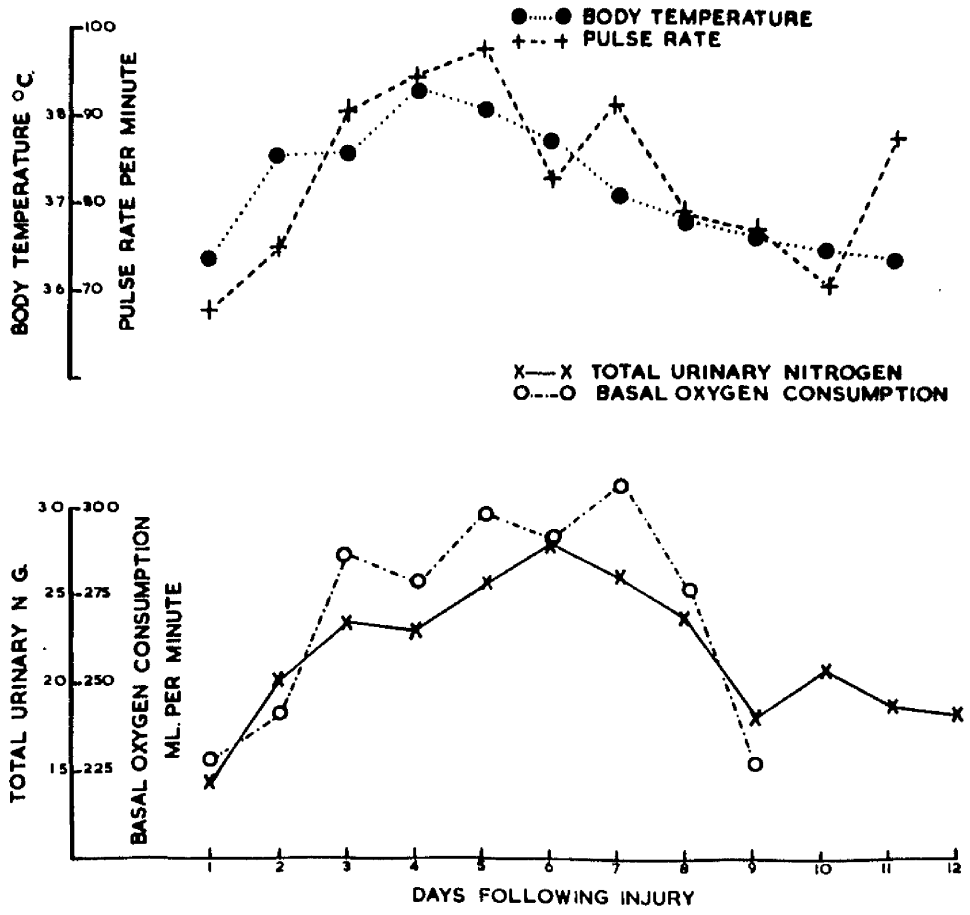


Fig 2. Daily urinary exoretion of total N, S and P of youth following fracture of both bones of one leg. Daily intake of N = 11.7 g. Drawn from metabolic data in Table VII, Cuthbertson (1930) in Biochem. J. 24, 1244.

Fig. 4. METABOLIC CHANGES IN INJURY - 2



Metabolic data on man aged 34 yr with fracture of tibia due to direct violence. No anaesthetic required. Daily nitrogen intake 15.36 g. Redrawn from Fig 1, Cuthbertson (1932) in Quart. J. Med. New Series 1, 233.

exerting a "permissive" effect (Ingle, 1951; Selye, 1954).

It has been suggested that the source of the nitrogen lost following injury is the "labile body protein" (Munro and Chalmers, 1945), and that this would explain the absence of the "injury response" in protein-depleted animals (Munro and Chalmers, 1945). However, in some human cases, more nitrogen has been lost after injury (Cuthbertson, 1964) than would correspond to 5% of the total body protein - the figure generally accepted as the very labile body protein (Munro, 1964).

Estimations of the heat and nitrogen lost following trauma indicate that the increased heat loss could be accounted for by the oxidation of the residual carbon chain of amino acids, the deamination of which had given rise to the increased nitrogen excretion (Cairnie, Campbell, Pullar and Cuthbertson, 1957). This is consonant with the data of fig.4 and indicates that injury leads to a specific effect on animal protein metabolism.

The plasma proteins may also be affected by injury: Cuthbertson and Thompsett (1935) reported a slight fall in serum albumin concentration and a marked rise in globulins following fracture or operation. Similar changes have been reported in patients suffering burning injuries (Bull, 1958). Further discussion of these observations requires data on the turnover of the plasma proteins.

The Turnover of Plasma Proteins

The term turnover refers to the continuous flux or breakdown and resynthesis of the constituents of the organism. Experimental

evidence for this concept is not old; the first being obtained by Borsook and Keighly (1935). From their investigations on nitrogen and sulphur metabolism in vivo, they concluded that there was a continuous metabolism to which food and tissue protein contributed. This was in contrast to the theory of Folin (1905) which described endogenous or tissue metabolism as constant, and exogenous or intermediate metabolism as variable with, for example, diet. This theory led directly to the concept of endogenous metabolism being solely due to 'wear and tear'.

Following the discovery of isotopes, more direct experimental evidence for continuous metabolic turnover was obtained by Schoenheimer, Rittenberg and others (Schoenheimer, 1942) and is adequately summarised in the title of Schoenheimer's posthumous book, "The Dynamic State of Body Constituents". Hopkins of course had referred to this in his aphorism "life is the expression of a particular dynamic equilibrium which obtains in a polyphasic system" (Hopkins, 1913).

Since the initial work of Schoenheimer, the use of isotopes in biochemistry and medicine has become increasingly common and the evidence for continuous turnover has accumulated in proportion.

There are numerous applications of isotopes in biochemistry but in metabolic studies there are three principal applications. These are:

1. The determination of metabolic pathways - best illustrated perhaps by the work of Wilson and Calvin (1955) on photosynthesis.

2. The demonstration of turnover.

3. The estimation of rates of turnover.

These are only general headings and there may be overlapping, for example, the knowledge of metabolic pathways is useful and may be essential for the determination of rates of turnover.

The theoretical aspects of turnover have provoked so large a literature in recent years that a comprehensive survey is outside the scope of this work. The best known article on this topic is that by Zilversmit, Entenman and Fishler (1943). This is unfortunate in that most of their treatment of the subject has been shown to be inadequate or has been superseded (Reiner, 1953). An indication of the possible complexity of experiments designed for the estimation of turnover rates is given by Reiner (1953); "Calculation of turnover rate for any component of a metabolic network requires a complete knowledge of that network as well as experimental study of the concentrations and specific activities of all its components. A reasonably complete time curve for each component must be obtained". However, the same author does suggest that techniques of successive approximations of a model system to experimental data may be used to obtain information about previously unknown metabolic pathways. This approach has, of necessity, been the one adopted in turnover studies on plasma protein metabolism.

The most general definition of turnover time is that of Robertson (1957):-

TURNOVER TIME is the time interval required for the amount of a substance transferred into or out of a compartment in the steady state to be numerically equal to the amount present in the compartment.

TURNOVER RATE is defined (Robertson, 1957) as:-

i . Reciprocal of turnover time or fractional turnover per unit time. In this definition turnover rate has the dimension of time^{-1}. It is suggested that this be referred to as "rate constant" of symbol "k"

or

ii. the amount of substance that is turned over in unit time; dimensions $\frac{m}{t}$ (where m is mass, t, time), referred to as "rate", symbol "ρ".

These definitions include the particular cases of the rate of turnover of substance X:-

a. The number of molecules of X which are newly formed per unit time, (equivalent to rate of appearance of X).

b. The number of molecules of X already present which are transferred per unit time, (equivalent to rate of disappearance of X).

In the use of radioactive isotopes in turnover studies, it is of course assumed that the labelled molecules behave in the same way as the unlabelled.

The STEADY STATE can be defined (Robertson, 1957) as the situation in which the rates of removal of substances are equalled by their rates of replacement, so that the concentrations and

amounts of the substances being studied are constant during the period of observation. In mathematical studies constant rates of transfer are usually implied, although recently it has been suggested that albumin catabolic rates could be calculated in the non-steady state (Franks, 1963).

In the steady state and with constant transfer rates for the unlabelled substance, the behaviour of a tracer is independent of reaction order and equations describing transfer all have the form of equations of first-order reactions (Robertson, 1957). The simplest demonstration of this is as follows:-

For a system without recycling of tracer consider a one compartment open system ($\longrightarrow$A$\longrightarrow$) into which a label is instantaneously introduced. If x is the specific activity of A at any time, t and x_0 the specific activity at zero time, then since the rate of disappearance of label at any instant is proportional to the concentration of label present at that instant,

$$\frac{dx}{dt} = -k.x \quad \text{and integration gives} \quad x = x_0 e^{-kt}$$

or, in logarithmic notation,

$$\log_e x = -kt + C \quad \text{(where C is a constant).}$$

Thus in the experimental situation where these conditions apply, the graph of the logarithm of the specific activity of A against time is a straight line of gradient equal to the turnover rate (rate constant) of A. The description of turnover rate in these conditions is analagous to the mathematical treatment of radioactive decay and has led to the expression of turnover rate in some cases

(notably the plasma proteins) as "half-life". The "half-life" of A is the time taken for the specific activity of A to fall to half its original value. It is obvious that then,

$$\log_e \frac{x}{x_0} = \log_e \frac{1}{2} \quad \text{and}$$

$$\text{"half-life of A"} = \frac{1}{k} \log_e 2 = \frac{0.693}{k}, \quad \text{and}$$

$$\text{'fraction turned over per unit time'} = k = \frac{0.693}{\text{half-life}}.$$

The experimental study of the turnover of plasma proteins

It is obvious that the isotopes of hydrogen (deuterium, tritium), carbon(^{13}C and ^{14}C), nitrogen (^{15}N), and sulphur (^{35}S) could be used in the study of plasma protein turnover. In practice, the isotopes of hydrogen have not been used, and their use could be objected to on the grounds that they may alter reaction rates. ^{13}C and ^{15}N are stable isotopes which require special assay methods and costly equipment. The rapid transamination reactions in which almost all amino acids enter vitiates the use of nitrogen as a label for plasma protein turnover (McFarlane, 1960).

^{14}C and ^{35}S remain and these have been used frequently in plasma protein turnover studies. The use of these isotopes is, however, open to one serious objection; that of the possibility of the recycling of the label. ^{14}C and ^{35}S are administered in the form of a labelled amino acid or are incorporated into amino acids before being incorporated into the proteins of the animal. In the process of breakdown of the protein in the animal, free amino acids are released and a significant amount of these is once

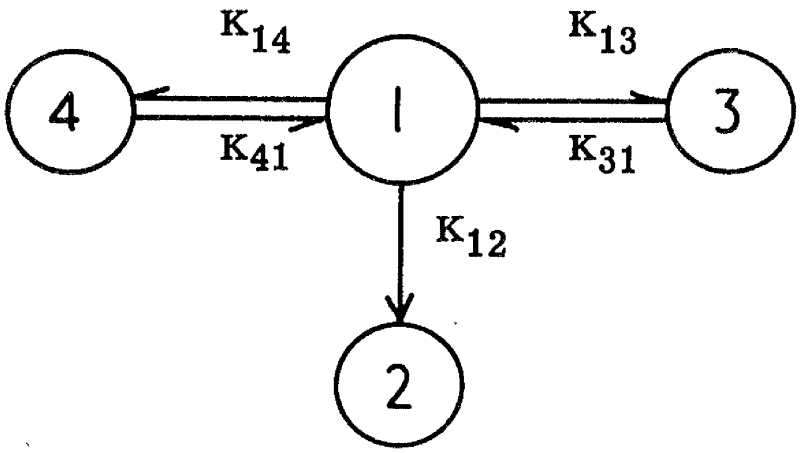
more incorporated into the proteins. Even if the initial amino acid is metabolised, unless it is broken down to CO_2, there is the possibility that the isotope could be re-incorporated into another amino acid and recycled.

Fortunately these objections can be overcome by the use of radioactive iodine; the isotope commonly used is ^{131}I - a β and γ omitting isotope of half-life of 8 days. Although iodine is not a normal constituent of plasma proteins such as albumin, under the appropriate conditions it can be readily induced to combine with the phenol ring of the amino acid tyrosine, and in the correct proportions and mild conditions does so without altering the properties of the plasma protein (McFarlane, 1956 & 1957).

Since the site and stages of breakdown of plasma protein are unknown, turnover studies have required that "model systems" be postulated and the experimental results tested for goodness of fit to the model. Thence by successive approximations the best model system can be selected.

Numerous model systems have been postulated. The earliest and simplest was that of Sterling (1951), who suggested an open single compartment system, which in the steady state, as shown above, gives first order kinetics for the rate of decay of plasma albumin activity. In normal human subjects the experimental results are in agreement with this (Sterling, 1951; Schwartz and Jarnum, 1960). A four compartment mammillary system (fig.5) was postulated by Matthews (1957). This may be an over-simplification of the biological system and does not give very good agreement with the experimental data.

Fig. 5. MODEL SYSTEM FOR PLASMA PROTEIN BREAKDOWN. 1.



Mammillary System of 4 Compartments

Compartment 1 = intravascular protein

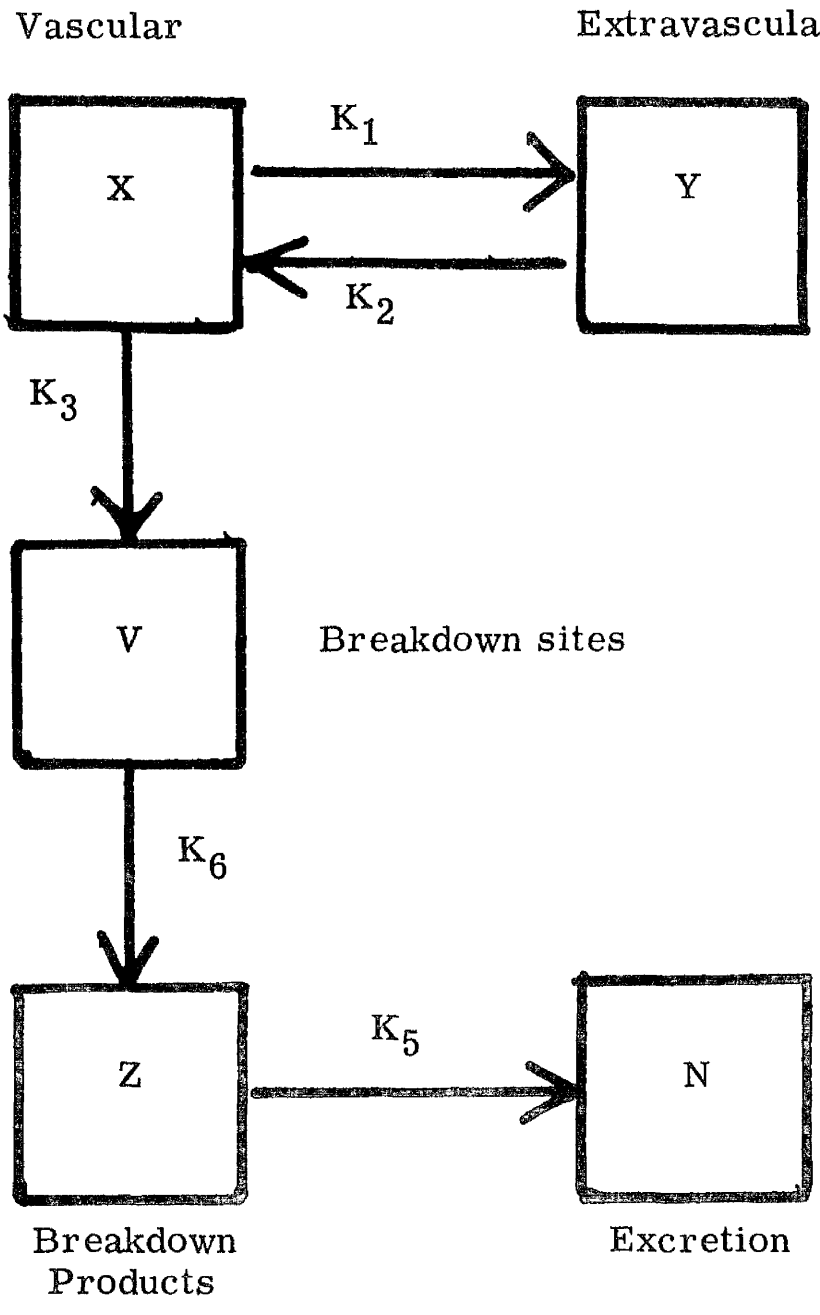
Compartment 2 = urine and faeces

Compartments 3 & 4 = extravascular protein

K_{12} is metabolic rate of protein

from Matthews (1956)

Fig. 6. MODEL SYSTEM FOR PLASMA PROTEIN BREAKDOWN. 2.



From Reeve and Roberts (1959)

Reeve and Roberts (1959) in a most comprehensive investigation suggested several model systems and, using the method of successive approximations and retrials, concluded that the best model system was a mixed (mammillary and catenary) system (fig.6). They also investigated the nature of the excretory products of ^{131}I labelled albumin and the kinetics of the excretory processes (Zizza, Campbell and Reeve, 1959). 80% of the ^{131}I label is excreted as ^{131}I-iodide, the remainder being organic ^{131}I compounds. The previous finding of Campbell, Cuthbertson, Matthews and McFarlane, (1956), that the administration of iodide in the drinking water increases the urinary excretion of ^{131}I was confirmed and in this case shown to be quantitative and without accumulation or attachment to protein. Reeve et al. (1959) also demonstrated that the plots of the logarithm of activity excreted in the urine daily and the logarithm of (1 - cumulative fractional activity excreted in the urine daily) versus time give straight lines of the same gradient (and therefore rate constant) as the semilogarithmic plot of serum albumin activity against time. This result was to be expected since k_6 and k_5 are much smaller than k_3 (fig.6).

Although the rate constants or half-lifes of the plasma proteins are most commonly used for expressing turnover, Matthews, (1957), and McFarlane (1960) recommend the use of "metabolic rate" which they define as plasma radioactivity at any time divided by the mean activity excreted in the urine over the corresponding period. Inspection of the results obtained with this method (Matthews, 1956 McFarlane, 1957) indicates that rarely in the graph of metabolic rate

Table 4

Plasma Protein Turnover

Species	Albumin	Globulins	γ-globulin	Fibrinogen
Cow	21	-	-	-
Man	10.4	13.9	22	11
	15	-	-	3.7
	25.6	-	-	-
Dog	8.2	-	-	-
Rabbit	5.8	-	5.3	-
	4.9	2.9	3.3	-
	8.0	-	6.3	2.0
Guinea-pig	2.7	-	-	-
Rat	2.7	2.1	2.4	-
	3.3	-	6.9	1.3
Mouse	0.69	-	-	-
	1.1	-	-	-

Figures are the half-lives of protein in days and are calculated from the fractional rates quoted by Anker (1960)

against time a straight line of gradient zero, as would be expected in the steady state. This deviation may be due to the error in the estimation of "metabolic rate" being greater than the error in estimates from regression line analysis of logarithmic urine or pla, plasma radioactivity curves; in the latter, the error is the experimental error of the single radioactive assay, in the former it is the error of two radioactive assays and the extrapolation of time. No statistical test of whether the deviation of the gradients from zero is significant or not has been published. It can be shown (Reeve and Roberts, 1959) that this "metabolic rate" is a composite of several rate constants and thus differs, although experimentally usually only slightly, from the true rate constant of serum albumin breakdown.

In an interesting discussion of turnover studies, Schwartz and Jarnum (1960) illustrate that in pathological conditions in the human, various methods of determining the turnover of albumin may give widely diverging results. These differences are probably due to the omission of the construction of an appropriate model for the particular case. The large scatter in figures quoted for the turnover rates of the plasma proteins - particularly albumin - in the normal human subject (table 4) illustrates the potential difficulty of determining the rate of turnover of a single easily accessible protein.

In determining the turnover time of serum albumin in an experimental animal by using ^{131}I-labelled serum albumin the following have been measured:-

1. The ^{131}I-activity of the proteins of the blood. If ^{131}I-labelled albumin was injected, it is only necessary to remove acid-soluble ^{131}I by precipitation of the proteins and washing several times to remove excess iodide. It is more elegant and technically more difficult to determine the serum albumin concentration and thence its specific activity. From this, in addition to turnover, it is possible to obtain an estimate of total body and circulating albumin, (Campbell et al., 1956; McFarlane, 1957). If the rat is used as the experimental animal a correction must be made for the amount of blood removed at each sample as the volume of the sample is a significant proportion of the total blood volume of the animal (McFarlane, 1957). In practice, it has been found necessary in this laboratory to anaesthetise rats in order to achieve reliability in the sampling of venous blood from the tail vein. This is a disadvantage in the type of experiment outlined below, as repeated anaesthesia might lead to liver or respiratory damage especially in animals deprived of protein.

2. The ^{131}I-activity excreted in the urine. When NaI is administered in the drinking water at least 80% of ^{131}I administered is excreted in the urine (Zizza, Campbell and Reeve, 1959). It is possible both from theoretical consideration of the kinetics (see p.18) and practically, (Reeve and Roberts, 1959; Lewallen, Berman and Rall, 1959) to obtain an estimate of ^{131}I-labelled albumin turnover from the urinary excretion of label alone. In practice this has rarely been done because the reported experiments were concerned with obtaining information on the stages of albumin breakdown and the relation between circulating and extravascular

albumin: they were not concerned as in the present work to relate turnover to other factors. In this method inadequate metabolic cages would lead to errors either due to loss of urine or oxidation of the iodide during collection of the urine.

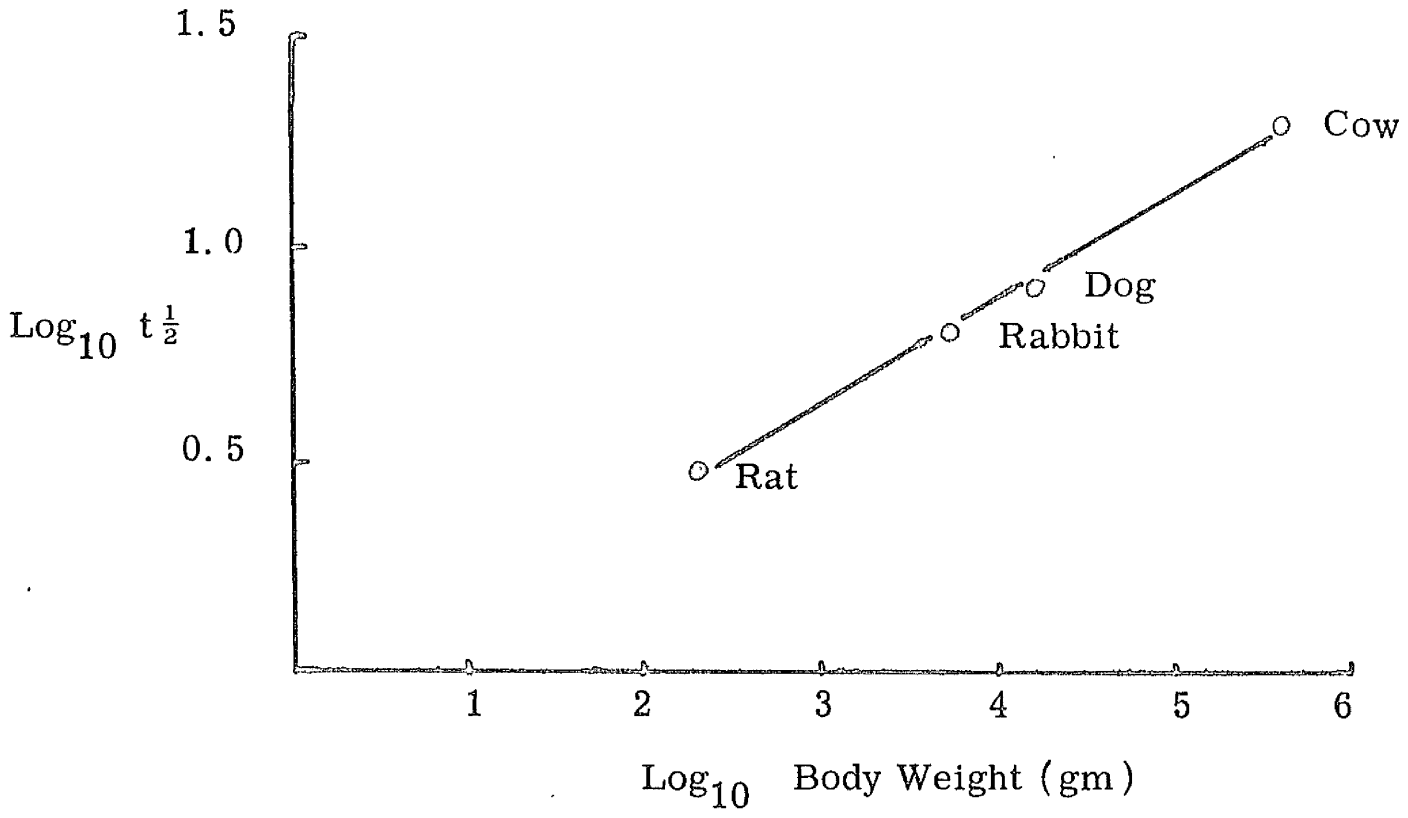
3. The total body ^{131}I-activity. This method requires special equipment - either a series of Gieger-Muller tubes arranged around a cylinder or a large well-type scintillator, both of which were unavailable at the time. In addition, the method depends on the fulfilment of the same kinetic conditions for the estimation of turnover as that depending on the excretion of urine activity alone - namely that the excretion of the ^{131}I-labelled breakdown products from the catabolised albumin is rapid and quantitative (Campbell et al. 1956).

In the series of experiments undertaken in this work, it was essential to carry out daily urinary nitrogen estimations in addition to the daily ^{131}I-activity determinations for the estimation of the half-life of serum albumin. Thus the daily manual work of the experiment had to be kept to the minimum consistent with reliable results. Because of this and the arguments given above, it was decided to utilise a method for the determination of the half-life of rat serum albumin which required only the estimation of the urinary ^{131}I-activity daily.

Factors modifying plasma protein turnover

The data of table 4 indicates that the rate of turnover of the plasma proteins varies considerably among the species. However, when the turnover of a protein such as plasma albumin is plotted on a logarithmic scale against body weight, a linear relationship

Fig. 7. THE RELATION BETWEEN ALBUMIN TURNOVER AND BODY WEIGHT



Data computed from Anker (1960)
cf. Allison (1960)

Table 5

The Relationship between
Body Size and Metabolism

Measurement (M)	Exponent "x" relating measurement to body weight (W), thus $M = W^x$
Basal Metabolism kcal/day	0.73
Urinary N mg N/day	0.72
F. Alb. turnover ($days^{-1}$)	0.66
Total Body Protein Synthesis	0.76
Muscle wt. (gm.)	0.99
Blood wt. (gm.)	0.99

The figures apply to rat (200 gm.), dog (10 Kg.) and man (70 Kg.)

and are obtained from Munro. (1964)

is obtained (Allison, 1960) - fig.7.

Albumin turnover expressed as days^{-1} can be related to body weight by the expression $M = W^{x}$ (see table 5) and the exponent obtained for 3 species is similar to that for basal metabolism and urinary nitrogen excretion.

The effect on plasma protein turnover of varying the protein content of the diet was investigated by Jeffay and Winzler (1958) using ^{35}S-labelled plasma proteins. Although the use of ^{35}S has not been recommended by McFarlane (1960) because of the problem of recycling of the label, Jeffay and Winzler (1958) claimed that an increase in the amount of protein in the diet of rats led to an increase in the rate of plasma albumin turnover. Iber, Nassau, Plough, Berger, Moroney and Fremont-Smith (1958) in studies on human subjects using ^{131}I-labelled albumin also showed that increase in dietary protein was related to an increased turnover of plasma albumin.

Previous reports on the effect of injury on plasma albumin metabolism have also appeared. In a survey of 10 surgical patients, Sterling, Lipsky and Freedman (1955) on the basis of plasma radioactivity curves, found that surgery was associated with a half-life of plasma albumin which was shorter than that of normal, control, subjects. In several of these cases, however, discrepancy between the semi-log plot of the plasma radioactivity and the per cent retained activity was observed; this discrepancy was absent in the normal controls. In addition, it is apparent from the data published, that little if any alteration in half-life would have been detected on the basis

of the per cent retained activity. Indeed, the authors suggested that the failure to recover the radioactivity in the urine in amounts comparable with the rapid plasma decline indicated that there may not be a greatly increased rate of complete degradation of albumin to amino acids. Further, the theoretical basis of the Sterling method of computing albumin turnover has been criticised (Schwartz and Jarnum, 1960). A preliminary communication by Davies, Ricketts and Bull (1959) presents 3 cases in which after the injection of ^{131}I labelled albumin, radioactivity data were followed for 16 days (case 1), 8 days (case 2) and 12 days (case 3). The rapid rate of albumin turnover of their case 1 (extensive burns with whole skin loss) could have been due to loss of albumin from the damaged areas for which apparently no correction had been made. In case 2 (fracture injuries), the experimental period was too short to justify any conclusions, as in the human subject linear exponential decay of plasma specific activity is not attained until about the 10th day after injection of the iodinated albumin (McFarlane, 1960; Schwartz and Jarnum, 1960). This criticism also applies to case 3, the results of which, however, are in agreement with those from patients with the same pathology (Schwartz and Jarnum, 1960).

That burning injuries are associated with an increase in plasma albumin turnover has been claimed by Birke, Liljedahl, Plantin and Wetterfors (1960); in some cases the increase in turnover has been observed for several months after the injury. The prolonged increase in albumin turnover in these cases may be due to a severe

stress effect with continued increased secretion of adrenal steroid hormones which are known to cause an increase in plasma albumin turnover (Rothschild, Schreiber, Oratz and McGee, 1958; Grossman, Yalow and Weston, 1960).

Finally, it has been observed that in human subjects, L-triiodothyronine causes an increase in the turnover of iodinated human plasma albumin (Iber et al., 1958).

In summary then there is evidence that:

a. a high protein diet leads to an increase in plasma albumin turnover

b. the effect of injury (apart from burning) on plasma albumin turnover is not clearly defined

c. burning injuries lead to an increase in plasma albumin turnover.

PART I

SECTION 2

EXPERIMENTS ON PLASMA PROTEIN METABOLISM

Introduction

In this section the aim of the experiments was to study albumin catabolism. This was done by varying the protein content of the diet and by investigating the metabolic sequelae of injury. It was hoped to decide whether albumin behaved metabolically as a liver protein, or in a different way, such as for example, whether it functioned as labile body protein.

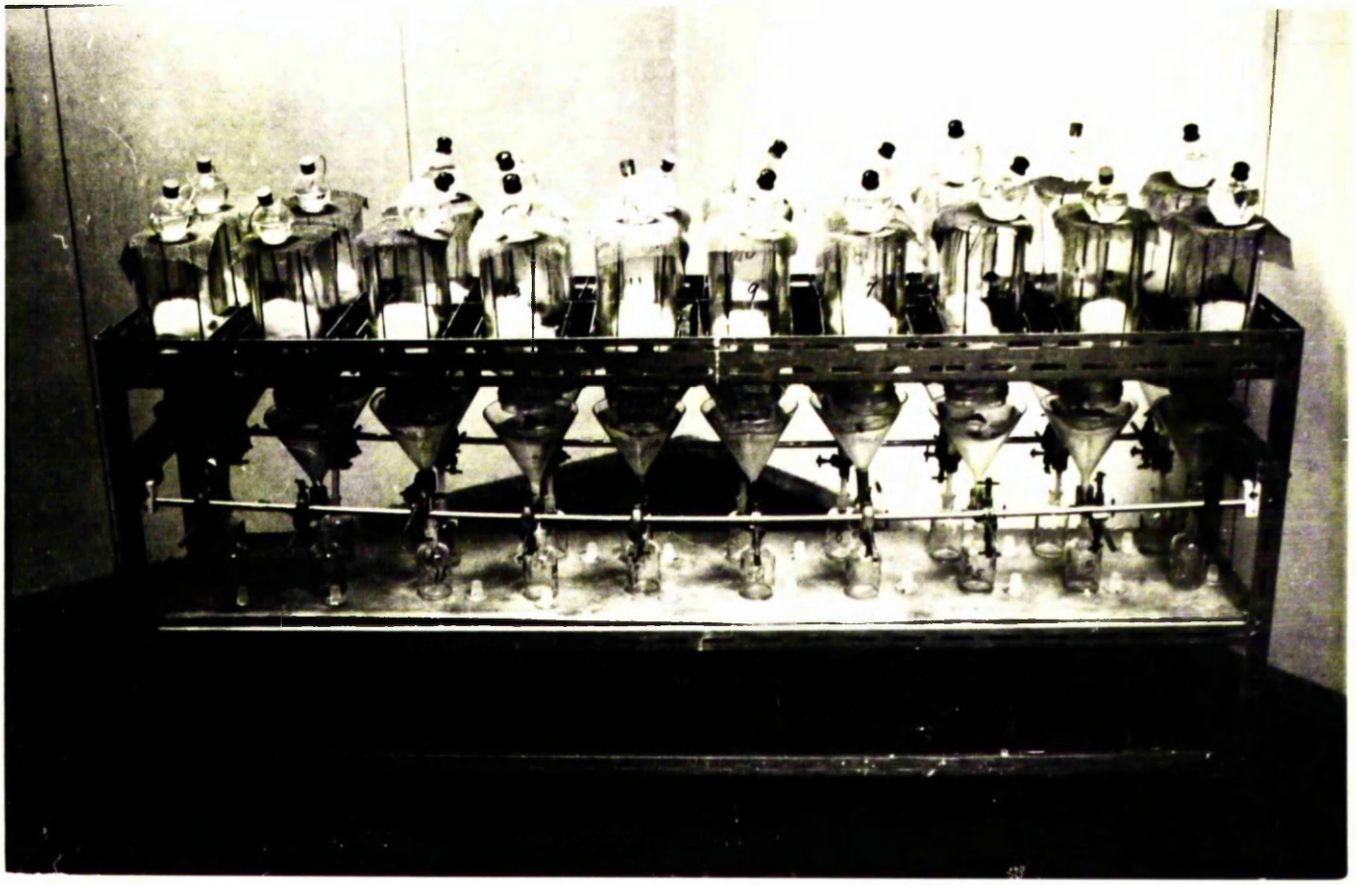
The general pattern of the experiments was that some animals were subjected to injury while others were retained as controls. During the experimental period, changes in body weight, urinary nitrogen excretion, and the half-life of serum albumin were noted, and at the end of the experimental period the livers were analysed for weight, protein, RNA and DNA content.

Since it is known that animals deprived of protein do not show the usual increase in nitrogen excretion following injury (Munro and Chalmers, 1945), it was decided to carry out the type of experiment outlined above on 2 groups of animals; one fed adequate protein and the other in which the animals were deprived of protein. By increasing the "stress" in this way it was hoped to eliminate any possible masking of the effects of injury on the liver by the relatively massive amount of non-liver protein present in the animal body.

Of the analyses carried out, some are self-explanatory (liver weight and protein content), but the nature of the information yielded by the remainder must be outlined. Since the relative weight of the liver and other viscera is small in relation to the carcass,

changes in body weight will reflect mainly changes in the carcass. The same argument applies to changes in urinary nitrogen excretion. The relation between RNA and protein metabolism is discussed in Section II, but at this point it is sufficient to say that changes in protein metabolism are usually accompanied by changes in the RNA content of the cell or organ. DNA estimations were carried out in order to determine whether any changes which might occur in the liver were due to a change in the number of cells per liver (see Davidson, 1957). The determination of the rate of turnover or half-life of serum albumin presented special problems which are discussed in the preceding section.

Fig. 8. METABOLISM CAGES



EXPERIMENTAL METHODS

Animals: Adult male albino rats from the departmental stock (Wistar strain) were used throughout.

Diet: Animals were given either a protein-free diet (LP diet) or a diet containing adequate protein (HP diet). The composition and calorific values of these are given in Tables 6, 7, 8 and 9.

When rats of approximately 250 gm. weight are fed adequate protein as casein, additional carbohydrate, fat, vitamins, minerals and roughage (VMR) to give a balanced diet such as that described in table 6, are required to bring the total energy level to approximately 1450 calories per square metre body surface area for nitrogen balance to be maintained (Naismith, 1955). The surface area of the rats was computed from the formula (Lee, 1929):

$$S = 12.54 \times W^{0.60} \times 10^{-4} \text{ square metres}$$

(S, is body surface area; W is body weight in gm.)

The animals were fed once a day (at 9.30 a.m.) either LP or HP diet as appropriate and at the energy level of 1450 calories per square metre.

Urine Collection: During the experimental periods the animals were housed in metabolism cages in an air-conditioned room at constant temperature and humidity. The metabolism cages had been previously designed and built in this department (Thomson and Munro, 1955) and were slightly modified for these experiments (see fig.8). The equipment consisted of:- 20 oval glass jars with the bottoms removed, held in the inverted position in a structure built of "Dexion": a removable wire mesh arrangement provided floor and top of the "cage" and allowed the animals to be kept in comfort and accessible: a filter funnel placed

Table 6

The Composition and Calorific Value of Rat Diets

	HP diet		LP diet	
Protein (casein)	20.5		none	
Carbohydrate				
Starch 42.5	56.5		64	85
Glucose 15			21	
Fat (margarine)	5		5	
VMR mixture*	10		10	

The figures refer to percentage composition by weight

Physiological calorie equivalents:

Protein	4 calories per gm.
Carbohydrate	4 calories per gm.
Fat	9 calories per gm.
VMR	3.5 calories per gm.

The diets as made up (both HP and LP) therefore had a calorie equivalent of 4.15 cals. per gm. of diet.

*The composition of VMR is given in Table 7.

Table 7

The Vitamin-mineral-roughage Mixture (VMR)

	(g.)
Sodium chloride	65
Salt mixture "446"	260
Vitamin supplement	500
Cod liver oil	125
Wheat germ oil	50
Agar	125
	1105

Table 8

Salt Mixture "446"

	(g.)
Sodium chloride	243.198
Potassium citrate	533.000
Dipotassium phosphate	174.000
Dicalcium phosphate	800.000
Calcium carbonate	368.000
Magnesium carbonate	92.000
Ferric citrate	36.000
Cupric sulphate (pentahydrate)	0.400
Manganese sulphate	2.800
Potassium aluminium sulphate (Alum)	0.200
Cobalt chloride (hexahydrate)	0.200
Potassium iodide	0.100
Zinc carbonate	0.100
Sodium fluoride	0.002
	2250.0 g.

Table 9

The Vitamin Supplement

	To prepare 1 kg. (&c.)
Calcium pantothenate	0.4
Choline hydrochloride	20.0
Pyridoxin hydrochloride	0.05
Riboflavin	0.05
Aneurin hydrochloride	0.05
Nicotinic acid	0.2
Biotin	0.01
p-Aminobenzoic acid	1.0
Folic acid	trace
Inositol	2.0
Menaphthone	trace

Starch to 1 kg.

below the neck of the glass jar with a "Mexican hat-shaped" gauze allowed urine to pass through but retained faeces and cast hairs etc: the urine was collected in 100 ml. reagent bottles placed below the filter funnels. Each day, immediately before feeding, the cages and funnels were washed down 3 times with distilled water and the washings allowed to drain into the urine-collecting bottle. Urine plus preservative (50% H_2SO_4) plus washings were then made to a suitable volume and samples taken for nitrogen estimation and ^{131}I activity determination.

Assay of ^{131}I Activity: This was carried out using Nuclear Enterprises scintillation counting equipment and a thallium activated sodium iodide crystal, (for details of the equipment and assay techniques etc. see appendix).

^{131}I-Labelled Rat Plasma Albumin: Using a heparinised glass syringe, or a disposable plastic syringe about 10-15 ml. of blood per rat was obtained from the Inferior Vena Cavae of 250-300 gm. rats kept under deep ether or ether-chloroform anaesthesia (the rats were then killed by thoracotomy and ventriculotomy). Plasma or serum was obtained by centrifuging.

(i) Preparation of Albumin - For a discussion of methods, see later section. To rat plasma or serum at 0°C one volume of ammonium sulphate (saturated at room temperature) was added slowly with stirring. After standing for 2 hours, the mixture was centrifuged at 10°C and 3,000 r.p.m. for 20 minutes. The supernatant was dialysed against cold running tap water in the cold room for 24 hours (i.e., until sulphate-free), then against 2 changes of distilled water, and finally was lyophilysed.

In this way 275 mg. of rat plasma albumin was obtained from 6 rats. A small sample of this preparation was subjected to starch-gel electrophoresis (Smithies, 1955) when a single band with the relative mobility of rat albumin was obtained. The nitrogen content of the albumin preparation was 14.7%.

(ii) Labelling of Albumin with ^{131}I. This was carried out using the Iodine Monochloride method of McFarlane (1958). 100 mg. of rat albumin were dissolved in 6 ml. glycine buffer B (pH 9). To this was added 0.5 ml. ICl solution†, 1.5 ml. glycine buffer A (pH 8.5) and 2 ml. of $Na^{131}I$ solution (thiosulphate-free) containing 2 mc. ^{131}I. Two samples of 0.05 ml. were taken in order to estimate the efficiency of labelling. The first sample was diluted to 100 ml. and the ^{131}I-activity of 10 ml. determined (total counts = a). To the second sample some bovine serum albumin was added to act as "carrier," the protein was precipitated with 30% Trichloroacetic acid (TCA), washed 3 times with 10% TCA, the precipitate dissolved in dilute alkali and made up to 100 ml. with distilled water. The total counts of a 10 ml. sample of this solution were then determined (= b). The ratio $\frac{b}{a} \times 100$ gave the efficiency of labelling albumin with ^{131}I by this method; in this case the figure was 60%, and in other labelling experiments the efficiency of labelling of albumin by this method was from 70 to 80%.

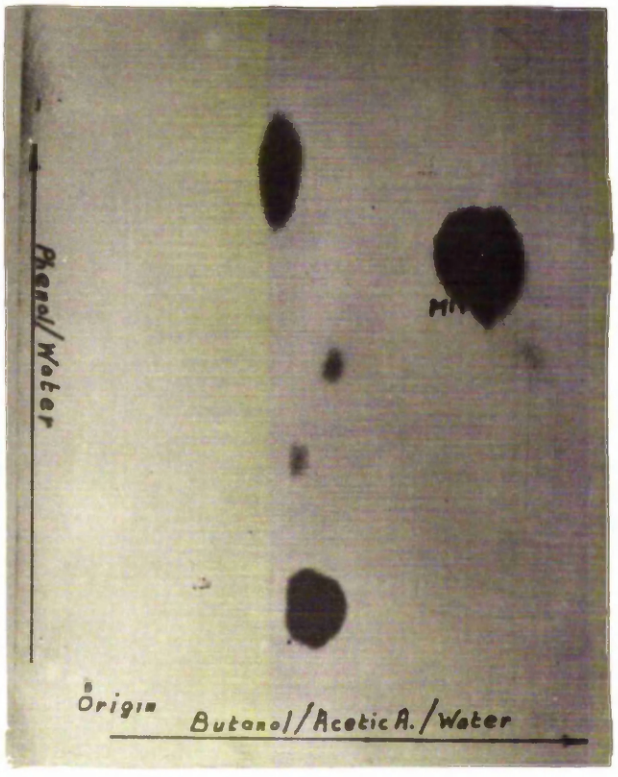
In order to remove excess $Na^{131}I$ from the labelled albumin it was dialysed in the cold against 0.9% NaCl until free from $Na^{131}I$. The experiments were arranged so that as soon as the labelled albumin was free from contamination with $Na^{131}I$, samples of the solution could be injected via a tail vein into suitably prepared rats for the determination

† 0.42mg I/ml; N/100 wrt HCl.

Fig. 9. CHROMATOGRAPHY OF ^{131}I - LABELLED HYDROLYSED RAT SERUM ALBUMIN

Hydrolysate + mono and di-iodotyrosine

Hydrolysate



Autoradiograph

of the half-life of serum albumin.

In the process of labelling, it is important that not more than 1 atom of ^{131}I be attached to each molecule of albumin (McFarlane, 1957). The amounts of albumin and $Na^{131}I$ utilised in the labelling procedure described above were adjusted so that, were the efficiency of labelling to be 100%, this ratio would be barely attained. At this level of labelling, it is unlikely that iodination of amino acids such as tyrosine would proceed beyond the insertion of one atom of iodine per molecule of tyrosine. This was crudely checked by subjecting the labelled protein to hydrolysis in 6N HCl at 100°C for 18 hours, followed by 2-dimensional chromatography of amino acids. Two such chromatograms were run; one of the labelled albumin hydrolysate alone, and the other of the labelled albumin hydrolysate to which had been added some mono- and di-iodotyrosine. An autoradiograph was prepared from one of the chromatograms. The results were photographed and are presented side by side in fig.9. It is apparent that as a result of the labelling process adopted, mono-iodotyrosine is produced, but, as expected, in insufficient amount to yield a ninhydrin-positive spot. No di-iodotyrosine has been produced. There is in addition, 2 other spots of radioactivity. One of these probably corresponds to mono-iodohistidine, and the other to inorganic iodide, some of which, it would be expected, would be released during the procedure of hydrolysis. Chromatography thus indicates that the amount of labelling of the protein with ^{131}I is not excessive.

Nitrogen Estimation: This was carried out by the micro-Kjeldahl

method using mercury as catalyst. Details of, and comments on, the estimation of nitrogen are given in the appendix.

Liver Analysis: At the end of the experimental period animals were deeply anaesthetised with ether, subjected to laparotomy, 10 ml. of blood removed in a heparinised syringe, and the liver removed. The liver was washed rapidly with ice-cold distilled water, blotted dry and clean, rapidly weighed, chilled and homogenised at $0^{o}C$ in 19 volumes of ice-cold distilled water in a Nelgo Blendor at full speed. Samples of the homogenate were taken for determination of: (a) total liver acid-precipitable nitrogen; (b) RNA, and (c) DNA. A modified Schmidt-Thannhauser procedure was used in the analysis of the nucleic acids (see appendix). Liver protein values were computed from: (total acid-precipitable liver nitrogen minus nucleic acid nitrogen) x x 6.25.

RESULTS

Following a preliminary experiment in which the method of determining the half-life of serum albumin was found to be satisfactory, the effects of diet and injury on protein metabolism were investigated in 3 separate experiments.

Experiment 1

A- Outline

Twelve animals of approximately 200 gm. weight were kept in metabolic cages in 2 groups of 6 animals; one group received a diet adequate in protein, while the other was fed a protein-free diet. The urine was collected at 24 hour intervals and nitrogen and ^{131}I output estimated. On the fourth day of the experiment,

Table 10

Change in Weight and Nitrogen Excretion of Animals of 200 g. wt

(Experiment 1)

	Change in body wt. (gm.)		N Excretion (mg.)	
	Period I	Period II	Period I	Period II
HP control (a)	+5	- 2	1253	1130
HP injured (b)	+8	- 8	1191	1317
LP control (c)	-2	-17	153	118
LP injured (d)	-2	- 9	120	143

Period I refers to 7 days immediately prior to day of fracture

Period II refers to 9 days immediately after fracture

Table 11

Activity in the Urine of 200 gm. Rats (Experiment 1) Excreted per Day from the 12th to 17th Days

Activity is expressed as logarithm of $\frac{\text{Total radioactivity excreted in the urine per day}}{\text{activity of standard}}$

Rats 1, 2, 6, 10, 11, 12 received adequate protein and rats 4, 14, 3, 9, 15 were maintained on a protein-free diet.

Rats 1, 10, 11, 3, 9, 15 were injured on the 9th day.

All animals received 5 mg. radioactive albumin by tail vein injection on the 4th day.

Day	Time (t) Hours	Adequate Protein Diet						Low Protein Diet				
		Controls			Injured			Controls		Injured		
		Rat 2	Rat 6	Rat 12	Rat 1	Rat 10	Rat 11	Rat 4	Rat 14	Rat 3	Rat 9	Rat 15
12	24	1.5128	1.5393	1.6155	1.5684	1.6756	1.6098	1.6202	-.	1.6086	1.6344	1.6676
13	48	1.4145	1.4415	1.4326	1.4559	1.5357	1.4228	1.4196	1.4602	1.3537	1.5005	1.4935
14	72	1.2553	1.2576	1.2636	1.2749	1.3535	1.2885	1.3219	1.3459	1.3696	1.4689	1.3526
15	96	1.1644	1.0781	1.1452	1.0920	1.3158	1.2143	1.2531	1.2065	1.3032	1.1989	1.2490
16	120	1.0744	0.9991	1.1271	0.9572	1.1649	1.1146	1.1342	1.1459	1.1626	1.2591	1.0952
17	144	0.8901	0.9399	1.0167	0.8442	1.0338	1.0228	1.0903	1.1364	1.0403	1.1507	0.9772
Regression Coefficient= (G)		-0.005019	-0.004998	-0.004764	-0.005052	-0.005178	-0.004683	-0.004255	-0.005532	-0.004144	-0.004063	-0.005655
Half-life of albumin ($t_{\frac{1}{2}}$)=		60.0	60.2	63.2	50.6	58.1	64.3	70.8	85.2	72.64	74.1	53.2
Intercept of Regression line on Y-axis (C)=		1.6407	1.6116	1.6655	1.6821	1.7812	1.6722	1.6640	1.5981	1.6544	1.7100	1.7809

Pooled Average Standard deviation of regression coefficients (S_G) = 0.000549

Table 12

Analysis of Variance

The figures used in this analysis are the values of the half-life of albumin for the individual rats of experiment 1, (see table 11).

	High protein diet			Low protein diet		
Control	60.0	63.2	60.2	70.80	85.2	
Injured	58.1	64.3	50.6	74.1	72.6	53.2

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance Ratio F
Between samples i.e. high and low protein diet	378	1	378	5.25
Within samples	649	9	72	
Total	1027	10	-	

This result is significant at the 5% level.

Table 13

The half-life of Serum Albumin in 200 g. Rats

(Experiment 1)

	(a) HP Control	(b) HP Injured	(c) LP Control	(d) LP Injured
Half-life of serum albumin expressed in hours	60	51	85	73
	60	58	-	74
	63	64	72	53
	61	58	78	67
Group mean				
Sx^2	6.43	94.1	103.7	261.8

t-tests

1. (a) v (c) $t = 3.054$

$0.05 > P > 0.02$

2. (a) v (b) $t = 0.8$ - not significant

3. (c) v (d) $t = 1.13$ - not significant

^{131}I-labelled rat serum albumin was injected via a tail vein to each animal. On the tenth day, all the animals were subjected to ether anaesthesia and one of the femora of each of three animals from both dietary groups were fractured. The half-life of serum albumin was calculated from the ^{131}I output in the urine from day 12 to day 17. At the end of the experimental period, plasma and liver analyses were carried out. All statistical tests of significance were based on the "t-test," except where otherwise indicated.

B - Results

The alteration in body weight and urinary nitrogen excretion in relation to diet and injury is shown in table 10. During the control period (period I), there was a significant loss of weight of the animals given the diet inadequate in protein whereas the protein-fed animals tended to gain weight (t-test, $0.02 > P > 0.01$). Subsequent to injury, injured animals receiving adequate protein lost weight as compared with the uninjured controls (t-test, $0.1 > P > 0.05$). In animals fed no protein, injury did not appear to influence the loss in weight. Injury is followed by a considerable increase in urinary nitrogen excretion by animals fed a diet adequate in protein (t-test, $P \ll 0.01$). In this experiment, the animals deprived of protein also showed an increase in urinary nitrogen excretion following injury (t-test, $0.05 > P > 0.02$). These changes in nitrogen excretion due to diet and injury are illustrated in fig.10.

Table 11 gives the data from which the half-life of serum albumin was obtained in this experiment. Tables 12 and 13 give alternative methods of analysing the results with summaries of the statistical treatments. One of the animals in the "zero protein"

Table 14

Summary of Results of Experiment 1 - The Effect of Diet and Injury on 200 g. Rats

	(a) HP Control	(b) HP Injured	(c) LP Control	(d) LP Injured
1. Change in* body weight (gm.)	- 2	- 8	- 17	- 9
2. Nitrogen*▲ excretion (mgm.)	-123	+125	- 35	+ 23
3. Liver weight (gm.)	6.3	6.5	5.3	5.1
4. Liver protein-N (mgm.)	161	171	112	107
5. Total plasma protein (gm./100 ml.)	5.9	5.8	5.0	4.4
6. Half-life of*† serum albumin (hours)	61	58	79	67
7. Total RNA per liver $\left(\frac{\text{mgm.}}{\text{RNA-P}}\right)$	4.25	4.22	3.70	3.62
8. Total DNA per liver $\left(\frac{\text{mgm.}}{\text{DNA-P}}\right)$	1.30	1.34	1.42	1.42

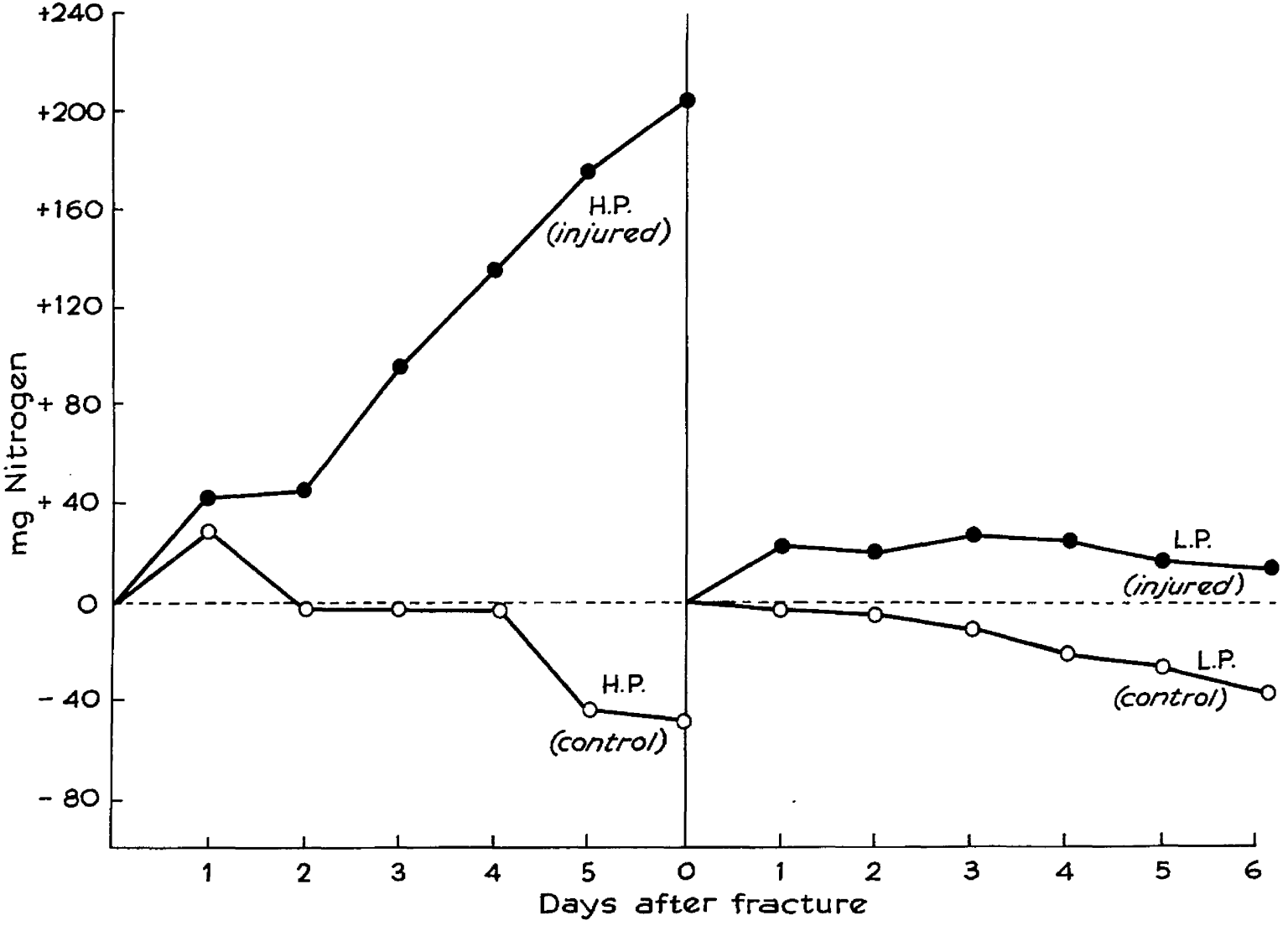
Figures for groups (a), (b) and (d) are the means of those obtained from 3 animals: group (c) are the means from 2 animals.

* see table 10

† see table 11

▲ see fig. 10

Fig 10. Cumulative Nitrogen outputs above or below Nitrogen output during pre-fracture period



control series (column c) developed a fatal respiratory infection during the final few days of the experiment. Injury does not influence the half-life of serum albumin, although diet produces a significant effect: the fractional turnover of serum albumin in the animals given adequate protein is greater than in those animals deprived of protein.

The remaining analytical data obtained in this experiment is presented in table 14, together with a summary of the results discussed above in order to facilitate comparison.

Summary of results from experiment 1 (Table 14)

1. On adequate protein diet injured animals lose weight to a greater extent than the controls, whereas injury does not affect the body weight of animals deprived of protein.

2.(a) The urinary nitrogen output of animals receiving adequate protein is greater by an order of magnitude than that of animals given a diet deficient in protein.

(b) Following injury, the output of nitrogen in the urine of animals fed adequate protein increases considerably. The nitrogen output of animals on a low protein diet also increases after injury.

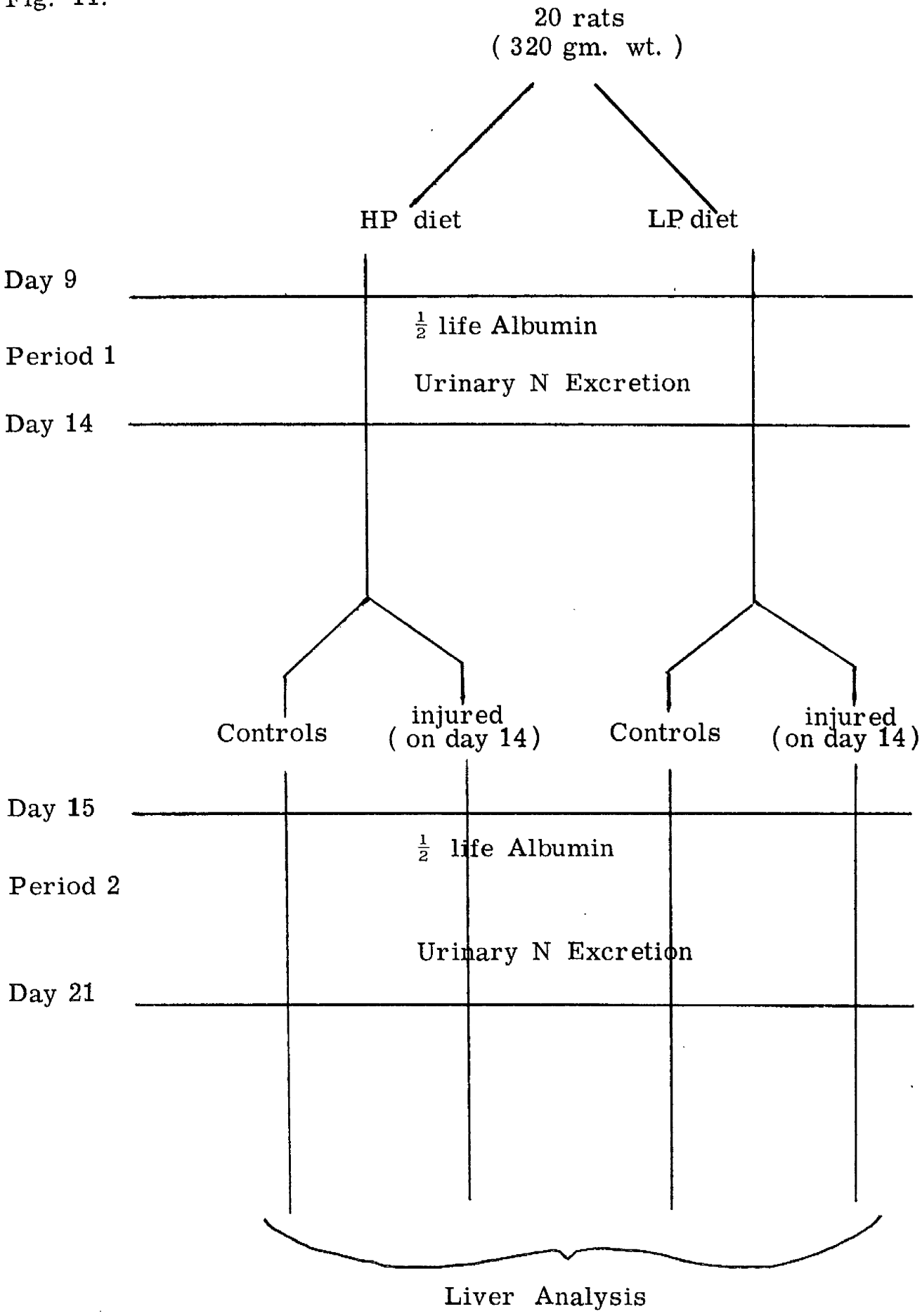
3. Livers of animals fed adequate protein are heavier than those of animals deprived of protein, but injury does not affect the weight of the liver (t-test, $P < 0.01$).

4. Injury does not influence the protein content of the liver, but the livers of animals fed adequate protein contain more protein than those of protein-deprived animals (t-test, $P < 0.01$).

5. There is no effect of injury on the plasma protein concentration,

EXPERIMENT 2 - SUMMARY OF PROCEDURE

Fig. 11.



but animals deprived of dietary protein have a lower plasma protein concentration than those fed adequate protein (t-test, $0.05 > P > 0.02$).

6. The half-life of serum albumin in protein-fed animals is significantly different from that of animals deprived of protein (t-test, $0.5 > P > 0.02$). Injury does not affect serum albumin turnover.

7. The RNA content of the liver is unaffected by injury but is influenced by diet. The RNA content of the liver is greater in protein-fed animals.

8. The total DNA content of the liver was not influenced by either diet or injury.

Experiment 2

In order to investigate further the effects of injury on protein metabolism and particularly on serum albumin turnover, a second experiment was carried out. In this experiment a larger number of animals was used, and by determining the half-life of albumin in 2 periods - before and after injury - it was expected that each animal could serve as its own "control." Thus it was hoped that a slight difference in serum albumin turnover due to injury could be detected.

A - Outline (see fig.11)

Twenty rats of approximately 320 gm. weight were housed in individual metabolism cages. Ten received adequate protein in the diet and ten were maintained on a protein-free diet. On the third day of the experiment ^{131}I-labelled rat serum albumin was injected via a tail vein to each rat and the half-life of serum albumin determined in 2 periods; the first (before injury) was from the ninth to the

Table 15

Change in Weight and N Excretion of

Animals of 320 G. wt. (Experiment 2).

	Change in wt. (gms.)		N excretion (mg.)	
	Period I	Period II	Period Ia	Period IIa
HP control	-15	- 3	761	764
HP injured	-13	-15	858	982
LP control	-23	-14	106	103
LP injured	-25	-18	89	91

Period I refers to 8 days immediately prior to fracture

Period II refers to 8 days immediately after fracture

Period Ia refers to 5 days immediately prior to fracture

Period IIa refers to 5 days immediately after fracture

Figures are group means.

Table 16

The Effect of Diet on the Half-Life of Serum Albumin in 320 g. Rats. Experiment 2 - Period I

	HP	LP
Half-life of serum albumin expressed in hours	57	67
	61	65
	57	79
	58	72
	67	59
	60	61
	58	79
	48	70
	45	77
	55	79
$\bar{x}$ =	57 ($\pm$ 4)* S.D.	71 ($\pm$ 6)* S.D.
Σx^2 =	354	526
	t = 4.5	$P \ll 0.01$

* This figure is the mean of the standard deviations from regression (standard errors of the estimates).

Table 17

The Effect of Diet and Injury on the Half-life of Serum Albumin in 320 g. Rats (Experiment 2 - Period II)

	(e) HP Control	(f) HP Injured	(g) LP Control	(h) LP Injured
Half-life of serum albumin in hours	61	73	77	92
	83	81	90	-
	83	69		-
		85		84
		75		81
		70		92
				76
	76	76	88	85

$t = 2.435$; degrees of freedom = 14; hence $0.05 > P > 0.02$

fourteenth day, the second (after injury on the fourteenth day) was from the fifteenth to the twenty-first day. Some animals from each dietary group remained uninjured to serve as controls. Urinary nitrogen output was estimated in 2, 3-day periods before and after injury. On the twenty-first day, plasma protein estimation and liver analysis of protein, RNA and DNA was carried out. Statistical analysis of the results was by the t-test throughout.

Experiment 2 - Results

The effects of diet and injury on body weight and urinary nitrogen excretion are shown in table 15, and are similar to those of the previous experiment (table 1). The increase in weight loss of the injured animals fed adequate protein is significantly different from the negligible weight loss of the animals of the control group (t-test $0.02 > P > 0.01$). Animals deprived of protein do not lose weight at a greater rate when injured. The urinary nitrogen excretion of the protein-fed group increased after injury (t-test, $0.05 > P > 0.02$); that of the protein-deprived group did not.

The data obtained for the half-life of serum albumin is given in tables 16 and 17. The figures of table 16 refer to the first period during which the half-life of albumin was determined. Since this period was prior to injury, the figures allow a direct comparison of albumin turnover in animals fed adequate protein with the turnover in animals deprived of protein. The half-life of albumin is obviously different in the 2 groups: the means differ widely and the standard error is small; by the t-test, $P \ll 0.01$. The turnover of albumin in protein-fed animals is more rapid than in animals deprived of protein. The data of table 17 shows that injury does

Table 10

Summary of the Results of the Effect of Diet and Injury on 320 g. Rats (Experiment 2)

	(e) HP Control	(f) HP Injured	(g) LP Control	(h) LP Injured
1. Change in body weight (gm.)*	- 3	- 15	- 14	- 10
2. Nitrogen balance (mgm.)†	+ 3	+124	- 3	+ 2
3. Liver weight (gm.)	8.8	8.9	7.9	8.2
4. Liver protein-N (mgm.)	238	240	182	178
5. Total plasma protein (gm./100 ml.)	5.8	6.0	4.8	5.5
6. Half-life of serum albumin (hours)*	76	76	88	85
7. Total RNA per liver (mgm. RNA-P)	6.4	6.4	5.8	6.2
8. Total DNA per liver (mgm. DNA-P)	2.2	2.1	1.8	2.0

* refers to period II (see tables 4 and 6)

† difference in nitrogen excretion of period II - that of period I

Column (e) figures are means from 4 animals

(f) figures are means from 6 animals

(g) figures are means from 3 animals

(h) figures are means from 8 animals

not influence the turnover of albumin, and also confirms the effect of dietary protein. The figures for the half-life of serum albumin given in table 17 are somewhat greater than those of table 16 (see also fig.13). There is no simple explanation for this: it may be due to contamination of the albumin with globulin in this case. However, McFarlane (1960) has remarked upon a similar apparent increase in the half-life of serum albumin when determined many days after the injection of the labelled albumin.

A summary of the results obtained in this experiment is presented in table 18.

Summary:

1. Injury does not affect the body weight of animals deprived of protein, although animals receiving adequate protein lose weight when injured.

2. (a) The urinary nitrogen output of animals deprived of protein is approximately 1/10 of that of animals maintained on adequate dietary protein.

(b) Subsequent to injury animals fed adequate protein excrete more nitrogen in the urine than control animals (t-test, $0.05 > P > 0.02$). The urinary nitrogen excretion of animals deprived of protein is not influenced by injury.

3. Animals fed adequate protein have heavier livers than those deprived of protein (t-test, $0.05 > P > 0.02$). Injury does not affect the weight of the liver.

4. The amount of protein in the liver is not affected by injury, but is determined by the protein content of the diet: feeding protein

leads to a higher liver protein content than deprivation of protein (t-test, $P < 0.01$).

5. The total plasma protein concentration of animals receiving adequate protein is greater than that of animals deprived of protein (t-test, $P < 0.01$). Injury has no effect on plasma protein concentration.

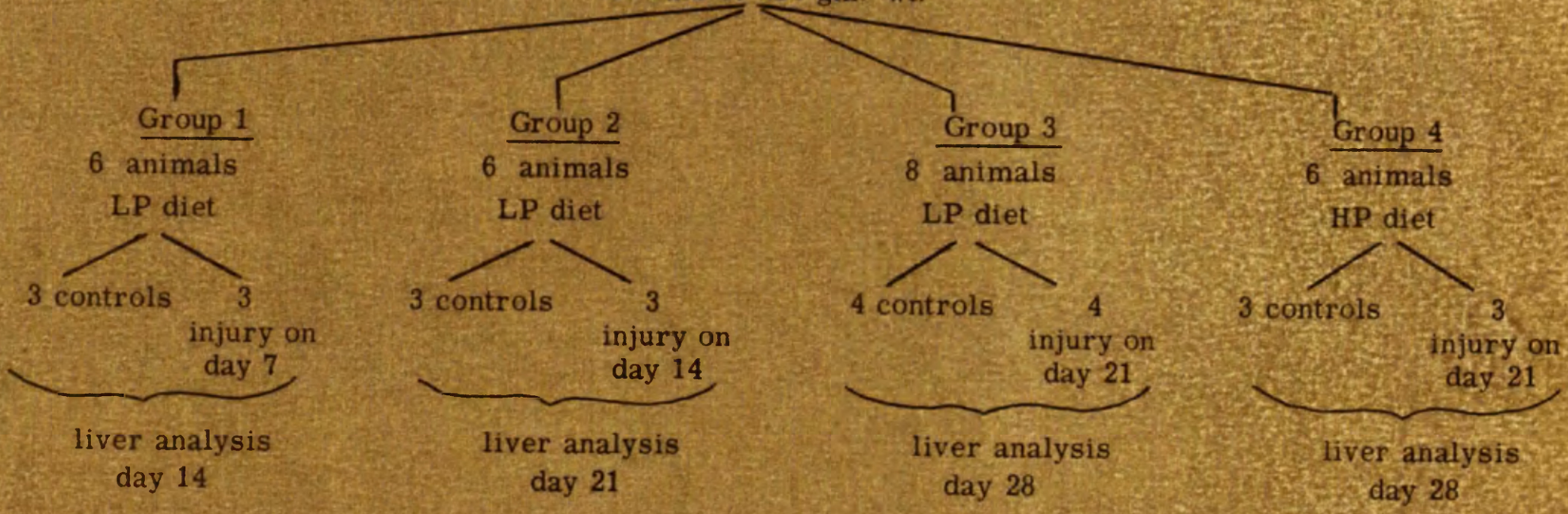
6. In both periods in which the half-life of serum albumin was determined, the half-life is greater when animals are deprived of protein than when fed adequate protein. Injury does not influence the turnover of serum albumin.

7. Probably due to the wide scatter of the experimental results, the apparent effect of diet on the RNA content of the liver is not statistically significant.

8. In this experiment, deprivation of protein is related to a significantly lower total liver DNA-P content than that of animals fed adequate protein (t-test, P approximately equals 0.02).

It is obvious that the results of experiments 1 and 2 are in good agreement and present the same general picture; namely, that injury does not affect the metabolism of the liver but does affect body weight and nitrogen excretion. However, in the first experiment, animals deprived of protein showed a small increase in the excretion of nitrogen following injury, while in the second experiment this was not observed. Since this effect might be related to the degree of deprivation of protein, that is to the length of time the animals had been maintained on a protein-free diet, a third experiment was carried out in order to test this.

Fig. 12. EXPERIMENT 3 - SUMMARY OF PROCEDURE



In each group N excretion was determined for a symmetrical period immediately before and after injury.

Table 19

Change in Weight and Nitrogen

Excretion of Animals of 250 G. wt. (Experiment 3)

	Change in wt.		Urinary Nitrogen Excretion	
	Period I 7 days before injury	Period II 7 days after injury	1st Period (3 days before injury)	2nd Period (3 days after injury)
Group I (LP)				
Control	-24	- 6	89	77
Injured	-18	-14	107	136
Group II (LP)				
Control	- 8	-10	65	59
Injured	- 7	-13	64	64
Group III (LP)				
Control	-13	- 4	51	47
Injured	-13	-4	48	54
Group IV (HP)				
Control	+ 7	+ 5	877	833
Injured	+ 6	- 2	767	888

Figures are group means.

Experiment 3 (see fig.12)

A Outline

Twenty-six rats of approximately 250 gm. weight were kept in metabolic cages. Six of these animals were maintained on adequate protein; 3 were kept as controls and the remaining 3 were injured on the twenty-first day of the experiment. The remaining animals were allocated to 3 groups, each of which received no dietary protein: in the first and second groups there were 3 control and 3 injured animals, injury being effected on the seventh day in group 1 and on the fourteenth day in group 2. The third group consisted of 8 animals; 4 controls and 4 injured; injury was carried out on the twenty-first day. In each case, injury, which was carried out under ether anaesthesia, consisted of fracture of a femur. Seven days after injury in all groups the animals were killed and the livers removed for analysis.

Experiment 3 - Results

The alteration in body weight and nitrogen excretion in relation to diet and injury is given in table 19. The general trend is similar to that of tables 10 and 15, despite a greater variation in the figures for individual animals which rendered statistical analysis unsatisfactory. When animals have received a diet lacking in protein for only a short period (group 1), it would appear that following injury they may lose weight at a slightly greater rate than the controls and also excrete somewhat more nitrogen in the urine than animals which have been maintained on a protein-free diet for 2-3 weeks prior to injury (groups 2 and 3). The animals of group 4 (fed adequate protein) show the expected loss in weight and increase in urinary

Table 20

The Protein Content of the Liver of Rats fed Low Protein Diets for a Variable Length of Time (Experiment 3)

Group	LP (I)	LP (III)	HP (IV)
Controls	119	90	148
	114	94	170
		100	
	122	104	188
Injured	110	93	159
	116	106	163
		109	
	124	102	175
Mean	118	100	167
Sx^2	139	298	955

$t_{(1)}$ $(LP_I - LP_{III})$ = 5.39 $P \ll 0.01$

$t_{(2)}$ $(HP_{IV} - LP_I)$ = 8.20 $P \ll 0.01$

Table 21

Summary of Effects of Diet and Injury on Animals of 250 g. wt. (Experiment 3)

	HP Control	HP Injured	LP Control GROUP			LP Injured GROUP		
			I	II	III	I	II	III
1. Changes in body weight (gm.)	+ 5	- 2	- 6	- 10	- 4	- 14	- 13	- 4
2. Change in nitrogen excretion (mgm.)	- 44	+121	- 12	- 6	- 4	+ 29	0	+ 6
3. Liver weight (gm.)	6.0	6.2	5.4	5.2	5.2	5.5	6.2	5.4
4. Liver protein nitrogen (gm.)	169	166	118	107	97	117	123	105
5. Total RNA per liver $\left(\frac{\text{mgm.}}{\text{RNA-P}}\right)$	4.77	4.97	4.08	3.55	3.74	4.21	4.36	4.01
6. Total DNA per liver $\left(\frac{\text{mgm.}}{\text{DNA-P}}\right)$	942	953	987	933	1001	904	954	1045

nitrogen excretion after injury.

In addition, it appears that the excretion of nitrogen in the urine decreased as the period of time during which the animals were deprived of protein increased.

From table 20 it appears that during the experiment, there was a progressive decrease in liver protein of animals deprived of dietary protein. The liver protein content of animals deprived of dietary protein was less than that of the protein-fed animals. Also, it was obvious that injury did not affect the protein content of the liver in any group of animals. The results of the experiment are summarised in table 21.

Summary:

1. Animals lost weight following injury only if they were fed adequate protein or deprived of protein for a short period.

2. Increase of nitrogen excretion subsequent to injury occurred in animals fed protein or deprived of it for only a short period.

3. Animals fed adequate protein had a greater liver weight than animals given a diet deficient in protein. There was no decrease in liver weight as the duration of deprivation of protein increased.

4. The livers of animals fed adequate protein contained more protein than the livers of animals deprived of protein. The liver content of protein decreased with time on the protein-deficient diet.

5. Injury did not affect either the weight of the liver or its protein content.

6. The RNA content of the liver is influenced by diet but not by injury.

7. The liver DNA content did not vary throughout the experiment.

Table 22

Effect of Injury on the Protein Metabolism of the Adult Male Rat

Dietary Protein Level	Group (Number)	Change at 7-9 days after injury		Liver Protein N mg./rat	Plasma Protein	
		Body wt. (gm.)	Change in Urinary N output		conc. gm. %	½ life hours
Adequate	Controls (10)	0	- 53	189	5.9	69
	Fractured (12)	- 8	+124	192	5.9	67
None	Controls (8)	-12	- 17	137	4.9	84
	Fractured (14)	-14	+ 18	134	5.0	76

A summary of the principal findings of the three experiments is given in table 22 (see also Fleck and Munro, 1963). From this it is seen that on adequate protein diet injury leads to:-

1. loss of body weight.
2. increase in urinary nitrogen excretion.

There is, however, no change in the protein content of the liver, the plasma protein concentration, or the half-life of serum albumin.

When animals were deprived of protein:-

1. there was little change in body weight after injury.
2. injury had little effect on the excretion of nitrogen.

The magnitude of the effects (if any) observed depended on the length of time the animals had been deprived of protein prior to injury (experiment 3). In animals deprived of protein, injury did not influence the protein content of the liver, the plasma protein content, or the half-life of serum albumin. It will be noted that the liver and plasma albumin were sensitive to the protein content of the diet although not to injury, indicating that the procedures used are adequate to detect differences in metabolism.

DISCUSSION

The results of the three experiments are consistent: animals deprived of protein lose weight, excrete much less nitrogen in the urine and have smaller livers containing less protein and RNA than animals fed adequate protein. Injury does not affect liver protein metabolism in either protein-deprived or protein-fed animals since the weight, protein and RNA content of the liver, and the half-life of serum albumin do not change after injury. Injury does, however, affect body weight and nitrogen excretion. This effect is considerable in animals fed adequate protein. In those animals deprived of protein, whether injury influences body weight and nitrogen excretion depends on the duration and extent of protein depletion; if the depletion is slight, then some effect following injury may be observed, but if severe or prolonged, no effect is detectable.

There is a considerable literature recently revieved by Munro (1964) dating back to Bischoff and Voit (1860) which relates to the effects of protein depletion on animals. When dogs are deprived of protein, the urinary urea excretion falls to a minimum in about 7-8 days; this is independent of the previous level of protein feeding (Voit, 1866). The human subject when deprived of protein also takes about 7 days to attain the minimal level of urinary nitrogen excretion (Martin and Robison, 1922), as does the rat (Campbell and Kosterlitz, 1948). These observations have led to the concept that there exists in the animal body a quantity of protein (about 3% of the total) which responds rapidly to changes in diet, and is known as the labile body protein. Although the work of Kosterlitz (1947)

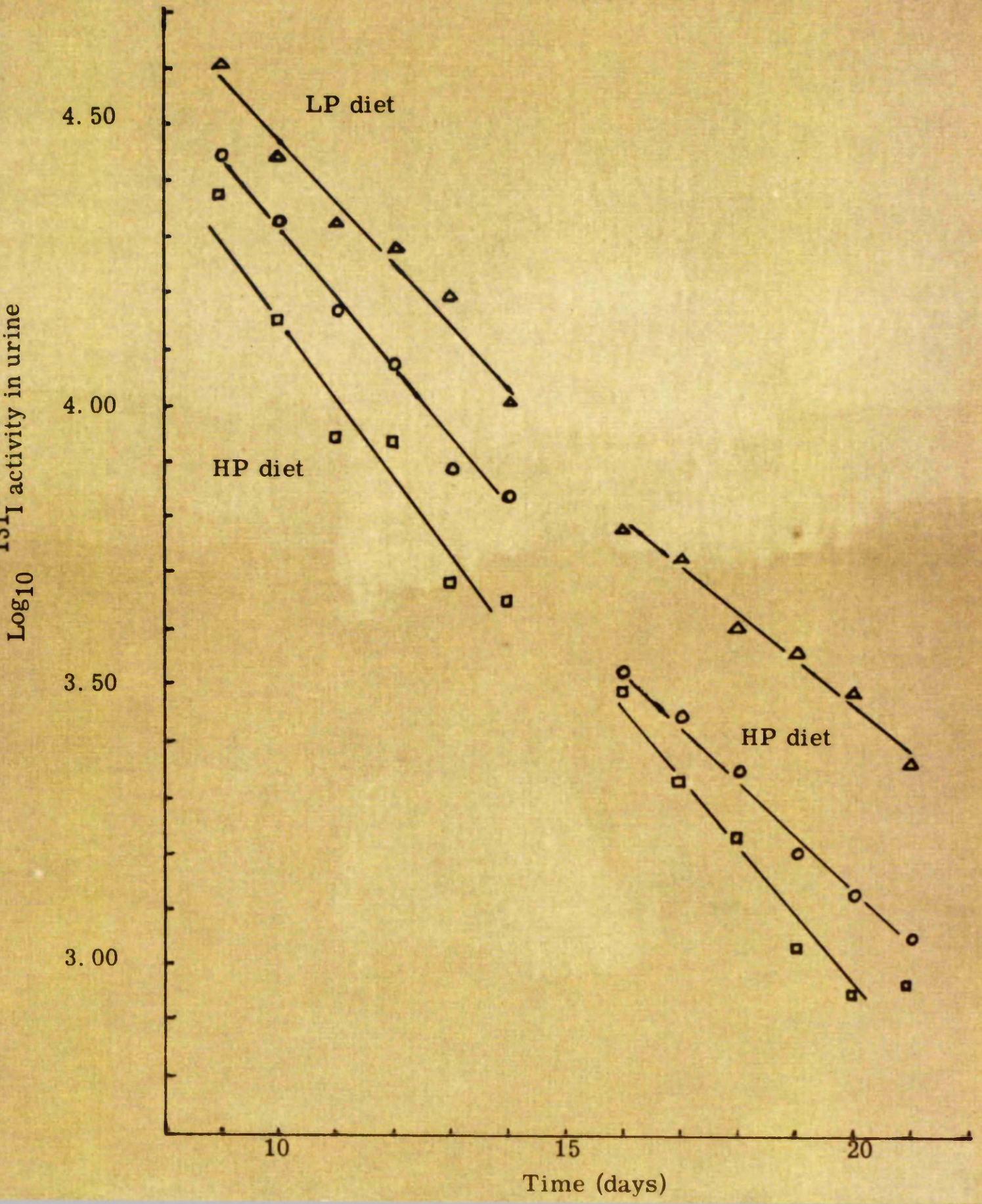
has shown that the protein, RNA and phospholipid content of the liver "cytoplasm" changes rapidly with diet, the liver cannot be the sole source of this labile protein because the weight of the liver varies from 4% of the body weight in a 200 gm. rat to about 2% of the body weight in a 70 Kg. man (Brody, 1945). For some time it has been shown that the enzymes of certain tissues such as brain and heart are more resistant to changes in the protein content of the diet than those of tissues such as liver and intestine (Fisher, 1954; Munro, 1964). The labile body protein is thus probably restricted in its distribution, but if the estimates of its relative quantity are correct, then it must occur in more than one tissue or organ. That the liver does contain a portion of the labile body protein seems to be generally accepted (Munro, 1964), and there is some evidence that there is an amount of labile protein in muscle (Munro, Black and Thomson, 1959). It follows that certain factors may affect the labile protein of one tissue or organ and not affect that of another tissue. Thus cortisone and other protein-catabolic hormones induce the loss of protein from muscle and an increase in the protein content of the liver, while growth hormone and other anabolic hormones induce the opposite changes (Munro, 1964).

Cuthbertson (1930) observed that human subjects showed an increase in the urinary nitrogen excretion after injury (or surgery). The effect is dependent on the previous level of protein nutrition (Munro and Chalmers, 1945) and is absent in experimental animals deprived of dietary protein (Chalmers, 1945). The source of the nitrogen lost in the urine is believed to be the labile body protein (Munro, 1964).

It will now be apparent that in the present series of experiments, the results of protein feeding and protein deprivation on nitrogen excretion and the composition of the liver are in agreement with the previously published work of other authors. The present results of the effect of injury (or the absence of it) on nitrogen excretion in protein-fed or protein-deprived animals is consonant with the work of Munro and Chalmers (1945). In addition, the present experiments indicate that the liver remains unaffected by injury on either the adequate or inadequate protein diets, as apart from the analytical data presented - (a) the weight of the liver is the same in injured and control animals; (b) the loss of body weight following injury in those animals fed adequate protein is greater than the total weight of the liver; (c) the increase in nitrogen excretion of protein-fed animals after injury is of such amount that it could not all have originated in the liver protein. It is of course possible, that in the course of seven days following injury the liver could have returned to its pre-injured state. However, it is difficult to accept that in animals deprived of protein some changes would not remain after 7 days. Also, the animals at 7 days post injury do not show a restoration of body nitrogen, nitrogen balance still being less favourable than that of controls. Thus, although injury is classified as a protein catabolic agent, it differs from the hormones quoted above in that it has no effect on liver protein metabolism.

The difference in half-life of serum albumin with diet is highly significant and in agreement with other work using

Fig. 13. DAILY URINARY ^{131}I EXCRETION



^{35}S (Jeffay and Winzler, 1958). Matthews (1961) observed that when 20-30% of the plasma albumin of rabbits was removed by plasmapheresis, this was compensated for by a reduction in the catabolic rate of albumin. In these experiments only the animals fed adequate protein were in the steady state so that the half-life of albumin calculated for the other experimental subjects may not be the true half-life. Nevertheless, it is difficult to see how this result could be explained except as being due to a true difference in turnover time of albumin. For example, it is unlikely that an animal losing weight would be able to retain the ^{131}I breakdown products of serum albumin for a sufficient time to account for the observed increase in half-life of albumin in animals deprived of protein. On the other hand, a change in the rate of breakdown of albumin due to injury, if short-lived, might not be observed. The duration of the effect of injury on nitrogen excretion is about 3 days, that is, it approximately equals the half-life of albumin. If the changes in albumin turnover were of the same order as the half-life of albumin this is a rather short period for the method of regression line analysis used to be sensitive to them. Also, the anaesthetic and surgery affect renal function for a short period (about 24 hours in the human subject, (Cuthbertson, 1932)) so that it was thought inadvisable to include the ^{131}I excretion during the first 24 hours after injury in the regression line calculation, (see fig.13).

Serum albumin, in the present series of experiments, behaved metabolically as a liver protein. If it had any function as a carrier of amino nitrogen to the carcass, as it may do in the extreme

conditions of plasmapheresis (Madden, George, Warsaich and Whipple, 1938) then following injury in protein-fed animals the turnover time of albumin should have decreased, due to the increased demand for nitrogen by the carcass.

SUMMARY

1. The effects of injury on protein metabolism in rats has been studied using animals deprived of protein and animals maintained on adequate dietary protein.

2. The effects of diet on body weight, urinary nitrogen excretion and the composition of the liver are in agreement with previously published work by other authors.

3. Injury leads to loss of body weight and increased urinary nitrogen output in animals fed adequate protein but no changes in these measures were observed in animals deprived of protein for 2 weeks or more.

4. Protein metabolism in the liver is unaffected by injury.

5. Serum albumin behaves metabolically as a liver protein.

The Site of Breakdown of Plasma Albumin

Introduction

The problem of the breakdown of plasma albumin has been investigated experimentally with some success only in recent years.

The plasma proteins are distributed in the blood, extravascular spaces of lymph, and catabolism follows first order reaction kinetics (Gitlin, 1957). Using ^{35}S-cysteine and lysine-^{14}C, Goldsworthy and Volwiler (1958) concluded from calculations based on the half-lives of the plasma proteins that in catabolism, the plasma proteins were completely degraded to amino acids. In a similar type of investigation, Fleischer, Liebzo, Walter and Haurowitz (1959) concluded that conversion of plasma proteins into tissue proteins involved the breakdown of the plasma proteins into amino acids or very small peptides.

More sophisticated kinetic studies of ^{131}I-labelled plasma albumin metabolism led to the conclusion that albumin breakdown occurred at an intravascular site (Matthews, 1957; McFarlane, 1957). This, of course, was recognised as being unlikely and the careful investigations of the kinetics of albumin breakdown in the rabbit by Reeve and Roberts (1959) indicated that breakdown took place in a separate extravascular compartment (fig.6).

In an early in vitro study of the metabolism of ^{14}C-labelled plasma protein fractions, Roberts and Kelley (1956) claimed that albumin could be rapidly and preferentially used for the production of energy and for gluconeogenisis by liver slices. Later, in an extensive investigation into the "site of catabolism of serum albumin,"

Gitlin, Klinenberg and Hughes (1958) studied the effects of 40% hepatectomy, dyes which blocked the reticulo-endothelial system, starvation, nephrectomy and resection of the intestine. When the reticulo-endothelial system was blocked, the half-life of albumin was prolonged. A similar effect though of lesser degree was observed in nephrectomy, 40% hepatectomy and starvation. Resection of the intestine did not alter plasma albumin turnover in these experiments on mice and the authors concluded that plasma albumin breakdown occurred in the reticulo-endothelial system.

The effect of enterectomy reported by Gitlin et al. (1958) is of some interest as the intestine as a possible site for albumin catabolism has been extensively investigated. Evidence for the presence of albumin and other plasma proteins, in the bile, stomach and gut contents has been presented (Holman, Nickel and Sleisenger, 1959). Armstrong, Margen and Tarver (1960), and Tarver, Armstrong, Debro and Margen (1960) from studies using labelled albumin, concluded first that the site of degradation of serum albumin was the gut, and later that some plasma protein was normally broken down in the gut.

Patients with gastrointestinal and cardiac disease investigated by Jeejeebhoy (1962) using the ^{131}I-labelled albumin turnover technique had a low turnover of albumin despite loss of albumin into the gastrointestinal tract. In these studies, Amberlite ion-exchange resin was administered orally in order to combine with inorganic ^{131}I in the gut, and this when estimated as faecal ^{131}I, gave a measure of the amount of albumin catabolised in the gut. It has been

shown subsequently that inorganic ^{131}I liberated from albumin anywhere in the body can pass into the gut, combine with the Amberlite resin and lead to a falsely high estimate of the breakdown of albumin in the gut (Jones and Morgen, 1963). In later studies, the ^{131}I-albumin: oral Amberlite resin technique was modified by the additional determination of the leakage of inorganic iodide into the gut. This was achieved by injecting ^{125}I-iodide and measuring the rates of appearance of the two isotopes in the faeces. From these experiments and a short review of related evidence Freeman (1963) concluded that only a few percent of the total daily albumin catabolism was due to intestinal leakage and breakdown of albumin. This conclusion is supported by studies in partially enterectomised (Franks, Mosser and Anstadt, 1963) and enterectomised (Franks, Edwards, Lackey and Fitzgerald, 1963) rabbits. In each case removal of the gut had little or no effect on albumin turnover.

The possibility that the liver is a main site of plasma protein breakdown has been investigated using in vitro methods with discrepant results. For example, Katz, Sellers and Golden (1960) using ^{35}S-labelled albumin and liver slices, failed to demonstrate in vitro catabolism of albumin. This is in agreement with the results of Armstrong and Tarver (1960). However, Roberts and Kelley (1956) claimed that albumin was used as a source of energy by liver slices and Cohen and Gordon (1958) and Gordon (1961) using a perfused rat liver system showed that a small amount of albumin (about 14% of the total in vivo breakdown) could be catabolised by the liver.

Perhaps the most interesting observation is that of Penn (1961) who found that rat liver mitochondria could catabolise serum albumin at a rate which could exceed the total body albumin catabolic rate.

From this short review of the experimental evidence, it would appear:

1. that in catabolism albumin is degraded to amino acids before these are further utilised.
2. that some breakdown of albumin may occur in the gut. This, if it takes place, accounts for only a small proportion of the total albumin catabolism.
3. that the main site of albumin breakdown may be the reticulo-endothelial system, so that the principal organs involved are the liver and kidneys.
4. In vitro and other evidence suggests that the liver can catabolise albumin.

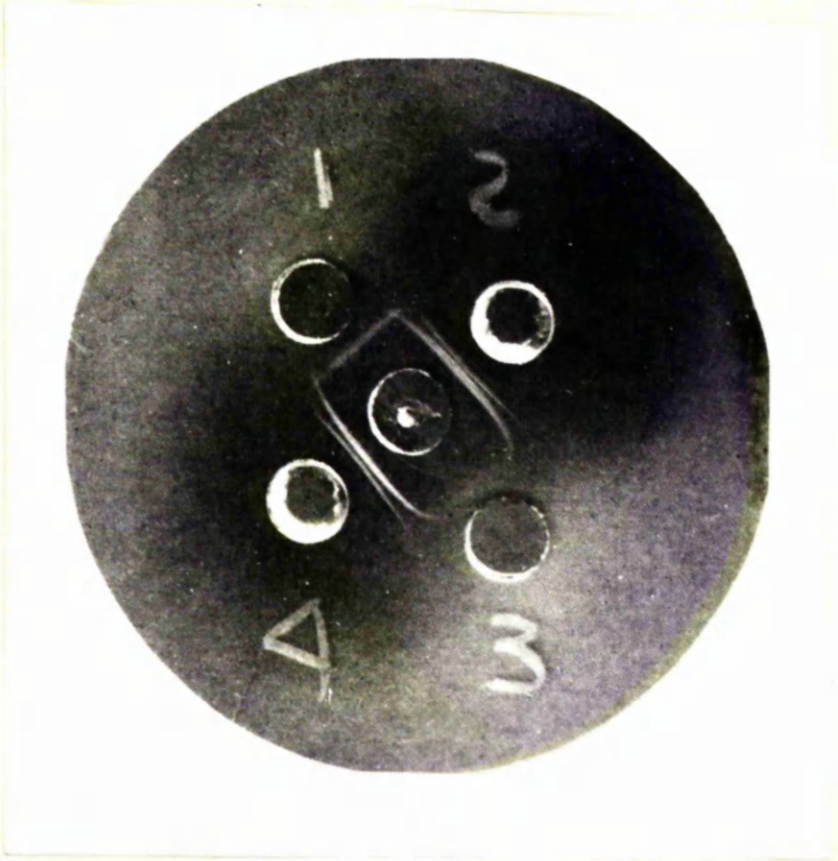
Experimental Section

During this investigation of albumin metabolism, the opportunity to investigate the proteins of bile was presented.

Methods

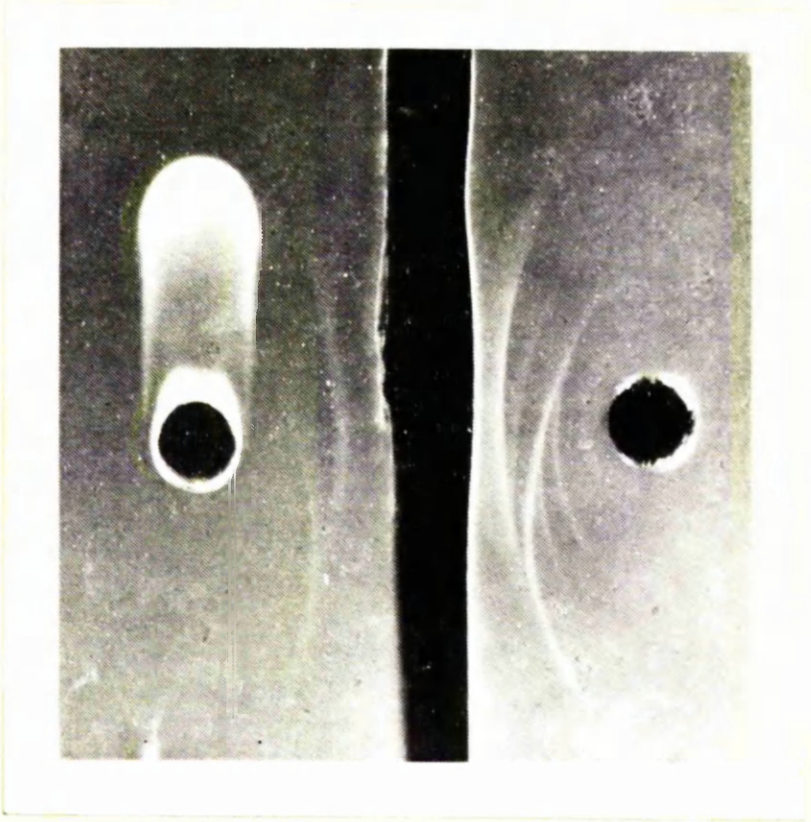
Bile was obtained during laparotomy from patients with a normal biliary tract and from patients with gallstones, by aspirating the gallbladder. The bile from normal gallbladders was clear and uninfected. Care was taken to avoid contamination with blood. Some specimens were freeze-dried to facilitate storage, others were concentrated by dialysis against "Aquax" (polyethylene glycol) at

Fig. 14. IDENTIFICATION OF THE PROTEINS OF BILE



Bile

Bile



Bile

Serum

4°C overnight.

The method of Lowry, Rosebrough, Farr and Randall (1951) was used for the direct estimation of protein. An attempt was made to estimate the protein-nitrogen content of bile by difference from the total nitrogen content (estimated by the micro-Kjeldahl method) minus the sum of the nitrogen of the bile salts, bile pigments and phospholipids. Bile pigments were estimated by the method of Malloy and Evelyn (1937); bile salts by the chromatographic method of Sjovall (1954); phospholipids by the method of Stewart and Hendry (1935).

Immunological methods: 1. Gel-diffusion

The technique of Ouchterlony (1949) was used with 1.5% Difco Bacto-Agar in 0.9 gm./100 ml. NaCl to which 0.1 gm./100 ml. sodium azide was added to prevent bacterial growth.

2. Immuno-electrophoresis

The method of Grabar and Williams (1955) was used. The supporting medium was 1.5% Difco Bacto-Agar and the buffer, 0.05 M borate pH 8.1.

For staining, the plates were fixed in 2% acetic acid for 3 hours, dried with filter paper and stained with Ponceau-S (Kohn, 1960), Sudan black or periodic acid-Schiff reaction (Culling, 1957).

3. Antisera

Rabbit antihuman serum and antibile serum was prepared by the method of Freund (1947).

Table 25

Identification of Bile Proteins by Ouchterlony Technique

Gall Bladder Bile

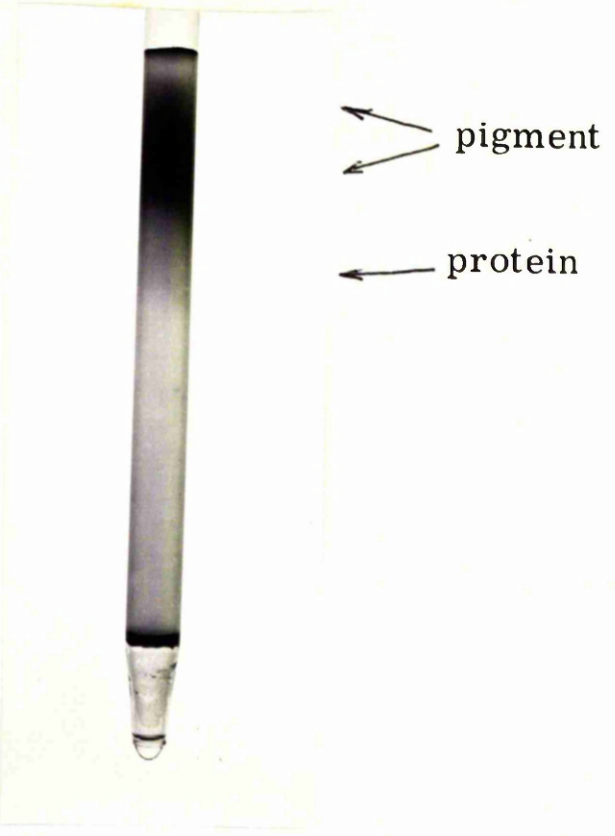
	No. of Specimens	Albumin	γ Globulin
Normal	20	20	12
Pathological	8	8	7
Total	28	28	19

Hepatic Duct Bile

Normal	8	8	3
Pathological	4	4	3
Total	12	12	6

From I.S. Russell and W. Dunnett, 1963.

Fig. 15. SEPARATION OF BILE PIGMENTS AND PROTEIN ON SEPHADEX



Results 1. Identification of the proteins of bile.

Typical results obtained with the gel-diffusion and immunoelectrophoretic techniques are shown in fig.14 and are summarised in table 23. The only proteins detectable in bile by these methods are serum albumin and γ globulin.

2. The estimation of the protein content of bile.

Since it is obvious that bile pigments interfere in the colorimetric determination of protein, attempts to remove the pigment were made by gel filtration on Sephadex G 25 medium using a barbital buffer pH 8.6, 0.06 M. The result is illustrated in fig.15. Although the greater part of the pigment is removed by this method sufficient pigment to interfere in colorimetric methods of protein determination remains adsorbed to the protein fraction. That the fastest running fraction corresponded to protein was confirmed by mixing a small amount of bile with a large amount of haemoglobin. On electrophoresis of the "protein fraction" on cellulose acetate by the method of Kohn (1960), a fast moving pigment band was obtained and on staining with nigrosin (Kohn, 1960) faint bands corresponding to albumin and globulin were occasionally obtained.

The Poncean-S staining method (Kohn, 1960) was insufficiently sensitive to detect protein, which indicated that the protein concentration of bile was probably less than 50 mgm./100 ml. bile.

The successful separation of protein and pigment by electrophoresis on cellulose acetate prompted its repetition on a small preparative column constructed from "Quickfit" apparatus. Polyvinyl acetate-polyvinyl chloride copolymer (obtained from I.C.I.)

Table 24

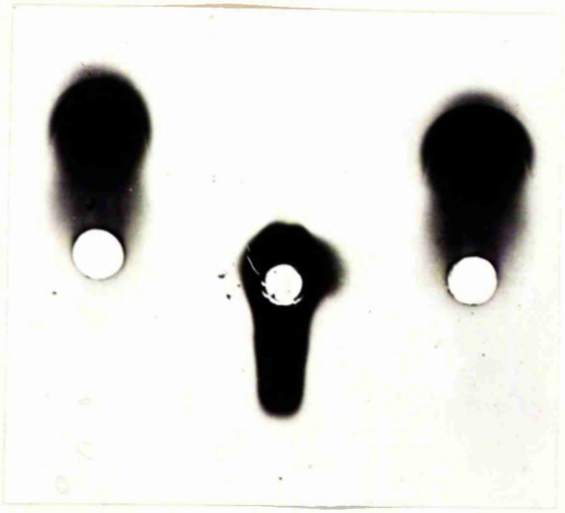
The Nitrogenous Constituents of Human Bile

Values are expressed as mg. N/100 ml. bile

	Specimen	Diagnosis	Phospho-lipid-N (a)	Bile salt-N (b)	Bili-rubin-N (c)	Non-protein-N (d = a + b + c)	Total nitrogen (e)	Protein-N (e-d)
Normal bile	1	Carcinoma stomach.	20	76	10	106	159	53
	2	Duodenal ulcer	53	258	13	324	368	44
	3	Duodenal ulcer	28	270	7	305	332	27
	4	Duodenal ulcer	56	232	23	311	303	(-8)
	5	Laparotomy	35	242	16	293	231	(-62)
					Mean	268	278	
Abnormal bile	6	Cholelithiasis.	10	35	6	51	115	64
	7		56	206	16	278	321	43
	8		31	113	13	157	238	81
	9		41	146	8	195	231	36
	10		27	196	14	237	209	(-28)
					Mean	183	223	

From Russell, Fleck and Burnett (1964).

Fig. 16. ELECTROPHORESIS OF BILE



Stained with
Ponceau S

was used as the supporting medium and the run was carried out in barbital buffer pH 8.6, 0.06 M. The separation of pigment and protein fractions was again achieved and the total protein eluted, estimated by the method of Lowry et al. (1951), was found to be approximately 30 mgm./100 ml. bile.

Finally, the albumin and globulin content of bile was estimated by an immunological method. For albumin, rabbit anti-human serum to which human γ-globulin had been added to neutralise the anti-globulin was used as the identifying antibody in the Ouchterlony gel-diffusion procedure. Quantitation was obtained by comparing the density and situation of the precipitate with that obtained with standardised serial dilutions of human plasma albumin. A similar technique was adopted for the estimation of globulin. The protein content of 19 normal and 9 pathological specimens of bile obtained in this way was : albumin 10 mg./100 ml, globulin 10 mg./100 ml.

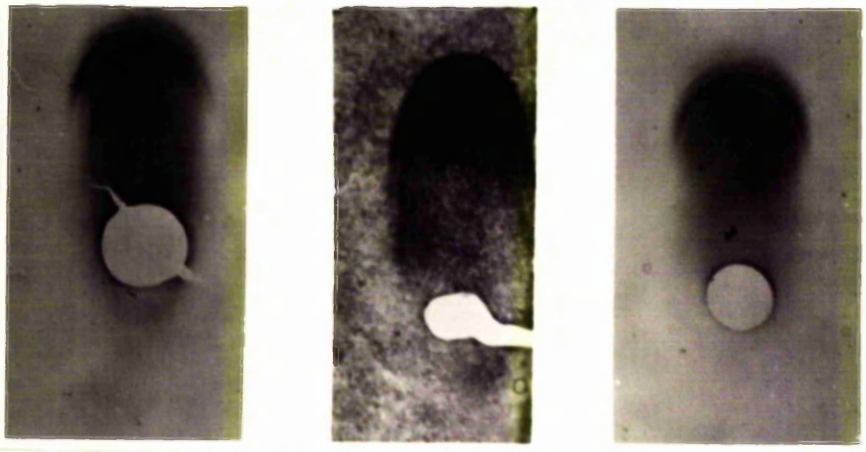
The protein-N content of bile, computed from the above figures is about 5 mg./100 ml. This is about 3.0% of the total nitrogen content obtained using the micro-Kjeldahl procedure on samples of whole bile (table 24). It follows that it is impossible to obtain an estimate of the protein-N content of bile by the difference method because of the errors involved.

3. Further investigations.

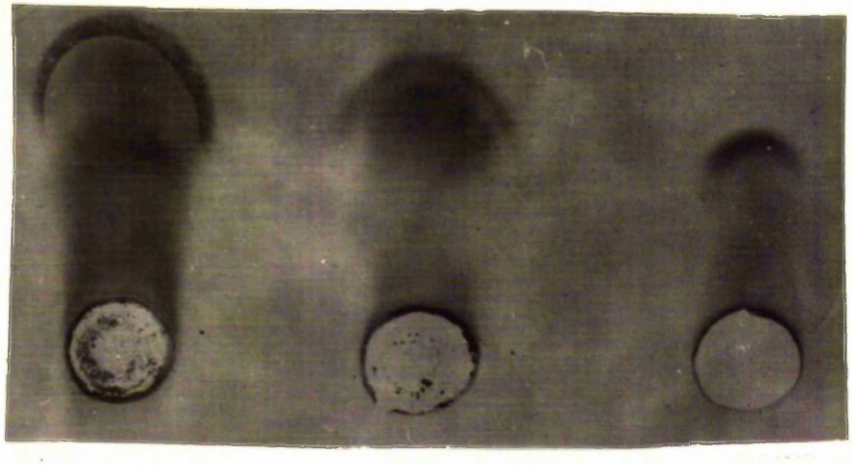
On agar gel electrophoresis of bile, the pigment bands run towards the anode whereas the proteins of serum are seen to run mainly towards the cathode (fig.16). Poncean-S and Sudan black stains some of the material which migrates most rapidly towards the

Fig. 17. ELECTROPHORESIS OF BILE AND BILE SALTS

Bile



Ponceau S | Sudan black | Unstained



Whole bile | bile salt mixture | pigment fraction from Sephadex column

Stained with Ponceau S

anode (fig.17). That this material is not necessarily protein is demonstrated by repeating the staining procedure and comparing the results obtained with whole bile, the pigment fraction from a Sephadex column, and a mixture of commercial sodium glycocholate and sodium taurocholate (fig.17b). The mixture of the bile salts behaves in a similar fashion to the bile pigment fraction and whole bile.

In summary, it can be concluded that:

1. Bile contains plasma albumin and globulin in small quantities.
2. The total protein content of human gall bladder bile is approximately 20-30 mg./100 ml.

Discussion

The relevance of the preceding investigation to the study of albumin breakdown is indicated by the following calculation -

Protein content of bile - 30 mg./100 ml

Output of bile per day - 500 ml (Black, 1957)

Hence, total plasma protein in bile per day = 150 mg.

In a 70 Kg. (standard) man there is 3.5 l plasma (Gamble, 1949; Edelman and Leibman, 1959). If the plasma albumin concentration is taken as 4.0 gm./100 ml. this gives a figure for the total circulating albumin - 140 gm. According to McFarlane (1957) the total body albumin (extravascular + intravascular) is approximately 1.5 times the intravascular albumin. Thus the total body albumin of a 70 Kg. man is approximately 200 gm. The catabolic rate of albumin is 0.046 per day (Anker, 1960). Therefore, the total daily breakdown of albumin in a 70 Kg. man is approximately 9.2 gm.

The total protein excreted in the bile is less than 2% of this. Hence, the breakdown of albumin due to its secretion in the bile makes a negligible contribution to the total daily albumin catabolism.

There remain two possibilities: first, that the albumin degraded in the gut per day is negligible in relation to the total daily albumin breakdown and second, that such albumin as is degraded in the gut is contributed by other sources than bile.

PART 2

STUDIES IN PROTEIN SYNTHESIS

INTRODUCTION

The two fundamental problems of protein synthesis are:-

1. the sequence of reactions in protein synthesis
2. the control of protein synthesis.

1. The sequence of reactions in protein synthesis

A vital role in protein synthesis is played by the ribosomal particle. This almost spherical particle consists of 40-50% RNA, and about 50% protein; the molecular weight is about 4×10^6, and diameter about 150 A^o. Usually, the particle occurs with sedimentation constant of 70-80 s, although 100s particles occur and in low magnesium concentration dissociation to 30 and 50 s particles takes place (see fig.20; Palade, 1958; Peterman and Hamilton, 1957; Gillchriest and Bock, 1958; Tissieres and Watson, 1958; Chantrenne, 1961).

The sequence of reactions in protein synthesis which has been elucidated in the last decade is summarised in fig.18 (see reviews by Hoagland, 1960; Korner, 1964). In the activation of the amino acid an amino-acid-adenylate-activating enzyme complex is formed (fig.19), in which the amino group is free and the carboxyl group is attached to the C_3 of the ribose (Hoagland, Keller and Zamecnik, 1956). The "activated" amino acid is then transferred to sRNA, or as it is occasionally known "transfer" RNA (Hoagland, Stephenson, Scott, Hecht and Zamecnik, 1958). It is now possible to prepare partially purified activating enzymes, and these enzyme studies, together with those on the fractionation of sRNA indicate that each amino acid has its own activating enzyme and specific sRNA (see Korner, 1963).

Fig. 18. SEQUENCE OF REACTIONS IN PROTEIN SYNTHESIS

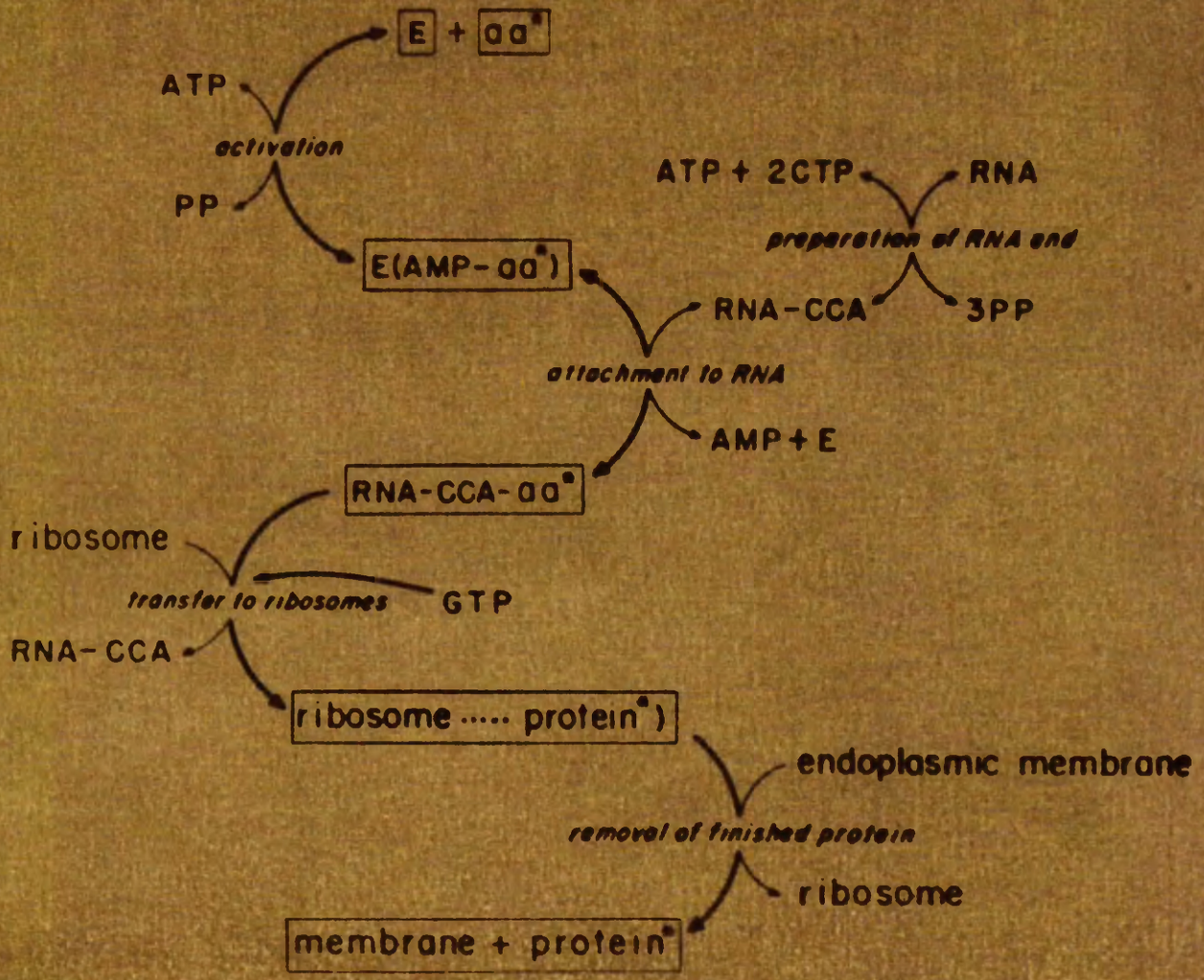
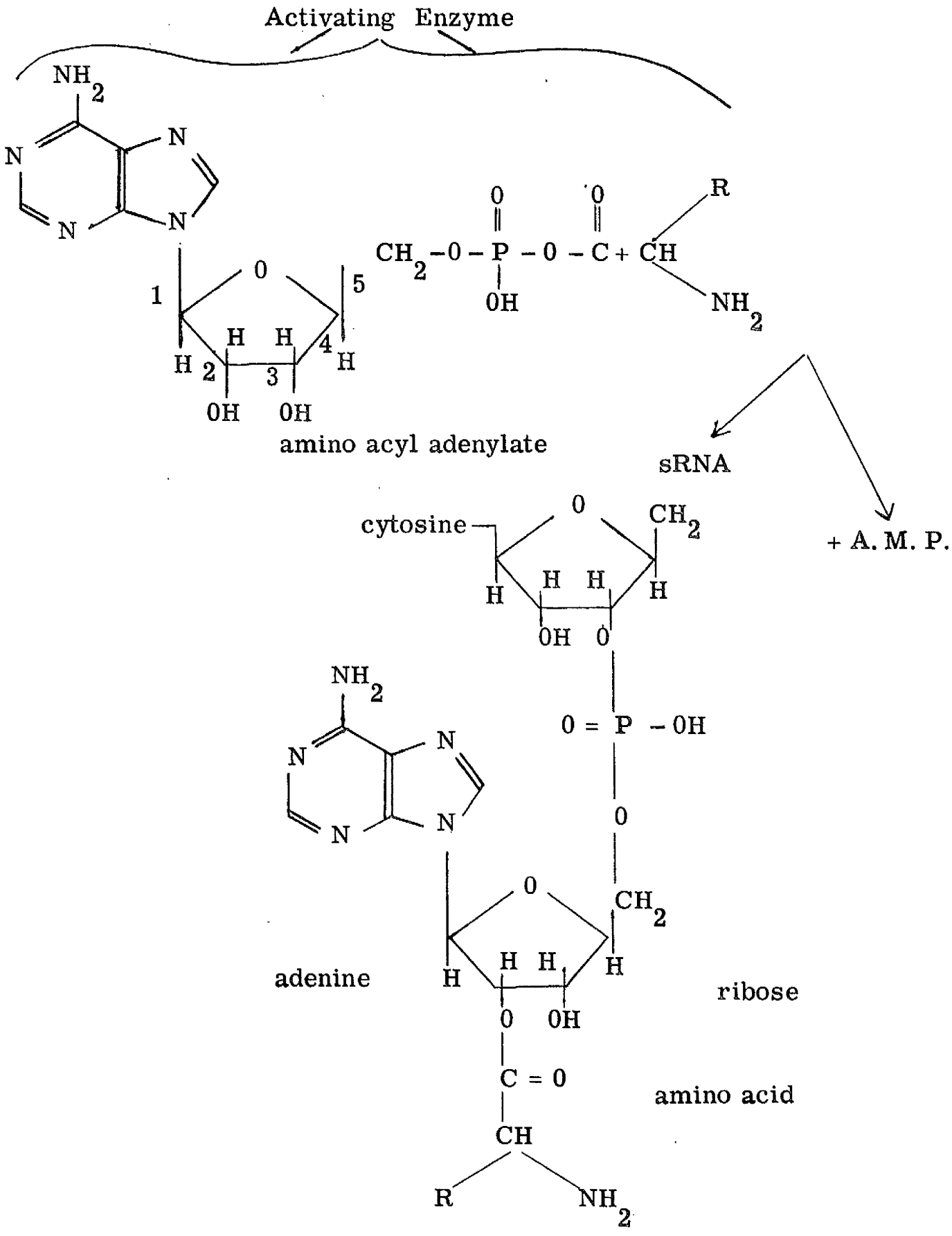


Fig. 19. TRANSFER OF AMINO ACID TO sRNA



Transfer of the s-RNA-bound amino acids to ribosomes appears to require a 'transfer enzyme' and GTP. Some progress has also been made in the isolation of this "transfer" enzyme which catalyses the transfer of the amino acid from sRNA to the ribosome. It appears that only one enzyme is required for the transfer of all amino-acyl sRNA's to the ribosome (Von der Decken and Hultin, 1960; Nathans, Ehrenstein, Monro, Lipmann, 1962). In this system, sRNA has a catalytic cofactor function (Bosch, Huizinga and Bloemendal, 1962; Nathans et al., 1962).

For the attachment of sRNA to and removal from the ribosome, the formation of the amino-acyl sRNA complex is not essential (Takanami, 1962). The role of GTP in the transfer reaction is unknown (Nathans et al., 1962). There is evidence to suggest that the protein chain is built up from the N-terminal end (Goldstein and Brown, 1961).

The final step is the release of the protein from the ribosome to the cell sap; this requires energy (ATP), and a heat-labile, non-dialysable factor (Hultin, Leon and Gerasi, 1961).

The stages in protein synthesis discussed above are common to both bacterial and mammalian systems. However, in mammals the situation is complicated by the occurrence of a cytoplasmic structure known as the endoplasmic reticulum.

Endoplasmic Reticulum

This cytoplasmic structure, alternatively known as ergastoplasm, has been defined by Porter (1954) as "a complete reticulum of strands and vesicles.....limited by a membrane similar in thickness

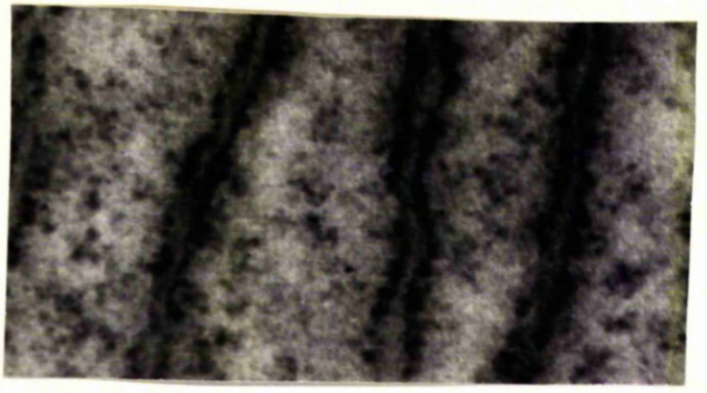
(ca. 80 A^{o}) to the plasma membrane of the cell."

Although there may be considerable variation in the appearance of the endoplasmic reticulum in various cells and even in the same cell type in different physiological conditions (Bernhard and Rouiller, 1956), it is possible to identify two types of endoplasmic reticulum in most cells. The granular, or rough-surfaced reticulum (fig.20) consists of the membrane studded with electron-dense particles of about 150 A^{o} diameter - ribosomes (Palade, 1956). The rough-surfaced membrane has been referred to by Sjostrand (1956) as the α-cytomembrane in order to distinguish it from the smooth-surfaced reticulum, or γ-cytomembrane, which occurs in the region of the Golgi apparatus (Dalton and Felix, 1956).

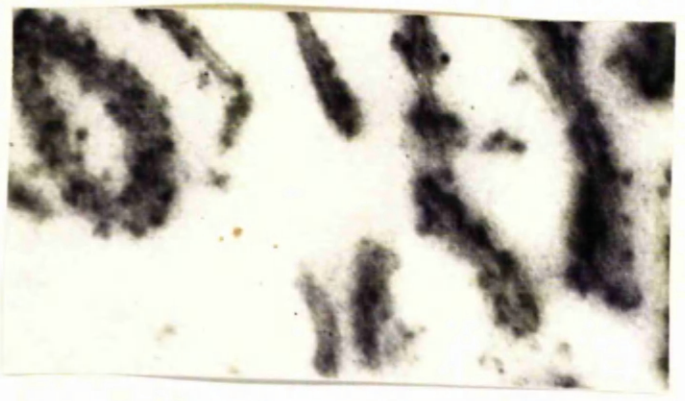
There exists close correlation among cytoplasmic basophilia, endoplasmic reticulum, ribosomes and microsomes (Porter, 1953; Palade and Siekevitz, 1956; Chantrenne, 1961) and it appears that microsomes are formed from the endoplasmic reticulum which during homogenisation breaks up or "pinches off" to form the vesicle of the microsome (Palade, 1958).

The discovery of microsomes by Claude (1940) preceded that of the endoplasmic reticulum; the microsomes were isolated from a tissue brei by differential centrifugation, and it was calculated that they consisted of particles of 50-200 mμ diameter (Palade, 1958). Microsomes are now commonly obtained by first homogenising a tissue gently in a buffered sucrose (0.15-0.44 M) medium, then centrifuging at 12-15000g for 5-10 minutes, and finally, centrifuging at 105,000g

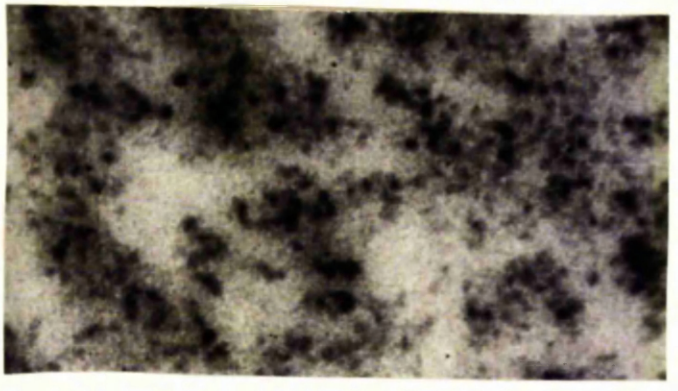
Fig. 20. ELECTRON MICROGRAPHS OF LIVER CELL AND CELL FRACTIONS



Endoplasmic reticulum



Microsome fraction



Ribosomes

(From, Palade and Siekewitz, 1956)

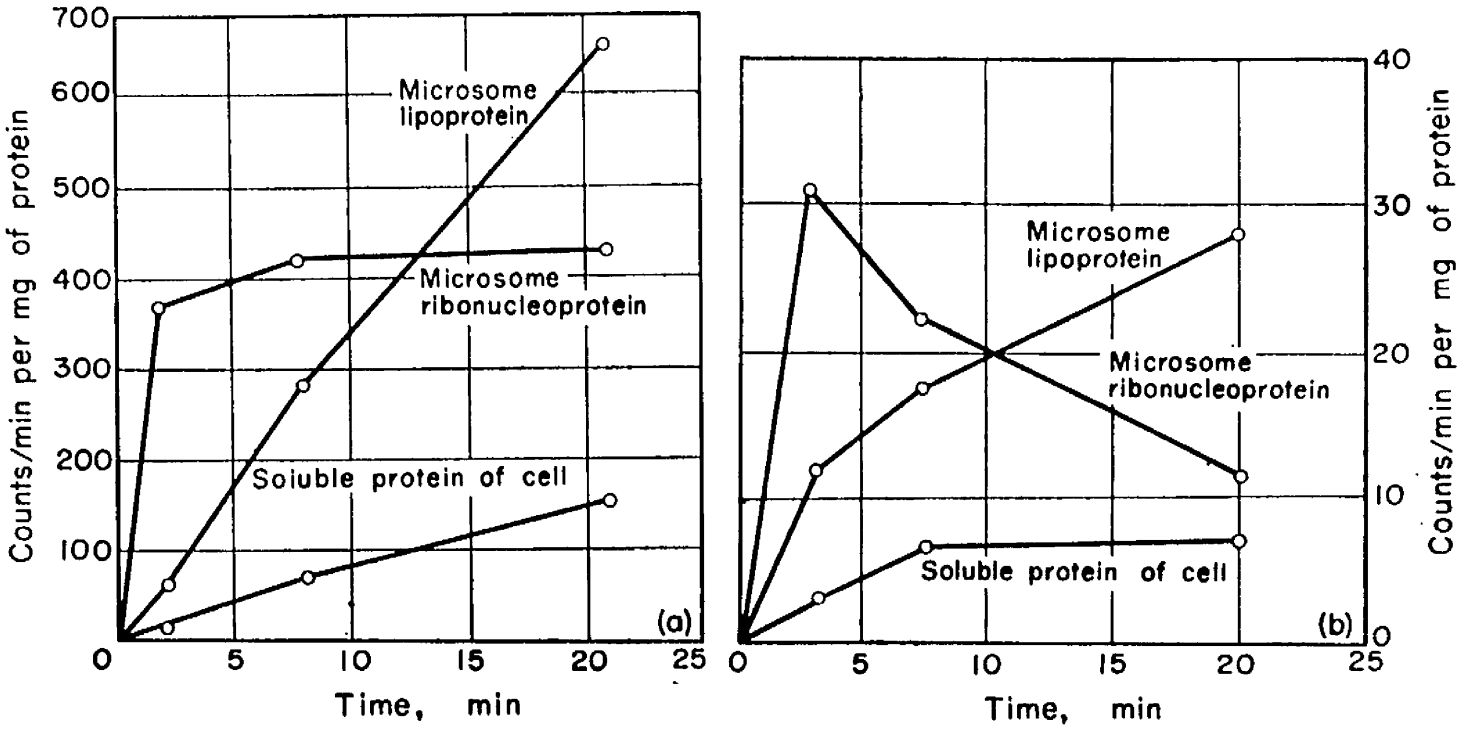
for 1 hour to obtain a pellet of microsomes (see fig.20). The microsome pellet so obtained is contaminated with small quantities of small lysosomes, free ribosomes, glycogen and ferretin (Novikoff, 1957; Duve, 1957; Chauveau, Moulé, Rouiller and Schneebeli, 1962). Chemical analysis of microsomes indicates that they consist of approximately 70% protein, 20% phospholipid and 10% RNA (Palade, 1958). The greater part of the RNA of microsomes is found in the attached ribosomes (Littlefield, Keller, Gross and Zamecnik, 1955). About 50% of the RNA, 50% of the phospholipid and 15% of the protein of the cell is found in the microsomal fraction (Palade, 1958). Microsomes also contain a large spectrum of enzymes (Duve, 1957; Dixon and Webb, 1958; Ernster, Siekevitz and Palade, 1962).

The complete function of the microsomes or endoplasmic reticulum in protein synthesis is not clear, but the following observations are of interest:-

1. Endoplasmic reticulum seems to be most well-developed in cells which produce specialised protein which is usually "for export" (Porter, 1953; Palade and Porter, 1954; Palade, 1958; Birbeck and Mercer, 1961), for example, the reticulum is abundant and well-defined in the cells of the salivary gland (Palade and Porter, 1954), pancreas (Palade, 1958) and liver (Palade and Siekevitz, 1956).

2. Liver cells from an animal maintained on a protein deficient diet show less reticulum than those from an animal maintained on adequate protein (Bernhard and Rouiller, 1956). Cells of the pancreas show similar changes (Weisblum, Herman and Fitzgerald, 1962).

Fig. 21. ^{14}C - LEUCINE UPTAKE BY MICROSOMES



In vivo incorporation of ^{14}C leucine into microsomes of rat liver fractionated with sodium deoxycholate.
(a) Saturating dose of ^{14}C leucine.
(b) Tracer dose of ^{14}C leucine.
(Littlefield *et al.*, 1955).

3. When a ^{14}C-labelled amino acid was administered to a rat (by intravenous injection), the protein of the microsomal ribosomes was the first to become labelled, followed by that associated with the microsomal membrane; finally, the "label" appeared in the cell sap proteins (Littlefield et al., 1955), (see fig.21).

4. Ribosomes which are capable of incorporating amino acids into protein can be obtained from microsomes by treatment with sodium deoxycholate (Kirsch, Siekewitz and Palade, 1960).

5. Following an attempt to obtain the smallest cytoplasmic particle which could incorporate amino acid into a complete protein molecule (in this case, rat serum albumin), Von der Decken and Campbell (1962) concluded that microsomes were the smallest particles capable of forming the complete albumin molecule and that ribosomes could not do so.

2. <u>The control of the rate and type of protein synthesised</u>

Currently, it is believed that the control of protein synthesis is genetic, and mediated by messenger RNA. Crick (1958) proposed that the genetic properties of cells were related to DNA that the genetic information carried by DNA was determined by its base sequence and was transferred to protein molecules by RNA, and that this process was irreversible. Later, experimental evidence was produced to show that the DNA "code" was linear and was based on nucleotide triplets (Crick, Barnett, Brenner and Watts-Tobin, 1961). The existence of a "messenger RNA" (mRNA) which acts as an intermediate between the DNA of the cell and the site of protein synthesis - the ribosome - is also supported by experimental evidence (Brenner, Jacob

and Meselson, 1961). Further work (on bacterial systems) supported the concept that mRNA is synthesised on DNA as primer (Hayashi and Spiegelman, 1961), that it subsequently complexed with a ribosome to produce an "active" or "heavy" ribosome (i.e. 10-15s heavier than the normal 70s bacterial ribosome), (Ishihama, Mizuno, Takai, Otaka and Osawa, 1962), and that the "heavy" ribosomes are the ones which synthesise protein (Risebrough, Tissieres and Watson, 1962).

The characteristics of mRNA are:-

1. It is rapidly labelled in "pulse" labelling experiments, (Gros, Hiatt, Gilbert, Kurland, Risebrough and Watson, 1961).
2. It is highly labile (Brenner et al., 1961).
3. It is heterogeneous (Monier, Naono, Hayes, Hayes and Gros, 1962).
4. It associates with 70-100s ribosomes (Ishihama et al., 1962; Risebrough et al., 1962).
5. It stimulates protein synthesis when attached to ribosomes - i.e. it acts as a template for protein synthesis (Risebrough et al. 1962).
6. It sediments with s-values in the range 8-30s (Ishihama et al., 1962; Monier et al., 1962).
7. The base ratios are assumed to be complementary to single strand (denatured) DNA or the same as native DNA (Jacob and Monod, 1961; Chamberlin and Berg, 1962; Hayashi and Spiegelman, 1961).

At present there seems to be two experimental approaches in the study of the control mechanism. One is the study of the synthesis, turnover, base ratios, and other related properties of mRNA. The other is the relation of mRNA to ribosomes; that is the study

of "activation" or attachment of mRNA to ribosomes.

An example of the first approach was the demonstration of a rapidly labelled RNA with complementary base ratios, the synthesis of which was DNA-primed (Gros, Hiatt, Gilbert, Kurland, Risebrough and Watson, 1961; Tissieres and Hopkins, 1961; Hayashi and Spiegelman, 1961).

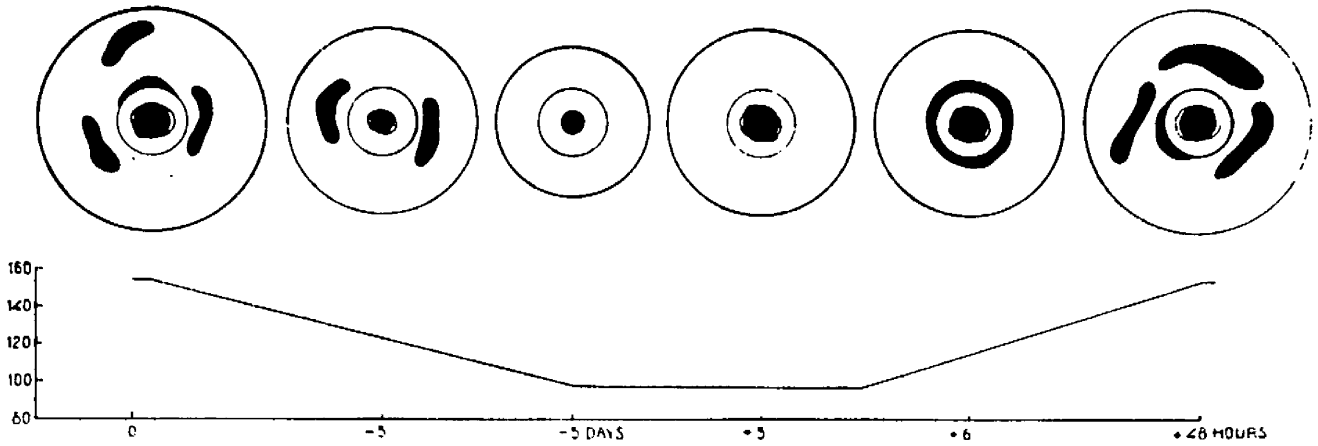
Evidence for the attachment of mRNA to ribosomes was presented by Risebrough, Tissieres and Watson (1962). They demonstrated that ribosomes to which messenger RNA had been attached sedimented slightly faster than normal ribosomes. Some fractionation of the RNA which can complex with ribosomes has been achieved by Ishihama, Mizuno, Takai, Otaka and Osawa (1962), using methylated albumin column chromatography. The experimental elucidation of the problem of which nucleotides code for a specific amino acid which was begun by Nirenberg and Matthaei (1961) and pursued by Speyer, Lengyel, Basilio and Ochoa (1962) is yet another example of the second type of approach mentioned above, and, incidentally, suggests that mRNA is the template for protein synthesis.

There is some recent evidence that the particle on which protein is synthesised is much larger than the "normal" 70s ribosome. The existence of ribonucleoprotein particles larger than 70s has been known for some time (Palade, 1958; Peterman and Hamilton, 1961), but recently it has been shown that the particles on which haemoglobin is synthesised are pentamers of the "normal" 70s ribosome, and that the chain of ribosomes is linked by a structure of 10-15 A° diameter

(Warner, Rich and Hall, 1962). In addition, Wettstein, Staehelin and Noll (1963) have shown that in rat liver ribosome preparations protein is not made by structures which are smaller than pentamers of the "normal" 70s ribosomes (as judged by ^{14}C-labelled amino acid incorporation). Wettstein et al. (1963) also obtained stimulation of incorporation of one (labelled) amino acid by the addition of a mixture of the remaining 19 amino acids. Both groups (Warner et al., 1962; Wettstein et al., 1963) suggest that the chain of ribosomes is held together by messenger RNA; if this is so, and the dimensions of the link as measured by electron microscopy (Warner et al., 1962) are correct, then the messenger RNA in this system must be single-stranded.

In an attempt to study the stability of mRNA in a protein-synthesising system using the synthetic "messenger" polyuridylic acid, Spyrides and Lipman (1962) observed that when almost all the polyuridylic acid was bound to the ribosomes they no longer sedimented at 70s, but at 150-200s; an observation which fits well with those of Warner et al. (1962) and Wettstein et al. (1963). The recent evidence therefore suggests that both in bacterial and mammalian systems, protein synthesis occurs not on the single 70s ribosome, but on a chain of ribosomes linked by mRNA. Further evidence in support of this hypothesis is accumulating (Warner, Knopf and Rich, 1963 and Gierer, 1963) and it has been suggested that the ribosomes move along messenger RNA and in this process peptide bonds are formed between amino acids (attached to sRNA) and the 'growing' polypeptide remains attached to a ribosome (Staehelin, Wettstein, Oura and Noll, 1964).

Fig. 22. CHANGES IN THE NUCLEOLUS WITH DIET



Graph illustrating the principal changes within the liver cells during starvation followed by feeding the high protein diet. Dark areas indicate ribonucleic acid-containing structures. The curve is a schematic record of the principal changes in the total nitrogen content of the liver. The ordinate scale gives the total nitrogen values in mg. N/100 g. initial body weight. Abscissa: time scale.

From Lagerstedt, 1949.

In the bacterial cell it appears that mRNA mediates directly between DNA and the ribosomes, as a bacterial ribosome protein synthesising system was stimulated by the addition of DNA which was shown to have "primed" the production of mRNA (Tissieres and Hopkins, 1961; Wood and Berg, 1962).

The situation in mammalian cells, however, appears to be more complicated. All the evidence to date suggests that mammalian mRNA when characterised as a rapidly labelled, labile RNA of base ratios similar to that of DNA, is only to be found in the nucleus and probably in association with the nucleolus (Sirlin, Kato and Jones, 1961; Sibatani, de Kloet, Allfrey and Mirsky, 1962; Hiatt, 1962). The latter observation is of interest when it is recalled that the nucleolus varies in size according to the supply of proteins or amino acids (Lagerstadt, 1949; Stenram, 1958), (fig.22). Although no very rapidly labelled RNA has been found in association with mammalian ribosomes, there is evidence for the existence of a messenger RNA attaching to mammalian cytoplasmic ribosomes. Arnstein, Cox and Hunt, (1962) have shown that polyuridylic acid influences the uptake of ^{14}C-labelled phenylalanine by reticulocyte ribosomes in the same way as bacterial ribosomes. More recently, Hoagland and Askonas (1963) have obtained a preparation of cytoplasmic post-microsomal RNA which stimulates the uptake of radioactive leucine by rat liver microsomes.

In summary, there is good evidence for the existence of a nuclear mRNA in the mammalian system. This mRNA can stimulate the uptake of labelled amino acid by E. coli ribosomes (Barondes,

Dingman and Sporn, 1962), although the significance of this observation for the intact mammalian system is not clear. That the mRNA of mammalian cytoplasmic ribosomes is not the same as the mRNA present in the nucleus is suggested by labelling experiments, so that there would appear to be two distinct types of mRNA in the mammalian cell. The tentative evidence is that the nuclear mRNA is somehow related to the nucleolus, and it is interesting to speculate whether the nuclear mRNA here influences the formation of the cytoplasmic mRNA or the production of cytoplasmic ribosomes or endoplasmic reticulum. In this context the isolated observation of Goswami, Barr and Munro (1962) that there exists in rat liver microsomes a RNA of similar base composition to nuclear RNA and of more rapid turnover than ribosomal RNA is of interest: was this RNA a "messenger" attached to membrane or to polysomes; or is it that membrane is "turned-over" more rapidly than ribosomes?

Finally, there remains the problem of the control of the production of the protein synthesising "machinery," that is the ribosomes or microsomes. The evidence obtained from the various types of in vitro experiments, as summarised above, suggests that mRNA activates the protein synthesising mechanism of ribosomes. Thus were the cell ribosome content to remain constant, the production of mRNA in response to, for example, amino acids, would result in activation of the ribosomes and a transient increase in the rate of protein synthesis. However, there is evidence that the amount of ribosomes (in bacterial cells) and endoplasmic reticulum (in mammalian cells) varies with the quantity of available amino acids

(Kjeldgaard, Maaloe, and Schaechter, 1958; Bernhard and Rouiller, 1956; Kennell and Magasanic, 1962). Bacterial cells previously grown on a medium containing only a minimal supply of amino acids rapidly synthesise RNA when tran amino acid-enriched medium, and conversely the breakdown of the RNA of bacterial cells previously cultured in an amino acid-enriched medium increases rapidly and to a considerable extent when the cells are transferred to a medium deficient in amino acids (Kjeldgaard et al. 1958). Hayashi and Spiegelman (1961) utilised this observation when they obtained a preparation of mRNA from E. coli cells which had been cultured on an amino acid-rich medium and transferred shortly before harvesting to a medium containing only slightly more than the minimal amino acid requirements of the cells: although protein synthesis continued in the poorer medium, it did so at a reduced rate and there was considerable breakdown of ribosomes with consequent enrichment of the cell RNA with messenger RNA.

Stent and Brenner (1961) suggested that amino acids act as inducers for ribosomal RNA synthesis in E. coli, and that in the absence of amino acids repressors inhibit the synthesis of ribosomal RNA. It would seem possible that a similar mechanism could operate in mammalian cells. It is also possible that in addition to this mechanism immediate control is exerted over the template for protein synthesis by a similar type of mechanism, in the analagous fashion to that by which the induction or repression of enzymes is controlled (see Jacob and Monod, 1961).

Section 1

Factors affecting the composition of the microsome fraction of rat liver

Introduction

In an early investigation of the influence of dietary protein on the composition of the liver cell, Kosterlitz (1947) found that the cytoplasm was more labile than the nucleus. This analytical approach was extended by Muntwyler, Seifter and Harkness (1950) when they obtained microsomes and supernatant fractions from rat liver and demonstrated that deprivation of protein for 3 weeks led to the loss of 34% nitrogen from the microsome fraction and 14% from the cytoplasmic fraction. Shortly thereafter, Keller (1951), investigating the turnover of protein in various cell fractions, found that the greatest turnover of protein occurred in the microsome fraction. Later studies by Mirsky, Allfrey and Daly (1954) confirmed the above-mentioned findings. Other investigations showed that RNA synthesis in rat liver (measured by rate of uptake of ^{32}P) was stimulated by protein feeding or by increasing the supply of energy from dietary carbohydrate or fat (Munro, Naismith and Wikramanayake, 1953). That the problem of the influence of diet on protein synthesis may be complex was illustrated by an investigation of the effects of diet on the changes in enzyme activity in rat liver by Niemeyer, Pérez, Garcés and Vergara (1962) following which they concluded that responses to diet were due to selective modifications in the pattern of protein synthesis.

The liver is not the only organ which may be influenced by the level of dietary protein. For example, Weisblum, Herman and Fitzgerald (1962) showed that following protein deprivation, the exocrine cells of the rat pancreas become depleted of zymogen granules, reticulum and ribosomes.

These effects described above on the protein synthesising mechanism are not confined to mammalian cells. This is apparent from the work of Pardee and Prestidge (1956) with E. coli, in which they showed that in amino acid deficient organisms, RNA synthesis was inhibited and that when amino acids were supplied, RNA and protein synthesis were stimulated. Similar results were obtained by Yčas and Brawerman (1957) with yeast, and more recently, Imahori and Kudo (1963) have shown that ribosomes prepared from proliferating yeast cells had a greater uptake of ^{14}C-leucine in vitro than ribosomes from starved cells. There was additional evidence suggestive of degradation of RNA in amino acid deficient cells (Imahori and Kudo, 1963).

The rate and type of protein synthesis of growing or developing cells appears to differ from that in the mature cell. Thus, Burraston and Pollak (1961) found that the incorporation of amino acids into the nuclear and cytoplasmic proteins of embryo rat liver was greater than in adult rat liver. In this respect, regenerating rat liver appears to be similar to embryo liver, although when the comparison of 'in vitro' uptake of labelled amino acids by the microsome fractions from regenerating and adult liver was expressed in relation to microsomal RNA, the difference was not more than 10%

(McCorquodale, Veach and Mueller, 1961). When the rates of synthesis of albumin and tissue proteins by regenerating liver was investigated it was concluded that cellular proteins are the first to be synthesised during regeneration (Braun, March and Drabkin, 1962). A further interesting observation is that of Plüchthun and Schreier (1960) that the half-life of albumin and γ-globulin in young rabbits is 50% shorter than in adult animals.

The influence of hormones on microsomes and protein synthesis has also been studied. Campbell and Kosterlitz (1949) showed that the liver cytoplasmic fraction of the mature male rat responded more markedly to changes in the protein content of the diet than did that of the mature female rat. Both growth hormone and cortisol administered in vivo to rats have a stimulating effect on the in vitro uptake of labelled amino acids by the microsome fraction from liver (Korner 1959, 1960; Wagle, 1963). Testosterone has a similar effect on the in vitro activity of prostatic ribosomes (Liao and Williams-Ashman, 1962).

From this brief survey and the preceding introduction, it will be apparent that several factors, including the protein content of the diet, may influence the chemical composition of microsomes. In this section, some preliminary investigations of the microsome fraction from rat liver are described, as well as the effects of diet on the chemical composition of the microsome fraction.

Fig. 23 PREPARATION OF THE MICROSOME FRACTION FROM RAT LIVER

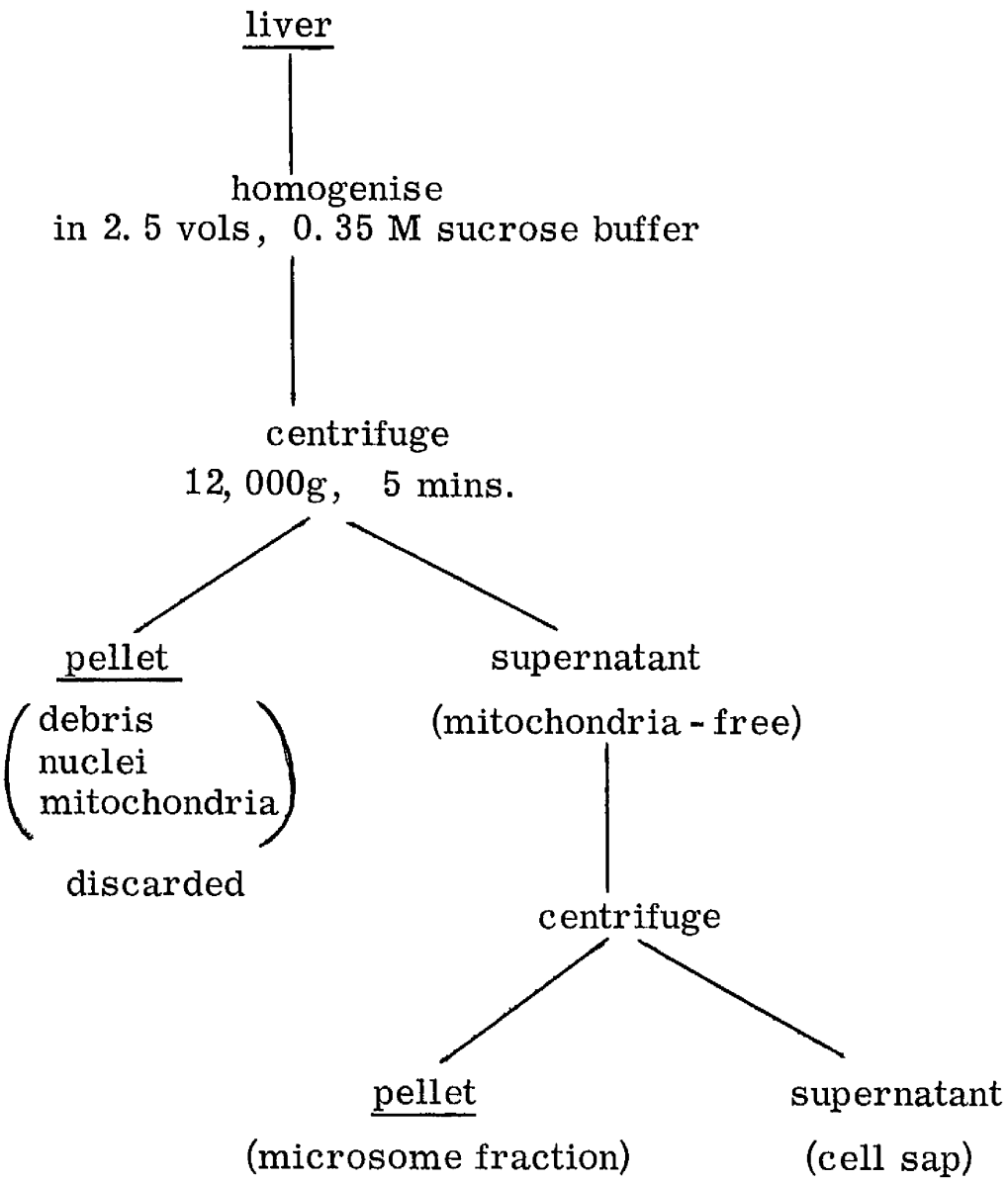


Table 25

Composition of Sucrose-Phosphate Buffer used in the Preparation of Microsomes

Sucrose	0.35 M
Potassium phosphate buffer pH 7.8	0.02 M
$KHCO_3$	0.03 M
KCl	0.025 M
$MgCl_2$	0.01 M

From Campbell, Greengard and Kernot (1960).

Experimental Methods and Results

Methods

Animals and diets . The animals and diets have been described in Part 1, page 30. In each experiment the diet was constant and protein-fed and protein-deprived animals had the same energy intake (1500 cals per square metre) for 5 days prior to sacrifice.

Preparation of microsomes. The preparation of microsomes follows in essentials the procedure of Campbell, Greengard and Kernot (1960) - see fig.23. The animals were killed by a blow on the head, the abdomen opened, hepatic vein severed, and the liver rapidly removed and immersed in ice-cold sucrose-phosphate buffer. (The composition of the buffered-sucrose is given in table 25). After blotting the liver dry on filter paper it was weighed and homogenised in 2.5 volumes of sucrose-buffer in a Potter-Elvejhem homogeniser. The homogenate was centrifuged at 12,000g for 5 minutes to remove cell debris, nuclei and mitochondria and the supernatant then centrifuged at 105,000g for 50 minutes to sediment the microsome fraction.

At each stage samples were taken for analysis, the pellets being resuspended by gentle homogenisation in sucrose-phosphate buffer before sampling. From the weight of liver, the total volume of the homogenate and the volume of the mitochondria-free supernatant used in the preparation of microsomes, it was possible to calculate that the yield of microsomes in each 5/8" x 3" Spinco tube was obtained from approximately 4.1-4.2 gm liver.

The exact figures in 3 successive experiments were within this range and differed by not more than 2%.

Table 26

Sampling Procedure for Analysis of Tissue Fractions

Tissue fraction	Volume of sample (ml) for analysis of:-			
	RNA	Protein	Total-N	Phospholipid-P
Whole homogenate	1	0.5	0.5	0.5
Mitochondria-free supernatant	1	0.5	0.5	0.5
Cell sap	2	1	1	3
Microsomes (from approximately 4.1 gm liver resuspended in 11 ml sucrose-phosphate buffer)	1	0.5	0.5	0.5

Table 27

Analysis of Liver Fractions

	RNA	Protein	Total-N	Phospholipid-P
Whole homogenate	3.94	93.1	30.2	1.20
Mitochondria-free supernatant	2.70	20.5	10.8	0.37
Microsome fraction *	2.4	-.	3.4	0.37
	2.5	24.3	3.5	0.35
	2.23	17.6	3.3	0.32
	2.09	-.	2.9	0.33

The figures are expressed as mg component per gm. wet weight liver.

* These figures were obtained in 4 separate experiments.

Analytical methods. These are discussed in Part 3 and detailed methods are presented in the appendix. Separate samples were taken for each analysis (see table 26).

RNA was estimated after acid precipitation and alkaline incubation from the extinction of the nucleotides at 260 mμ at pH 1-2 (Fleck and Munro, 1963a).

For protein estimation, the sample was suitably diluted usually to 100-200 ml in faintly alkaline solution and a 1 ml sample taken for determination of protein by the method of Lowry, Rosebrough, Farr and Randall (1951). Another sample of the same diluted material was taken for the determination of nitrogen by the modified Nessler procedure of Paul (1958). Alternatively, nitrogen was estimated by the micro-Kjeldahl procedure after precipitation of the sample with ice-cold 10% TCA, and washing twice with 5% TCA to remove sucrose, and dissolving the precipitate in dilute alkali for sampling.

Phospholipid was extracted by the chloroform-methanol procedure of Folch, Lees and Sloane-Stanley (1957), following which the phospholipid-phosphorus was determined by the method of Allan (1940).

In addition to the chemical analyses, the mitochondria-free supernatant and the resuspended microsome fractions were examined in the analytical ultracentrifuge (Spinco model E).

Results

1. Preliminary Investigations. The RNA, protein, total nitrogen and phospholipid-phosphorus content of the various cell fractions are given in table 27. The composition of the microsome fraction

Table 20

Relative Amounts of the Components Present in Microsomes

(a) Percentage composition of microsomes

	RNA	Protein	Phospholipid
Present experiments	8	66	22
Data of Palade (1958)	10	70	20

(b) Ration of components to total N of microsomes

	µg RNA-P/mg N	µg PL-P/mg N
Present experiments	66	96
Data of Moulé Rouiller and Chauveau (1960)	65 ± 5.9	111.5 ± 9.4

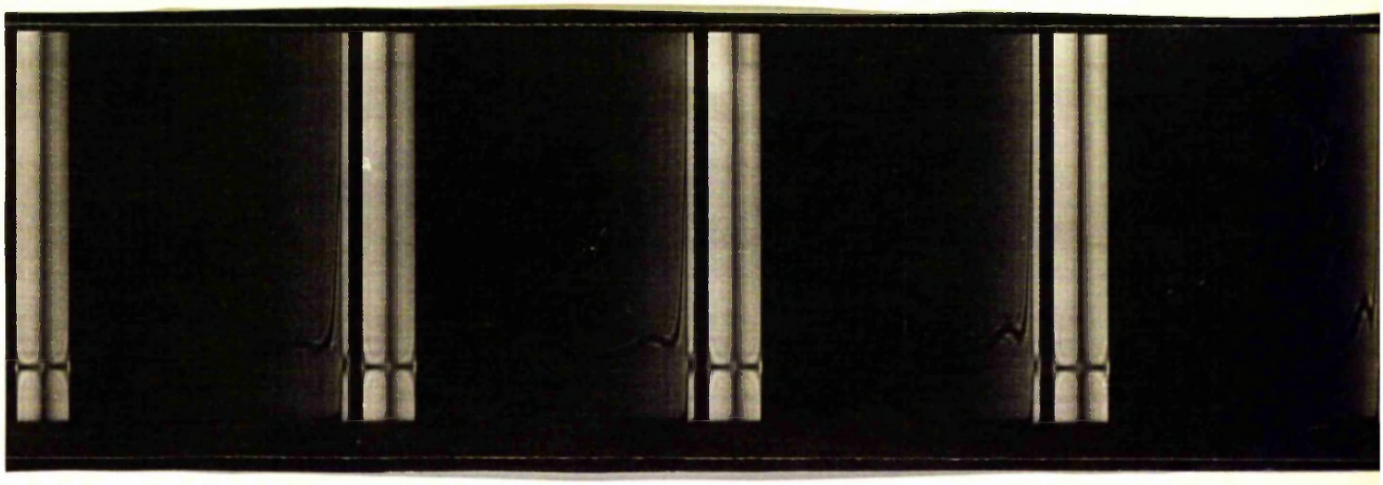
Table 29

Experimental Yield of RNA, Protein and Phospholipid-P

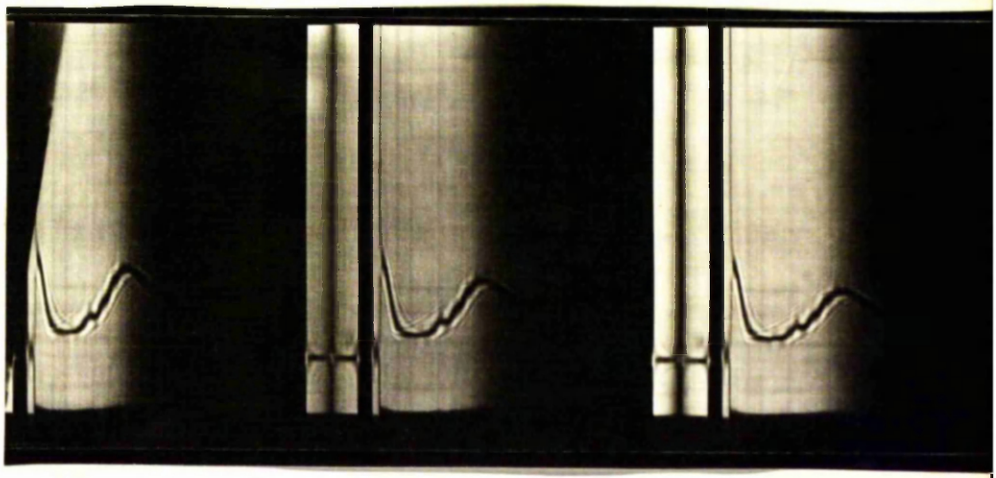
	RNA	Protein	Phospholipid-P
Mitochondria-free supernatant	68	31	31
Microsomes	57	19	26
Data of Palade (1956) for microsomes	50	15	50

The figures are % of the amount in whole liver.

Fig. 24. ULTRACENTRIFUGAL ANALYSIS OF MICROSOMES



mitochondria-free supernatant



resuspended microsome pellet

expressed as mg component per gm wet weight of tissue is seen to be reproducible. When the percentage composition and the composition relative to nitrogen is computed, the figures obtained show good agreement with those previously published by Palade (1958) and Moulé, Rouiller and Chauveau (1960) (table 28).

When the yield of the microsome components is expressed in relation to the amount of the component in whole liver, good agreement with the data of Palade (1958) is obtained for RNA and protein, but not for phospholipid (table 29). Since the method used for the estimation of phospholipid was carefully checked (see part 3 and appendix) and is known to extract all the phospholipid of the cell, it may be that the method used by Palade did not extract all the cell phospholipid.

When the mitochondria-free supernatant was examined in the analytical ultracentrifuge on two occasions single peaks were observed. When the "s" values were corrected for temperature, viscosity and specific gravity, the S_{w20} values were 106 and 130. No smaller peaks were detectable (fig.24).

The resuspended microsome fraction however, consisted of particles of several "s" values. The principal peak and the largest 'minor' peak, when corrections were made for temperature and viscosity gave "s" values of 132 and 80 respectively (fig.24). It seems reasonable to assume that the 80s peak corresponds to free ribosomes. It is impossible to say from this investigation whether some free ribosomes are normally to be found in rat liver.

From these investigations it appears that the microsome fraction obtained by the method described is similar to that obtained

Table 30

Changes in Body Weight and Liver Weight with Diet

		HP diet	LP diet
Initial body weight	a	279 (4)	286 (5)
	b	171 (4)	181 (5)
	c	143 (4)	142 (5)
Final body weight	a	265	257
	b	161	162
	c	140	128
Liver weight	a	9.54	8.14
	b	5.84	5.55
	c	6.66	5.54

Results are expressed in gm. and are the means from each group of animals.

The figure in brackets indicates the number of animals in each group.

"a" indicates the results obtained from rats of 280 gm initial body weight

"b" indicates the results obtained from rats of 170 gm initial body weight

"c" indicates the results obtained from rats of 140 gm initial body weight

Table 31

Changes in the Composition of Liver Cell Fractions with Diet

		Whole liver		Microsome Fraction		Cell sap	
		HP	LP	HP	LP	HP	LP
RNA-P	a	2.32	1.87	0.78	0.60	0.25	0.25
	b	2.20	1.94	0.95	0.66	0.40	0.30
	c	4.20	2.76	1.48	1.00	0.56	0.43
Protein	a	739	504	65.4	61.4	320	240
	b	894	697	97.1	67.6	349	243
	c	987	725	131	89	411	292
Phospholipid-P	a	3.99	2.81	0.85	0.87	-.	-.
	b	4.41	3.13	1.44	0.86	-.	-.
	c	6.32	3.72	1.82	1.21	-.	-.

Figures are expressed as mg/100 gm initial body weight.

"a", "b" and "c" refer to experiments with animals of different body weights - see Table 30.

Table 32

The Effects of Diet on the Composition of the Liver Cell

		Whole liver	microsomes	cell sap
RNA-P	a	19	13	0
	b	15	22	25
	c	35	32	23
Protein	a	21	6	25
	b	22	30	30
	c	27	32	29
Phospholipid-phosphorus	a	30	0	-.
	b	29	40	-.
	c	41	34	-.

The figures are the percentage losses in the low protein fed animals expressed in relation to the components of the high protein fed animals as 100%.

"a", "b" and "c" refer to experiments with animals of different body weights - see Table 30.

by Palade (1958) and Moulé, Rouiller and Chauveau (1960).

2. The effects of diet on the microsome fraction. As a preliminary check that the diets were having the appropriate effects on the animals, and to eliminate animals with infection and excessive experimental variation, the initial and final body weights and liver weights were carefully noted (table 30). In experiment (a) there was wide variation in the initial body weights of the animals - from 255-300 gm - which may in part explain the occasional anomalous results of tables 31 and 32. The initial body weights of the protein-deprived animals of experiment (b) were somewhat greater than the protein-fed animals but the variation within each group was very small. For this experiment the dietary differences, the mean figures of which are given in tables 31 and 32 were all statistically significant, in most cases at the level of $P < 0.01$. This was also the case in experiment (c), in which the initial body weights of both protein-fed and protein-deprived groups were the same. The expected changes in body weight and the liver weight between HP and LP animals was observed in all experiments (table 30, and previous experiments - Part I).

The changes in RNA-P, protein and phospholipid-phosphorus induced by diet are described in table 31, in which the figures given refer to mg of component (RNA-P etc) per 100 gm initial body weight. Deprivation of protein results in loss of RNA, protein and phospholipid from the liver cell and from the microsome fraction. There is no phospholipid in the cell sap fraction, but the expected loss of RNA and protein occurred on protein-deprivation. It may be noted in

Table 33

Changes in the Percentage Composition of the Microsome Fraction with Diet

		HP	LP
RNA	a	9	8
	b	6	7
	c	8	8
Protein	a	71	70
	b	70	72
	c	70	70
Phospholipid-phosphorus	a	20	22
	b	24	21
	c	22	22

"a", "b" and "c" refer to experiments with animals of different body weights - see Table 30.

passing, that the younger the animal (i.e. the lower its initial body weight) the greater the RNA, protein and phospholipid content of its liver.

The results of table 31 are summarised in table 32, in which the changes are expressed as a percentage of the appropriate component of the high protein fed liver. From this table it is apparent that the younger the animal, the greater the loss of RNA and protein from its liver when it is deprived of protein. It is also apparent that the microsome and cell sap fractions lose RNA approximately in proportion to the whole liver, but that the loss of protein from these fractions is proportionally greater than those from whole liver.

That the composition of the microsome fraction itself is little affected by diet is seen in table 33.

Discussion

The composition of the microsome fraction of the present series of experiments (8% RNA, 22% phospholipid, 70% protein) is in good agreement with that obtained by other authors (Palade 1958; Moulé, Rouiller and Chauveau 1960) and indicates that contamination with other particles, for example mitochondria, is probably insignificant.

The discrepancy between the present results and those of Palade (1958) in the estimate of the contribution of the phospholipid of the microsomes to the whole cell phospholipid may be due to analytical error. It may be computed from the data of Biezanski and Spaet (1961) that the phospholipid of the microsomes is

approximately 30% of the whole cell phospholipid. This is in agreement with the present experiments. In addition, it is claimed that the chloroform-methanol extraction procedure is quantitative (Folch, Lees and Sloane-Stanley, 1957); a claim which has been confirmed in this laboratory (see Part 3).

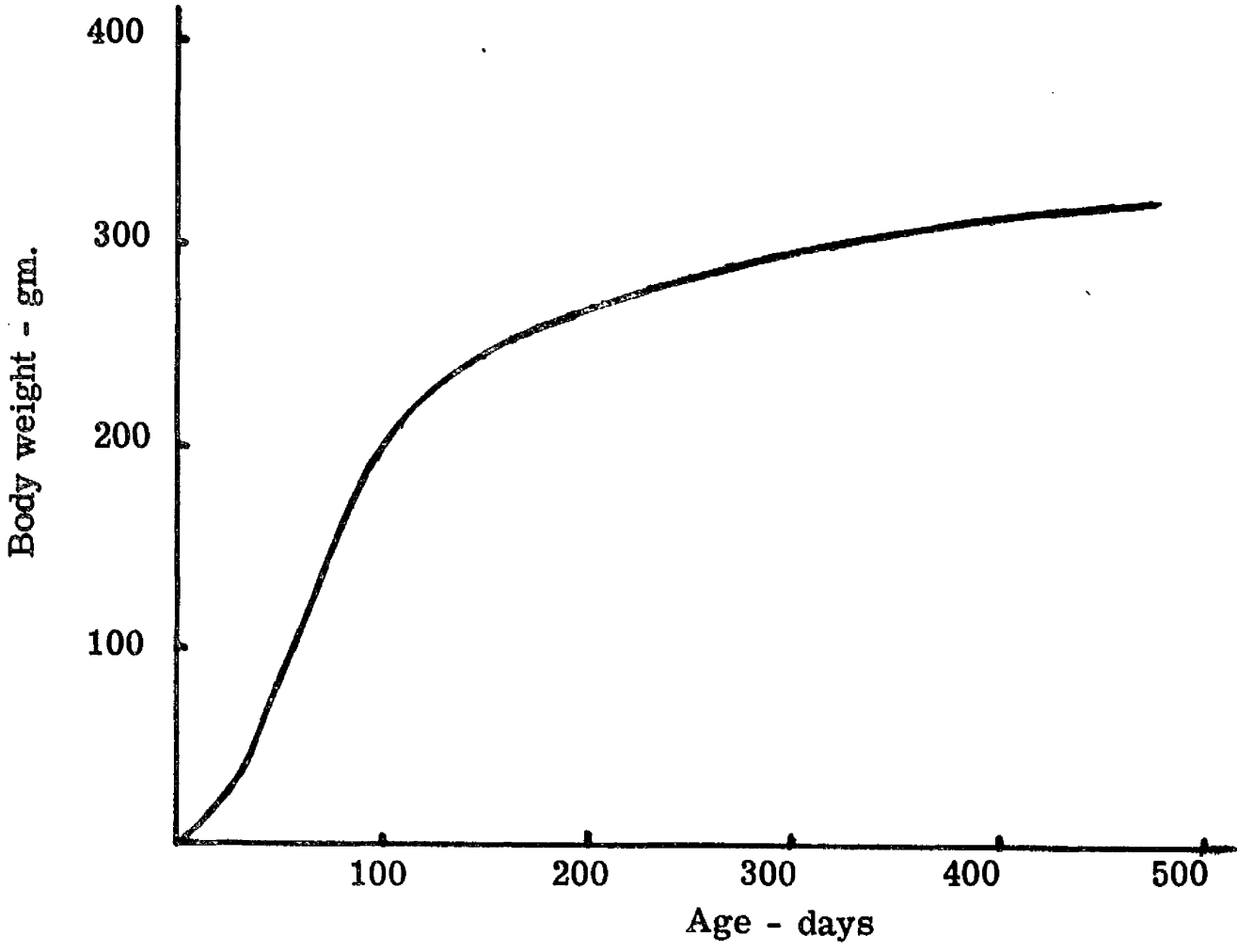
The S_{w20} values of 80 obtained for the largest minor peak from the analytical ultracentrifuge probably indicates the presence of a small amount of free ribosomes in the microsome fraction. Apart from the obvious fact that the main peak, probably related to the presence of rough-surfaced vesicles (RSV), consisted of particles much larger than ribosomes, no useful information is gained from the calculation of the 's' value since the particles, as the name implies, are not spherical and must have a density considerably different from ribosomes. The conclusion drawn from the analytical ultracentrifuge evidence obtained from the resuspended microsome fraction and the mitochondria-free supernatant fraction that there are very few free ribosomes in rat liver is disputed by Chauveau, Moulé, Rouiller and Schneebeli (1962) and by Hallinan (1964). It is of course possible that some free ribosomes are detached from the rough-surfaced vesicles (RSV) or endoplasmic reticulum during homogenisation, but it must be conceded that both Hallinan (1964) and Chauveau et al. (1962) have obtained direct evidence for the existence of free ribosomes in rat liver. The issue is, for the present, a minor one.

In protein-deprived animals the loss of RNA, protein and phospholipid from the whole cell, the microsomes and the cell sap is in agreement with expectations, following the results of

Kosterlitz (1947), Muntwyler, Seifter and Harkness (1950), Munro Naismith and Wikramanayake (1953), Bernhard and Rouiller (1956) and Weisblum, Herman and Fitzgerald (1962) with mammalian systems and Pardee and Prestidge (1956) with E.coli, and Yčas and Brawerman (1957) and Imahori and Kudo (1963) with yeast. The results of the present experiments indicate that following protein deprivation, in addition to losing protein from the cell, the relative composition of the microsomes remains unchanged and the total amount of microsomes per liver is reduced, indicating that degradation of the protein synthesising mechanism has occurred. These results are consonant with those of Munro and Clark (1960) who observed increased breakdown of RNA in animals deprived of protein and with the observations of Imahori and Kudo (1963) on yeast. This reduction in the quantity of the protein-synthesising mechanism following on the administration of a protein-free diet would lead to the supposition that in protein-deprived animals the turnover of protein would be reduced, a hypothesis which is supported by the experiments on ^{131}I-albumin turnover (Part 1. See Fleck and Munro 1963b).

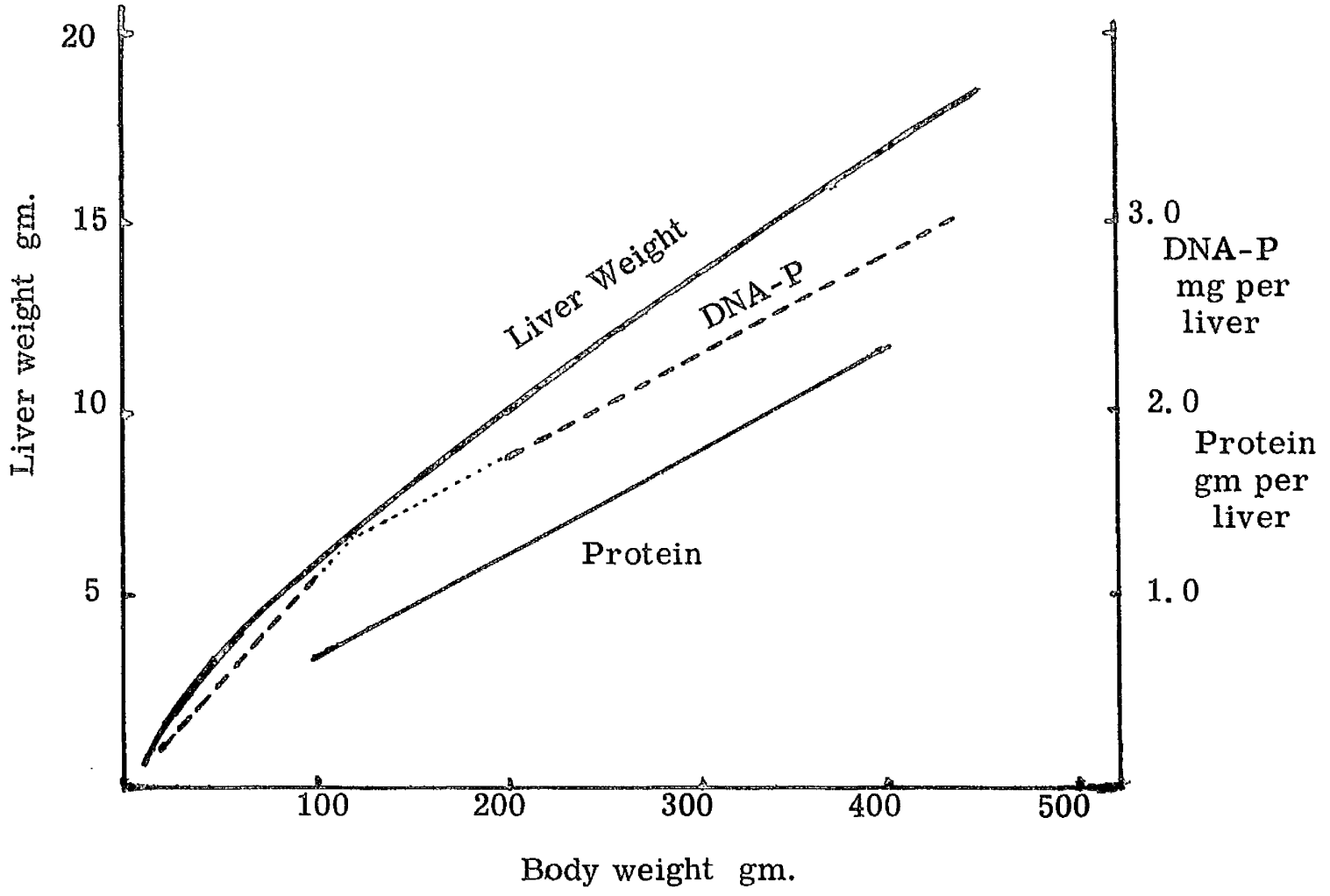
A further hypothesis is required to explain fully the results of the present series of experiments. It is that in young animals the turnover of the protein and RNA of the liver cell is more rapid than in old animals. The evidence for this has already been presented (see introduction to this section) by Burraston and Pollak (1961) who compared the rates of protein synthesis of embryo and adult rat liver and McCorquodale, Veach and Muellar (1961) in their studies of regenerating rat liver. Other contributions to

Fig. 25. CHANGE IN BODY WEIGHT WITH AGE OF WISTAR RATS



Data from Donaldson (1924)

Fig. 26. CHANGE IN LIVER WEIGHT, DNA-P AND PROTEIN CONTENT WITH BODY WEIGHT OF WISTAR RATS



Data from Donaldson (1924) and
Campbell and Kosterlitz (1949)

the hypothesis have been made by Braun, Marsh and Drabkin (1962) and Plüchthun and Schreier (1960). In the present experiments the effect of lack of dietary protein was more marked in the younger (140-170 gm) animals than in the older (280 gm) animals, an observation which although not directly confirmatory of the hypothesis, is in agreement with it.

Finally, it may be noted that in younger animals the liver protein, RNA and phospholipid content in relation to body weight is greater than in older animals. This observation is in keeping with the data of Donaldson (1924) and Campbell and Kosterlitz (1950). Donaldson (1924) - see fig.25 - has shown that the body weight of rats increases rapidly up to 200 gm weight (100 days old), the rate of increase then gradually declines but growth continues till death at a weight of more than 300 gm (450-500 days old). The increase in liver weight does not change quite so dramatically but the gain in weight with age slowly declines (fig.26). Campbell and Kosterlitz (1950) investigated the relation between liver protein content and liver DNA-P and age of rats and found that in rats above 200 gm weight, there was a linear increase in total liver protein with age and that the liver DNA-P content increased in two linear stages, one very rapid, up to about 200 gm body weight and the other more slowly from 250 gm weight upwards (fig.26). The increase of DNA with age and body weight may explain the occasional observation of significantly different liver DNA content of protein-deprived and protein-fed groups of animals of the same initial body weight. For example, the DNA content of the livers of the protein-fed and protein-deprived animals

of group c of the present series of experiments and those of experiment 2, part 1 was different. For a possible explanation, it is necessary to suppose that the duration of the experiment was sufficient to deplete the reserves of the animal to such a level that the synthesis of purine and pyrimidine bases from amino acid precursors would cease or be very much reduced and that in normal conditions the animals would have gained significantly in weight and liver DNA content. In this situation the DNA content of the animals receiving the HP diet would increase in the normal way, while that of the LP diet animals would not increase, leading eventually to a measurable or significant difference in the DNA content of the livers of the two groups of animals.

Summary

1. The method adopted for the preparation of microsomes from rat liver is satisfactory in comparison with the data available from the literature.

2. Protein deprivation leads to a loss of microsomes from rat liver, as well as losses of protein, RNA and phospholipid from all cell fractions studied.

3. The results of the experiments described are consonant with the hypothesis that in young animals the turnover of protein, RNA and phospholipid is greater than in old animals, and that the turnover of protein in animals deprived of protein is less than that of protein-fed animals.

Section 2

Attempts to Solubilise Microsomes

For the isolation of a protein such as albumin from microsomes it is necessary to disrupt the structure of the particles and release the protein, preferably in solution. A similar problem has been encountered in the attempts to isolate oxidative enzymes and cytochromes from mitochondria. For example, Järnefelt, Basford, Tisdale and Green (1958) and Basford and Green (1959) used potassium deoxycholate to solubilise a suspension of mitochondria in their studies of the succinic dehydrogenase complex. Kreinzer and Wainio (1961) also used deoxycholate in the preparation of cytochrome c oxidase from mitochondria. Both groups of workers found that extensive dialysis (40-150 hours) was required in order to remove the deoxycholate and Kreizner and Wainio (1961) found that complete removal of the deoxycholate led to precipitation of the enzyme.

Attempts to disrupt microsomes may be classified in two groups:

(a) Those aimed at separating the ribosomes from the vesicle in order to study the properties (e.g. ability to synthesise protein) of the individual particles and

(b) attempts to solubilise completely microsomes or independently, ribosomes and the vesicles in order to study their constituent proteins.

(a) Littlefield, Keller, Gros and Zamecnik (1955) in their early studies on protein synthesis in rat liver used deoxycholate to solubilise the lipoprotein membrane material (vesicles) of the microsome in order to demonstrate that labelled amino acid was first incorporated into the ribonucleoprotein particle - the ribosome (fig.21).

Subsequently, Kirsch, Siekewitz and Palade (1960) showed that ribosomes which are capable of incorporating amino acids into protein in vitro can be obtained by treating microsomes with deoxycholate. This has been confirmed by Korner (1961) and Moulé, Bouvet and Chauveau (1963) using rat liver. In addition, Acs, Neidle and Waelsch (1961) have used a similar procedure in the preparation of active ribosomes from brain tissue. Deoxycholate also finds extensive use in the preparation of polysomes from rat liver (see Wettstein, Staehelin and Noll, 1963). Other studies on the separation of ribosomes and vesicles from microsomes include those of Sachs (1958a) who used pyrophosphate to remove the ribosomes from the microsome particle, leaving a smooth membrane vesicle (Sachs, 1958b). There is also an extensive study of the morphology and distribution of enzymes in the particles separated from microsomes by deoxycholate treatment by Ernster, Siekewitz and Palade (1962). Hawtrey and Schirren (1962) have prepared active ribosomes from microsomes by using an iso-octane extraction procedure. This method has been investigated and modified by Hallinan (1964) to yield a rapid procedure for the separation of smooth membrane vesicles, rough-surfaced vesicles and free ribosomes from the microsome fraction of rat liver. Finally, it may be noted that the proteolytic enzyme trypsin has been used in the separation of ribosomes from rough-surfaced vesicles (Lust and Drochmans, 1963).

(b) Attempts to solubilise ribosomal or microsomal protein have been less successful. Following an extensive investigation, Bolton, Aronson, Britten, Cowie, Duerkson, McCarthy, McQuillen and Roberts (1959) concluded that treatment of E.coli ribosomes with distilled

water was the most effective method and released considerable amounts of protein and enzyme activity from the ribosomes.

The formation of complex ions of EDTA (ethylene-diamine-tetra acetic acid) and bivalent cations was studied by Care and Stavelay (1956) and since then EDTA has been used to effect the release of soluble protein from yeast ribosomes (Kihara, Halvorson and Bock (1961)). These authors (Kihara et al. 1961) had tried several substances including 0.5 M phosphate and 0.5 M NaCl, as well as EDTA in order to disrupt ribosomes: they do not give complete data on the relative effectiveness of these substances.

Wallace, Squires and Ts'o (1961) investigated the effects of the treatment of ribosomes with sodium dodecyl sulphate and concluded that although it caused the release of protein from ribosomes, it formed a complex with protein which subsequently prevented good separation of the proteins on electrophoresis.

An extensive investigation of the amino acid composition of E.coli ribosomal protein has been conducted by Spahr (1962). Two methods of preparation were used; the first was extraction with acetic acid and the second utilised the latent ribonuclease of the ribosomes to solubilise the RNA. Both methods have apparent disadvantages in relation to the preparation of proteins in an undenatured state.

It is thus apparent that there is no previously published method which achieves solubilisation of microsomes. However, several substances with either detergent properties (e.g. deoxycholate) or magnesium complexing ability (pyrophosphate and EDTA) have been used

with some success. Other possibilities are distilled water and 0.5 M phosphate.

Experimental methods and results

Methods. The experimental animals, the method of preparing the microsomes from rat liver and the analytical methods have been described in the previous section.

The disruption of microsomes: The microsome pellet was obtained, as before, in a 3/8" x 3" 'Spinco' centrifuge tube and was resuspended in 5 ml of the disrupting solution by gentle homogenisation. Another 5 ml of the solution was then added and the solution carefully and thoroughly mixed. Samples were withdrawn for analysis of protein, RNA and phospholipid phosphorus. The volume withdrawn was replaced by disrupting solution and the mixture was allowed to stand for 10 minutes, then thoroughly but carefully mixed once more before being re-centrifuged for 1 hour at 105,000g (average). After centrifuging, the supernatant was carefully removed without disturbing the pellet and sampled for analysis as before. After correction for volume adjustment the degree of disruption was expressed as a percentage for each component.

Solutions used in disruption: All substances used (except de-ionised water) were made up in the previously described (table 25) sucrose-phosphate buffer. The pH was 7.4 except when 0.11 M sodium pyrophosphate was used, when it became 8.2.

The following materials were used, either singly or combined: sodium pyrophosphate, the disodium salt of EDTA, water, potassium phosphate, sodium deoxycholate (Merck) and potassium laurate.

Table 34

Solubilization of Microsomal RNA

Treatment	Percent Solubilization of RNA Experiment No. 1	2	3
control	10	10	8
0.11 M sodium pyrophosphate	71	71	70
0.003 M sodium pyrophosphate	41	37	-.
0.001 M EDTA	33	35	-.
0.01 M EDTA	45	48	-.
0.10 M EDTA	62	62	49
0.5 M potassium phosphate	61	-.	-.

Table 35

Solubilisation of Microsomal RNA, Protein and Phospholipid

		Percent solubilisation		
		RNA	Protein	PL-P
Control		8	27	0
Ultrasonics*		19	33	0
Sodium deoxycholate	0.5%	44	101	64
	1%	39	78	100
	2%	45	93	100
Sodium laurate	0.5%	89	83	110
0.11 M PP		71	28	0
0.1 M EDTA		56	30	0

* The buffer contained no Mg^{++} and the mixture was subjected to ultrasonic waves for 3½ minutes.

In each experiment several methods of disruption were compared and a control, in which the microsomes had been resuspended in sucrose-phosphate buffer alone, was run. It was possible by inspecting the pellet obtained after re-centrifugation to obtain an approximate idea of the degree of disruption from the size and colour of the pellet. It was observed, however, that a white semi-translucent material was invariably packed at the foot of the tube. Later tests, including hydrolysis, and estimation of glucose by the glucose oxidase method (Huggett and Nixon, 1957), indicated that this material was glycogen.

Results

Early experiments showed that treatment with de-ionised water alone did not yield much soluble material, this procedure was therefore discarded.

In table 34 are presented the results of 3 experiments in which the effects of sodium pyrophosphate, EDTA and 0.5 M potassium phosphate on the microsomal RNA was investigated. Obviously the most effective agent is 0.11 M sodium pyrophosphate.

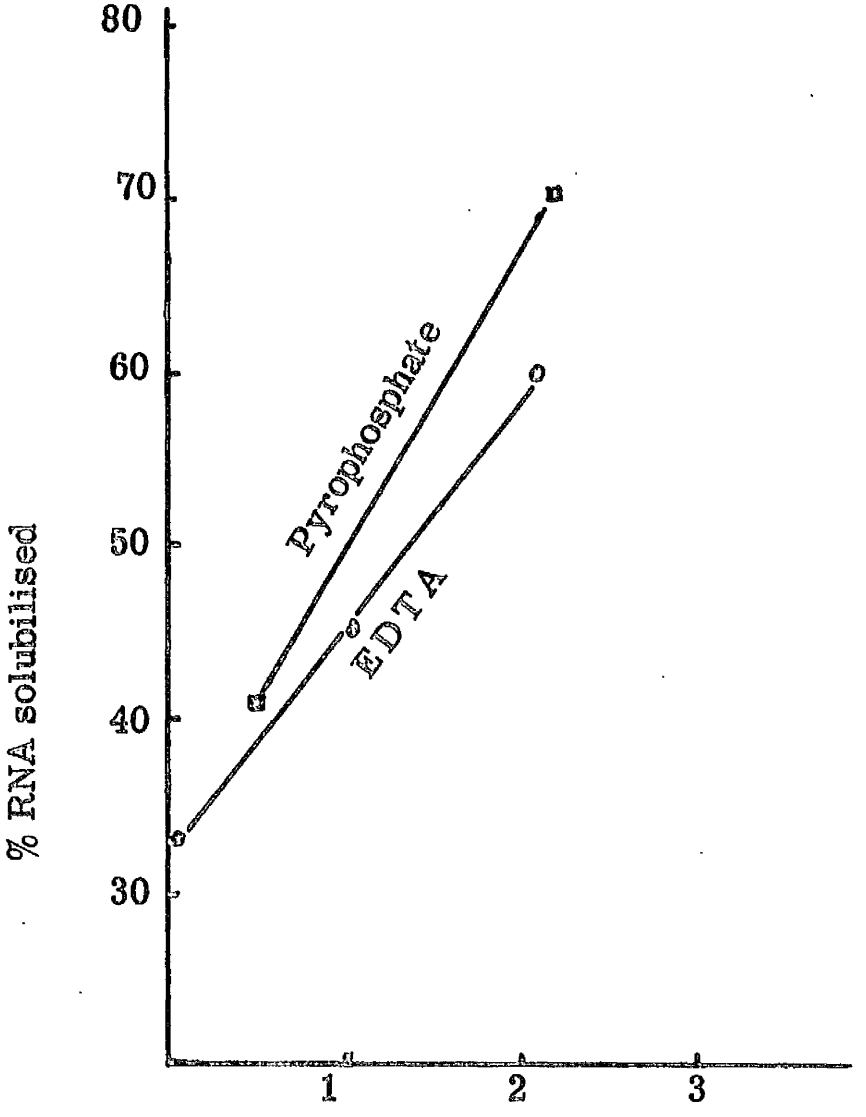
The investigation was extended to include the effects of ultrasonic disintegration and detergents on the protein and phospholipid of the microsomes as well as the RNA. The results, summarised from a series of experiments, are given in table 35. Ultrasonic waves, even in the absence of magnesium, have little effect on microsomes. Sodium deoxycholate at various concentrations extracts most of the protein and phospholipid but little of the RNA. Another detergent, sodium laurate, seems to extract more RNA but less protein than the

Table 36

Solubilization of Microsomal Components by Mixed Reagents

DOC	PP	Percent Solubilization RNA	Protein	PL-P
0.5%	0.11 M	90	95	92
1%	0.11 M	84	92	100
2%	0.11 M	71	94	100

Fig. 27. RELEASE OF MICROSOMAL RNA BY PYROPHOSPHATE AND EDTA



$\log_{10}$ (c x 10^2)

"c" is concentration in moles of pyrophosphate or EDTA

same concentration of sodium deoxycholate. A possible disadvantage of sodium laurate was that it formed a copious white precipitate, probably of magnesium laurate. Pyrophosphate and EDTA extract much less protein than RNA from microsomes. No single agent, so far, had produced 100% extraction of all components of the microsome; the subsequent experiments were therefore directed to determining whether a mixture of complexing agent and detergent might achieve this.

Table 36 gives the results of a preliminary experiment using two types of substances, in this case, deoxycholate and pyrophosphate. They were selected because individually they seemed to be the most effective substances. In order to achieve complete extraction of phospholipid, the concentration of deoxycholate must be 1%, whereas the extraction of protein is approximately constant (94%) with from 0.5 to 2% deoxycholate. The most interesting effect is that increasing the concentration of deoxycholate appears to decrease the extraction of RNA.

From these and the preceding results it was expected that the determination of the optimal concentrations of deoxycholate and pyrophosphate for extraction of RNA, protein and phospholipid of microsomes would be straightforward. Accordingly, since RNA seems to be the limiting material, a graph of extraction of RNA against concentration of sodium pyrophosphate and EDTA was drawn (fig.27). From this it was concluded that 0.05 M sodium pyrophosphate would extract approximately 60% of the microsomal RNA. Since 0.5-1% deoxycholate alone extracts 40% of the RNA, a mixture of 0.5 or 1% deoxycholate with 0.05 M pyrophosphate should give complete solubilisation

Table 37

The Effects of Varying Concentrations of Deoxycholate and Pyrophosphate in Disrupting Microsomes

Treatment		Percent Solubilization		
Sodium Deoxycholate	Sodium Pyrophosphate	RNA	Protein	Phospholipid
0.1%	--	17	49	8
0.25%	--	30	78	52
0.5%	--	43	97	101
1.0%	--	54	97	95
0.25%	0.05 M	83	88	63
0.5%	0.05 M	95	91	97
1.0%	0.05 M	95	101	100
0.1%	0.11 M	74	76	49
0.25%	0.11 M	97	96	80
0.5%	0.11 M	92	95	90

of microsomal RNA. It was expected that the extraction of protein would be similarly additive.

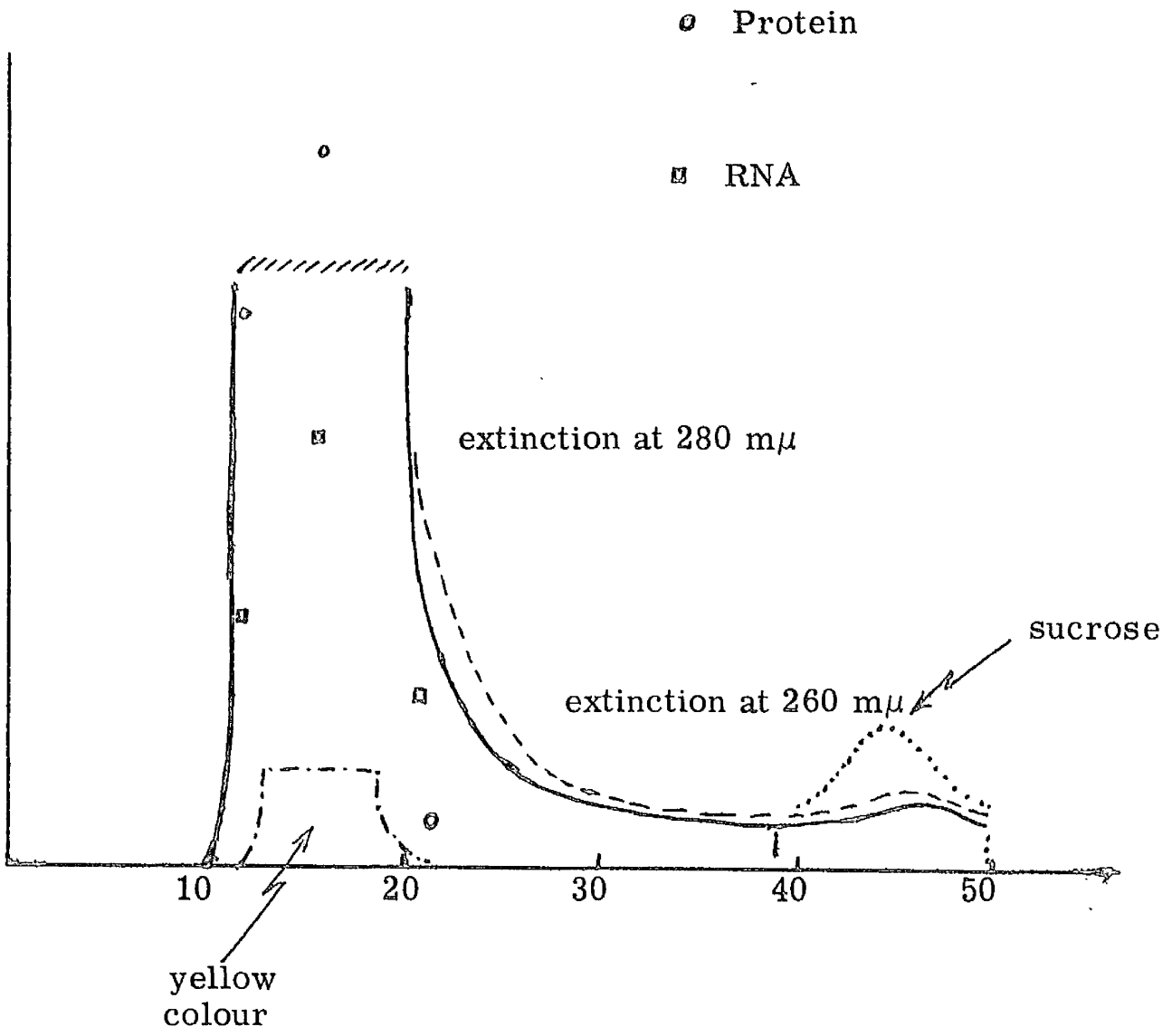
The extraction of RNA, protein and phospholipid from microsomes using various concentrations of deoxycholate alone or in combination with pyrophosphate was investigated with the results summarised in table 37. Maximal extraction of RNA, protein and phospholipid, in the case of protein and phospholipid, 100%, is achieved with a mixture of 1% sodium deoxycholate and 0.05 M sodium pyrophosphate at pH 7.4. This result, namely that treatment of microsomes with an excess of a solution of 1% sodium deoxycholate and 0.05 M sodium pyrophosphate renders 100% of the protein and phospholipid and 95-100% of the RNA of microsomes non-sedimentable at 105,000g for 1 hour in the ultracentrifuge, has been confirmed in numerous subsequent experiments.

Since the aim in disrupting microsomes is to be able to fractionate their constituent proteins etc., preliminary investigations of the number of components resulting from the treatment of microsomes with 1% sodium deoxycholate and 0.05 M sodium pyrophosphate were carried out. However, it was first necessary to remove deoxycholate, pyrophosphate and sucrose. It was found during attempts to prepare albumin from microsomes (see later section) that the sodium deoxycholate obtained from BDH was not readily removed by dialysis but that the Merck product was, and that at least 36 hours dialysis was required to effect approximately complete removal of deoxycholate. Attention was then turned to "Sephadex," a cross-linked dextran gel

with the useful property of separating high and low molecular weight materials (see Porath and Flodin, 1959). A large column (3.4 cm diameter and 32 cm long) of 'Sephadex' G 50 fine grade (available from Pharmacia (GB) Ltd.) in a buffer of pH 7.4 was used. The composition of the buffers, 0.02 M sodium phosphate or 0.05 M ammonium bicarbonate, was selected according to the projected treatment of the eluate. The ammonium bicarbonate buffer was used when the protein-containing portion of the eluate was to be lyophilised in order to give a dry, salt-free protein preparation (Cohen and Dollum, 1961).

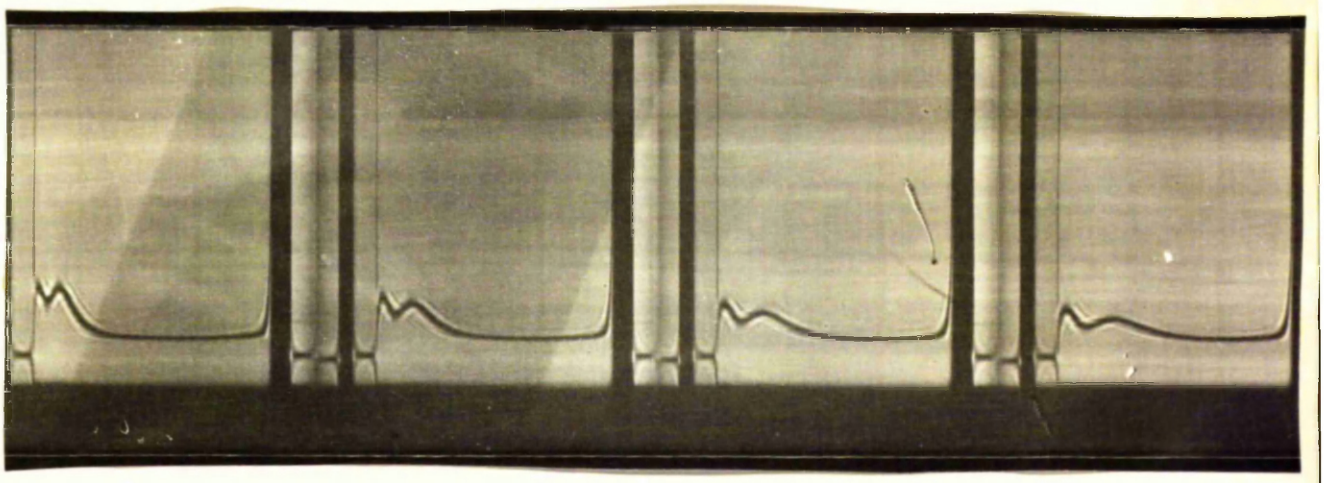
The contents of one Spinco tube (10 ml) of microsomes (from 4.2 gm liver) dissolved in 1% sodium deoxycholate, 0.05 M sodium pyrophosphate-sucrose buffer were carefully applied to the column and washed with three applications of buffer. Elution was carried out with the same buffer. Detection of the microsomal material on the column and in the eluate was simple due to the brown-red colour of the microsome protein (presumably cytochrome b_5 - Palade, 1958). However, to establish that the protein and RNA obtained in the eluate was in solution in the desired buffer and entirely free from deoxycholate and sucrose was more complex. Attempts to estimate deoxycholate in the eluate by heating with 65% H_2SO_4 for 15 minutes (the method of Mosbach, Kalinsky, Halpern and Kendall, 1954) failed due to the fact that the presence of protein and sucrose caused the solution to become dark brown in colour and opaque. Deoxycholate

Fig. 28. REMOVAL OF DEOXYCHOLATE AND SUCROSE FROM MICROSOMES ON SEPHADEX

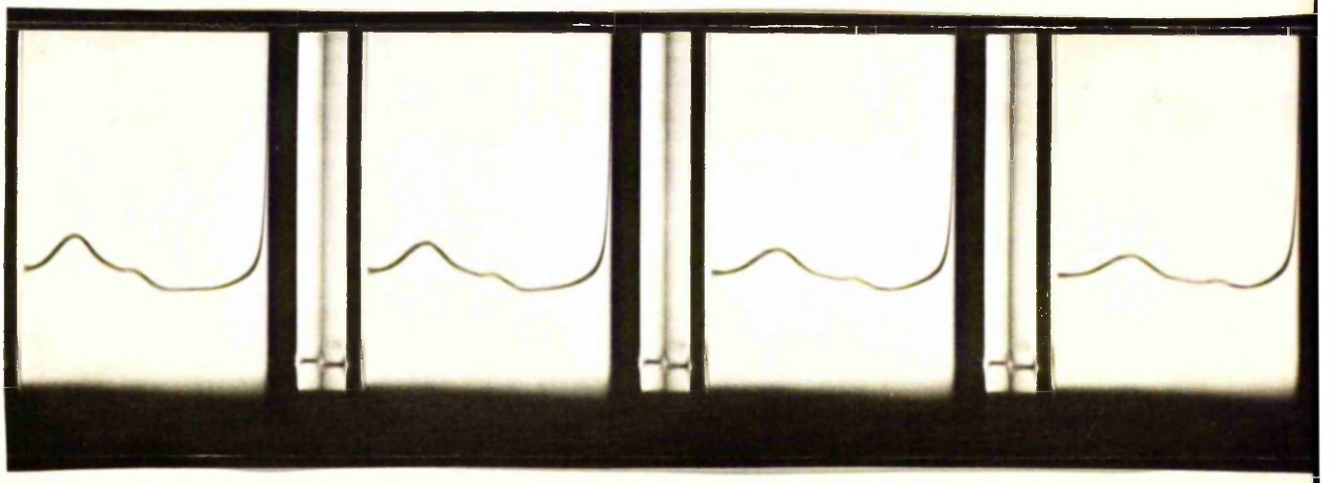


Sephadex G50 column 31. 5 cm x 3. 4 cm diam.
Ammonium bicarbonate buffer pH 7. 3

Fig. 29. ULTRACENTRIFUGAL ANALYSIS OF "DISRUPTED" MICROSOMES



Microsomes after treatment with deoxycholate and pyrophosphate.



Microsomal material remaining after dry ether extraction of deoxycholate-pyrophosphate treated microsomes.

has some 'end-absorption' in ultraviolet wavelengths at 260 and 280 mμ which renders ultraviolet spectrophotometry unsatisfactory as a method of detecting its separation from protein. A semi-quantitative procedure was then devised. This depended on a visual estimate of the red colour obtained in Selivanoff's test for sucrose. The final evidence for the satisfactory separation of deoxycholate and sucrose from protein etc. by the Sephadex column procedure depends on the estimation of protein by the method of Lowry et al. (1951), ultraviolet spectrophotometry at 260 and 280 mμ, the estimation of RNA by the modified Schmidt-Thannhauser procedure of Fleck and Munro (1963a) and on Selivanoff's test (see fig.26). The protein-containing portion of the column eluate is slightly cloudy and pink in colour: when freeze-dried a fluffy pink solid, similar in appearance to freeze-dried protein, was obtained.

When the dissolved microsomal material obtained, free from deoxycholate and sucrose by the Sephadex column procedure in 0.02 M sodium phosphate buffer was examined in the analytical ultracentrifuge only 2 peaks of S_{w20} values 36 and 4.3 were observed (fig.29). The material corresponding to these 's' values could be readily separated by centrifuging at 36,000 r.p.m. (82,000 x g) for 20 minutes in the Spinco model L using the '40' rotor. The pellet material (36s) and the supernatant (4s) when the protein, RNA and phospholipid content was determined, did not differ significantly.

Similarly electrophoresis on cellulose acetate of the lyophilised microsome material dissolved in 0.06 M barbital buffer pH 8.6 after the method of Kohn (1960) was not satisfactory. Typical

Fig. 30. ELECTROPHORESIS OF EXTRACTS OF MICROSOMES

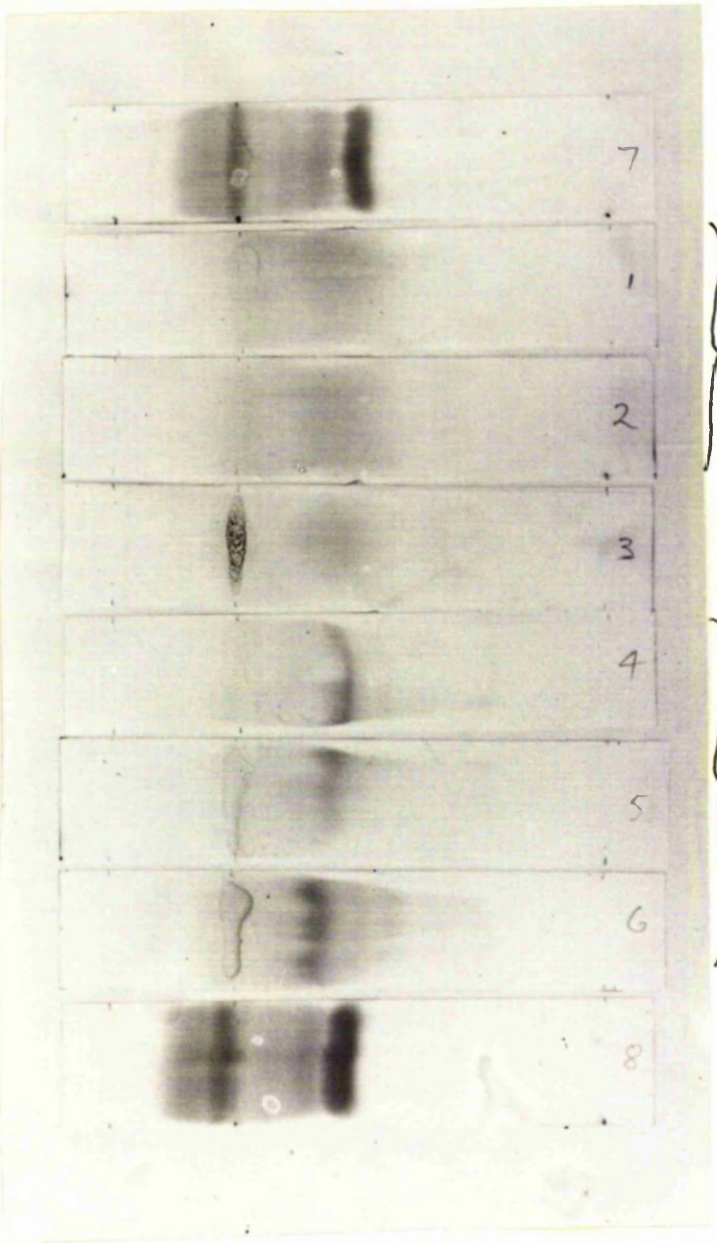


Table 38

Summary of Preparation of Soluble Microsomal Material - Quantitative Aspects

Total weight of rat liver used	51.90 gm
Total volume of homogenate	176 ml
Volume for preparation of fraction	**Volume recovered**
171 ml homogenate	130 ml mitochondria-free supernatant
97 ml mitochondria-free supernatant	a. 97 ml cell sap b. microsome fraction obtained in 90 ml sucrose-deoxycholate-pyrophosphate

Table 39

Recovery of protein and RNA in the Preparation of Soluble Microsomal Material

Fraction	Protein		RNA	
	mg/ml fraction	mg/gm liver	mg/ml fraction	mg/gm liver
homogenate	94.4	185	1.22	4.14
mitochondria-free supernatant	37.2	96	1.09	2.81
original microsomes	7.6	18.2	0.769	1.84
microsomes in solution	7.6	18.2	0.760	1.82
microsomes in solution after removal of deoxycholate and pyrophosphate and sucrose on Sephadex column	6.0*		0.605*	
cell sap	28	72.3	0.252	0.65

* There was a dilution factor of 4/5 so that no protein or RNA was lost at this stage.

Note: after dissolving the microsomes, the residue contained glycogen corresponding to 4.8 mg glucose per gm liver.

results are shown in fig.30 and in comparison with those obtained from serum and cell sap the microsome fraction reveals only one main band which when unstained is red-brown in colour.

At this stage it seemed possible that phospholipid, or lipoprotein and RNA could be causing the formation of aggregates with the protein of the microsomes (see Putnam, 1948 and Jordan, 1960). Accordingly some microsomes were dissolved in deoxycholate and pyrophosphate as before, these disrupting agents removed by passage through a Sephadex column, and the microsomal material freeze-dried from ammonium bicarbonate buffer. The lyophilised material was then extracted three times with dry ether, taken up in 0.02 M phosphate buffer pH 7.4 and the particulate material which remained was removed by centrifuging at 105,000g for 30 minutes. The resulting solution, which was pale yellow in colour, was examined in the analytical ultracentrifuge and found to contain material with S_{w20} values of 7.4, 5.9 and 3.5 (see fig.29). The yield of this material from the microsomes in terms of protein was poor.

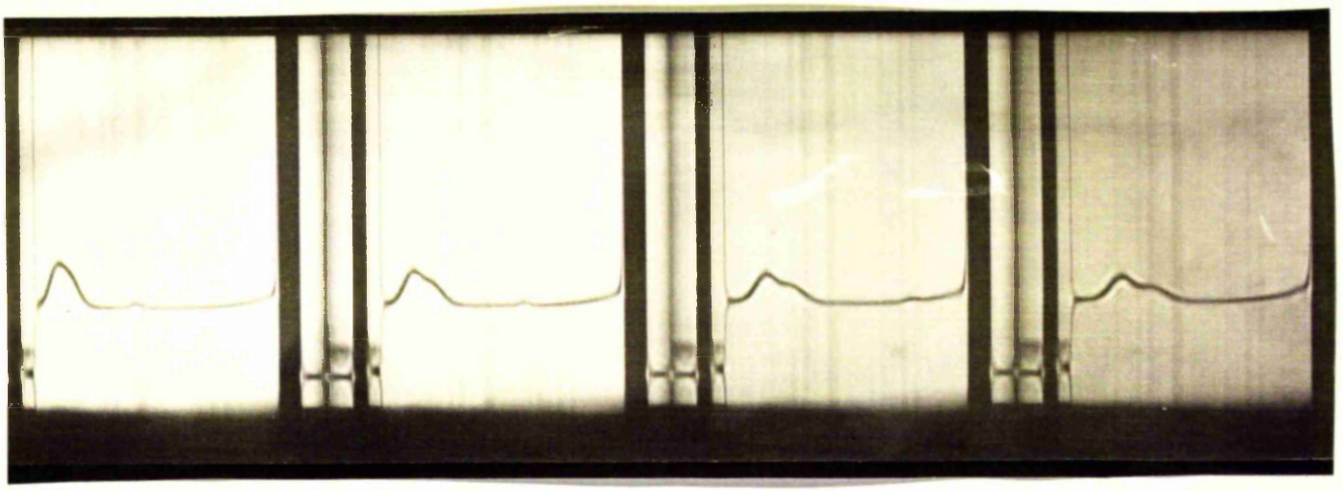
Since the process of obtaining microsomes in solution was preliminary to an investigation of the proteins of the microsome and the preparation of rat albumin from microsomes, it was important that the procedure should be quantitative. The results of a typical experiment presented in tables 38 and 39 demonstrate that the recovery of microsomal protein and RNA in solution and free from deoxycholate is 100%, so that the quantitative aspects of the procedure are satisfactory.

Discussion

The results of this investigation of the effects of deoxycholate and pyrophosphate on microsomes are in general agreement with previously published work. The detergent would be expected to act mainly on the phospholipid and protein (Putnam, 1940) and the pyrophosphate (and EDTA) to cause breakdown of ribosomes by complexing with magnesium (see page 58). On this basis, Littlefield, Keller, Gros and Zamecnik (1955) used deoxycholate to dissolve the vesicles of the microsomes so that they could obtain a preparation of ribosomes, and Sachs (1958b) utilised pyrophosphate to disrupt ribosomes and obtain a preparation of the vesicles of the microsomes in the form of smooth membranes.

The present experiments show that deoxycholate alone does extract all the phospholipid, almost all the protein and less than half the RNA from microsomes. Although it is impossible to say exactly how much of the microsomal RNA and protein is associated with the ribosomes, most if not all the RNA is believed to be found in the ribosomes (Hallinan, 1964) and since the protein:RNA ratio of ribosomes is approximately 1:1, only 11% of the microsomal protein can be associated with the ribosomes. Thus, although deoxycholate is acting principally on the membranous material of the microsome, it must also have a slight effect on the ribosomes. To what extent this effect is due to the formation of a magnesium deoxycholate complex and precipitate is unknown: certainly a precipitate does form when sodium deoxycholate is mixed with magnesium salts and it is the usual practice to add deoxycholate fresh immediately before homogenising

Fig. 31. EXAMINATION OF RAT SERUM IN THE ANALYTICAL ULTRACENTRIFUGE



to disrupt particles in order to minimise this precipitation.

Sodium pyrophosphate does not dissolve all the RNA of the microsomes and its effect on the protein is difficult to evaluate due to the fact that even gentle homogenisation may release up to almost 30% of the microsomal protein (table 35). There is thus no evidence that sodium pyrophosphate has an effect on the vesicles of the microsome.

To achieve complete solution of microsomes, a mixture of the appropriate concentrations of deoxycholate and pyrophosphate was necessary. Salting out effects could be avoided by matching the amounts of the two agents so that together they achieved complete solution of RNA and protein. The ratio of deoxycholate to protein was approximately 5:4.

Despite the observation that this procedure could be carried out quantitatively, there is some evidence that aggregation of some molecules has occurred so that further fractionation of the proteins is not yet a simple procedure. The evidence was obtained in the analytical ultracentrifuge, by electrophoresis, and later, by chromatography.

The occurrence of only two peaks in the ultracentrifugal run of microsomes in solution, possibly indicates incomplete disruption of the microsomes into individual protein molecules. It must be noted that serum also gives only two peaks in the ultracentrifuge (see fig.31), but in the case of the microsomes the S_{w20} values of the peaks were approximately 36 and 4s; the occurrence of the large 36s particle possibly indicates the presence of a large molecular

aggregate. The production of three fractions following dry ether extraction is most probably due to the disaggregation of a lipo-protein complex, the poor yield in this case however indicated that the method was unsatisfactory. Further evidence for the occurrence of a molecular aggregate was obtained by electrophoresis. In this case only one significant band was seen. In later attempts to isolate albumin by column chromatography (see Part 3) more evidence suggestive of incomplete disaggregation of protein and RNA was obtained.

Thus it would appear that although quantitative solubilisation of microsomes can be achieved, disaggregation of the protein and RNA molecules has so far not been attained.

Although these results are somewhat disappointing, it may be possible to utilise the findings to give a rapid method for the estimation of total microsomes by ultraviolet spectrophotometry.

However, despite the evidence of incomplete resolution of microsomes by deoxycholate-pyrophosphate treatment, it was thought worth while studying the isolation of albumin by column chromatography from disrupted microsomes. This work is described in the next section.

Section 3

The Preparation of Rat Serum Albumin

Reviews of the properties and methods of preparing the plasma proteins have appeared recently (Putnam, 1960). However, a short discussion of methods is required at this stage in order to select the best and most convenient method of preparing albumin from serum and microsomes. The aim in preparing albumin from microsomes was the study of the influence of diet on albumin synthesis. Thus, methods considered for further study were those for which the apparatus was available or which could be carried out readily on a small scale, and included: salt fractionation, electrophoresis, precipitation with cold ethanol, extraction with acid ethanol and column chromatography.

Salt fractionation: An excellent review and summary of salt fractionation methods has been published (Howe, 1925). This method is now rarely used as a single procedure in the preparation of a pure protein. However, a standard procedure used to obtain albumin from serum is to half-saturate the serum with ammonium sulphate; the globulins are salted out leaving albumin in solution. This method was adopted for the preparation of rat serum albumin used in studies on ^{131}I-labelled albumin turnover. The material was pure by the criteria used in its assessment (see Part 1). It may be that this was due to the long period of dialysis against de-ionised water which was necessary to remove the ammonium sulphate. This process would probably lead to denaturation and precipitation of any α and β globulin which had not been removed by the preliminary salt precipitation step.

The converse problem, namely, the precipitation of some albumin at half-saturation with ammonium sulphate, has been reported by Mosse (1959), but is not important here, as a quantitative yield was not required, only a pure sample of albumin.

Separation of serum into albumin and globulin fractions using sodium sulphate forms the basis of a routine clinical laboratory procedure for the estimation of serum albumin and globulin (Cornall, Bardawill and David, 1949).

Electrophoresis: The applications of electrophoresis in protein biochemistry are numerous and include the preparation of proteins and identification and assessment of purity of protein fractions. Various procedures are available, for example, the moving boundary method of Tiselius (Longsworth, 1942), electrophoresis on filter paper (Wolstenholme and Miller, 1956), cellulose acetate membrane (Kohn, 1960), starch gel (Smithies, 1955), polyacrilamide gel (Raymond and Wang, 1960) and agar gel (Crowle, 1961). In addition, continuous electrophoresis (Block, Durrum and Zweig, 1955; Lederer, 1957), and electrophoresis on columns (Flodin and Porath, 1954) or blocks (Fønss-Bech and Li, 1954) of suitable supporting media have been used.

Among these procedures, preparative methods of electrophoresis are restricted to moving boundary, column, block and continuous electrophoresis.

The moving boundary method requires the Tiselius apparatus, some modifications to which and the techniques required have been described by Longsworth (1942, 1947). The procedure may be used as a

qualitative or as a preparative technique but the apparatus is complex, the method tedious and time-consuming and it has been replaced largely by the other techniques mentioned.

Column and block electrophoresis are less time-consuming and do not require expensive fixed apparatus. Various supporting media such as starch granules, ethanolised cellulose (Flodin and Kupke, 1956), Sephadex and copolymers of polyvinyl acetate and polyvinyl chloride (Müller-Eberhard, 1960) may be used. Electrophoresis on columns requires special equipment, see Hill, Spackman, Brown and Smith, 1958 (such as the excellent apparatus produced by LKB of Sweden) although it is possible to build an assembly from readily available "Q and Q" laboratory apparatus, as will be described later. Following the column separation, it is usual to elute the material by passing buffer through the column and to collect the eluate as fractions of a suitable volume in a fraction collector. Although the frequently used barbital buffer pH 8.6 absorbs ultraviolet light at 280 mμ, it is possible by measuring the extinction in the region of 290 mμ to obtain an estimate of the quantity of protein in each fraction.

In preparative electrophoresis on trays or blocks the main difficulties arise at this stage, that is in detecting and then obtaining the desired fraction from the block. Probably the best technique is to cut the block into a large number of rectangles and elute the protein from each rectangle separately. Cutting the block in two parts, staining one and eluting from the other is another method, but some gels are liable to shrinkage on staining with

resultant difficulty in matching the stained and unstained sections. The process of eluting the protein may also pose problems depending on the supporting medium used for electrophoresis. For example, elution from starch gel is usually regarded as being difficult, although elution from polyacrilamide gel may be simpler.

In both column and block electrophoresis the problem of maintaining a satisfactory low temperature arises. This is rather more easily solved in column electrophoresis, by enclosing the column containing the supporting medium in a water-jacket, whereas this is not possible when a tray or block is in use: in this case it is usual to resort to the cold room.

Yet another method of preparative electrophoresis is the continuous flow method. Specially constructed apparatus is required which is usually made of perspex and contains an arrangement similar to that of a tank used for descending paper chromatography in which the individual 'tails' at the foot of the paper dip into test tubes into which, during the run, the fractions drip. In addition, the paper has two large 'tails' about two inches wide, dipping into buffer troughs on either side of the test tubes. There is a continuous descending flow of buffer and a simultaneous, continuous application of the sample to one spot on the paper. The whole apparatus is air tight to prevent disturbances due to convection, and during the application of the sample a current is passed via the large side 'tails' on the paper. Thus electrophoresis takes place in a horizontal direction while the proteins are washed down the paper by the buffer, to give after staining, a wedge or triangular

arrangement of bands reaching from the point of application to the test tubes. The process is as the name implies, continuous and has been used in the preparation of serum protein fractions (Lewis, Walters, Didisheim and Merchant, 1956).

The remaining forms of electrophoresis are mainly qualitative or may be quantitative but are not usually used as preparative techniques. Electrophoresis on filter paper is now less popular due to the greater resolving power of the more recently developed methods, particularly starch gel electrophoresis (Poulik and Smithies, 1958; Espinosa, 1961; Deaton, Selby and Wright, 1961). The technique described by Kohn (1960) making use of cellulose acetate paper is very rapid and convenient - the separation of serum proteins at pH 8.6 in a barbital buffer in this procedure requires only 1-1½ hours against the 12-18 hours required for the separation on filter paper. Successful quantitative electrophoresis on cellulose acetate paper has been described (Albert-Recht, 1959), and improved resolution when using a tris/EDTA/boric acid buffer has been claimed by Aronsson and Grönwall, (1957.) Where high resolving power is required, starch and polyacrilamide gels are very useful. They are not so convenient or rapid as the cellulose acetate method, but polyacrilamide gel has several advantages in addition to the high resolution. These are: the gel can be 'cleared' to give a completely transparent background; elution of stained (or unstained) proteins from it can be easily carried out; it is easier to handle than a starch gel.

A discussion of techniques of electrophoresis would be incomplete without mention of the very useful method of immuno-electrophoresis. This highly sensitive method is most commonly carried out with agar gel as the supporting medium (Crowle, 1961).

Using this technique, Rejnek, Bednarik and Kolc, (1963) have shown that preparations of albumin which were purified by electrophoresis or ethanol fractionation contained two fractions and they suggested that albumin might be composed of molecules of different antigenic structures. The method has been applied to the examination of subcellular components by D'Amelio, Mutolo and Barbarino (1963) and the albumin of rat liver microsomes has been investigated by Von Der Decken (1963).

From this short summary it appears that when using electrophoresis for the preparation of proteins a column technique may be the most convenient and for identifying the fractions obtained, the cellulose acetate paper method of Kohn would be the most suitable procedure.

Ethanol precipitation: The procedure using buffered ethanol solutions at low temperatures was developed by Cohn and his colleagues (Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor, 1946) for preparative large scale fractionation of plasma. The crude albumin fraction (Cohn fraction V) from bovine serum contains amounts of α and β globulin which are easily detectable on starch gel and cellulose acetate electrophoresis. However, albumin prepared from dog plasma by this method had a molecular weight of 66,000 (chemically determined) - 70,400 (determined in the analytical

ultracentrifuge) and an 's' value of 4.24 (Allerton, Elwyn, Edsall and Spahr, 1962) and so must have been of a high standard of purity.

In the preparation of albumin for ^{131}Iodine-labelled albumin studies McFarlane (1957b) states that methods using fractionation with alcohol are to be avoided because of the possibility of producing albumin with an increased rate of degradation. Although this has been disputed (Porath, Pazmandy, Pliethun and Schreier, 1960) it was decided to avoid the ethanol fractionation technique in this study.

The use of acid ethanol: In 1956, Korner and Debro reported that albumin was soluble in alcohol after it had been precipitated from serum with trichloroacetic acid. A method for the estimation of albumin in serum, based on this finding, was then devised (Debro, Tarver and Korner, 1957). The albumin obtained by this method of precipitation with TCA and extraction with organic solvents (ethanol or acetone) was carefully compared with crystalline bovine serum albumin and found to be identical in sedimentation, electrophoresis and solubility in ammonium sulphate, despite the observation of the presence of a small quantity of a large molecular weight component (Schwert, 1957). The acid/ethanol method of isolating serum albumin was further investigated by Peters (1958) and using a glycine or phosphate buffer pH 2.35, ionic strength 0.1, applied to the extraction of serum albumin from specific precipitates with rabbit antibodies. The method was further modified by Michael (1962) by substituting methanol for ethanol and precipitating the extracted protein by adjusting the pH of the solution in methanol

to pH 6 by the addition of NaOH. It was found that in this method, gelatin, globulin and protamine were soluble as well as albumin. Charlewood (1961) however, had used the method of acid/ethanol extraction to obtain samples of albumin which were pure and homogeneous when examined in the ultracentrifuge.

The method has been applied to the extraction of albumin from the rat liver microsome fraction by Campbell, Greengard and Kernot (1960). When the preparation was partially hydrolysed and the resulting peptides compared with those from a pure preparation of rat serum albumin, patterns which were similar but not identical were obtained (Campbell and Kernot, 1962). That this interesting procedure for the isolation of albumin requires further investigation is indicated by the report of Venkataraman and Lowe (1959) that a fraction of rat liver ribonucleoprotein previously precipitated using cold TCA became soluble on the addition of cold ethanol. This observation has been confirmed by Hallinan, Fleck and Munro (1963) and it has been shown (see Part 3) that considerable quantities of microsome protein may be dissolved in acid/ethanol.

Chromatography: The application of chromatographic techniques to the fractionation of proteins is a fairly recent development and was to some extent, dependent on the production of the diethyl-amino-ethyl (DEAE) and carboxy-methyl (CM) cellulose or dextran (Sephadex) derivatives, since the widely used Amberlite and Dowex ion-exchange resins had proved generally unsuitable for application to proteins due to irreversible binding of most of the protein to the resin.

The general aspects and problems of the chromatography of proteins have been reviewed by Peterson and Sober (1960) and Porter (1961). Techniques using ion-exchange cellulose and applications of them to studies on plasma protein fractionation have been described by Peterson, Wyckoff and Sober (1961). Tombs, Cooke, Burton and Maclagan (1961) investigated the fractions obtained from serum following electrophoresis on starch grains (in a block) followed by chromatography on DEAE or CM cellulose columns and obtained a pure preparation of albumin from DEAE cellulose columns. Although preliminary reports of chromatography of proteins on DEAE cellulose paper (Curtain, 1961) and on a thin layer of hydroxyl-apatite (Hofmann, 1962) have appeared, the principal application of chromatography of proteins has been preparative column chromatography. Porter (1961) has stated that in the preparative fractionation of proteins, ion-exchange chromatography is the method of choice. A recent, useful development in this field is the development of 'Sephadex' in ion-exchange and normal forms. In 1956 Lathe and Ruthven showed that it was possible, using columns of starch in water to separate substances in order of their relative molecular sizes. This principle was later applied and extended by Porath (1959) who developed the use of dextran gels (Sephadex). Later, ion-exchange forms of Sephadex were produced (Porath and Lindner, 1961). A Sephadex column may be used in desalting or changing the buffer of a protein solution and is more rapid and convenient than dialysis.

Important properties of albumin in relation to its isolation

There are some properties of serum albumin which are specially important in considering methods of isolation. The general properties of albumins from various species have been summarised on page 4 and table 2.

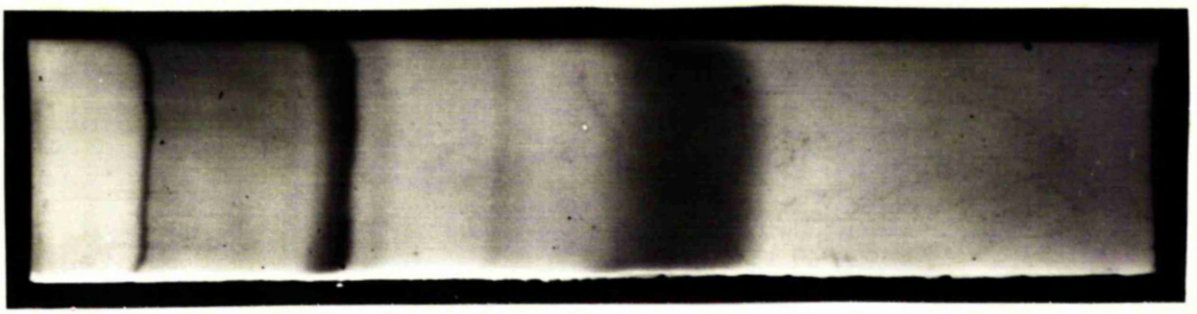
One very interesting property of albumin is its solubility in aqueous ethanol at low pH. This is believed to be due to isomerism which is due to the equilibrium between folded and unfolded forms of the molecule (Foster, 1960).

Isomerism at low pH has been investigated using bovine (Luzzati, Witz and Nicolaieff, 1961) and human (Schmid, Polis and Takahashi, 1962) plasma albumin. The change from folded to unfolded forms occur at pH 5-3 depending on the salt concentration of the buffer used. There is a suggestion by Foster (1960) that a similar type of change may take place above pH 9. This is of interest following the observation of splitting of the albumin band during paper electrophoresis at pH 8.9 (McLoughlin, 1961).

It appears to be generally accepted that albumin has a greater ability than most other proteins to complex with various molecules. This has led to difficulties in quantitative paper electrophoresis (Wolstenholme and Millar, 1956). The ability of albumin to form complexes with charged dextran derivatives (Thompson and McKernan, 1961) and lipids (Blix, 1941 and Goodman, 1957) has been investigated.

These observations on the properties of complexing with charged molecules and lipids which albumin possesses, may have a bearing on some

Fig. 32. ELECTROPHORESIS OF RAT SERUM ON STARCH GEL



technical problems in the isolation of albumin from microsomes.

Experimental methods and results.

The isolation of albumin from serum and microsomes was investigated using the following methods:- 1. salt fractionation, 2. electrophoresis, 3. column chromatography, 4. extraction with acid/ethanol.

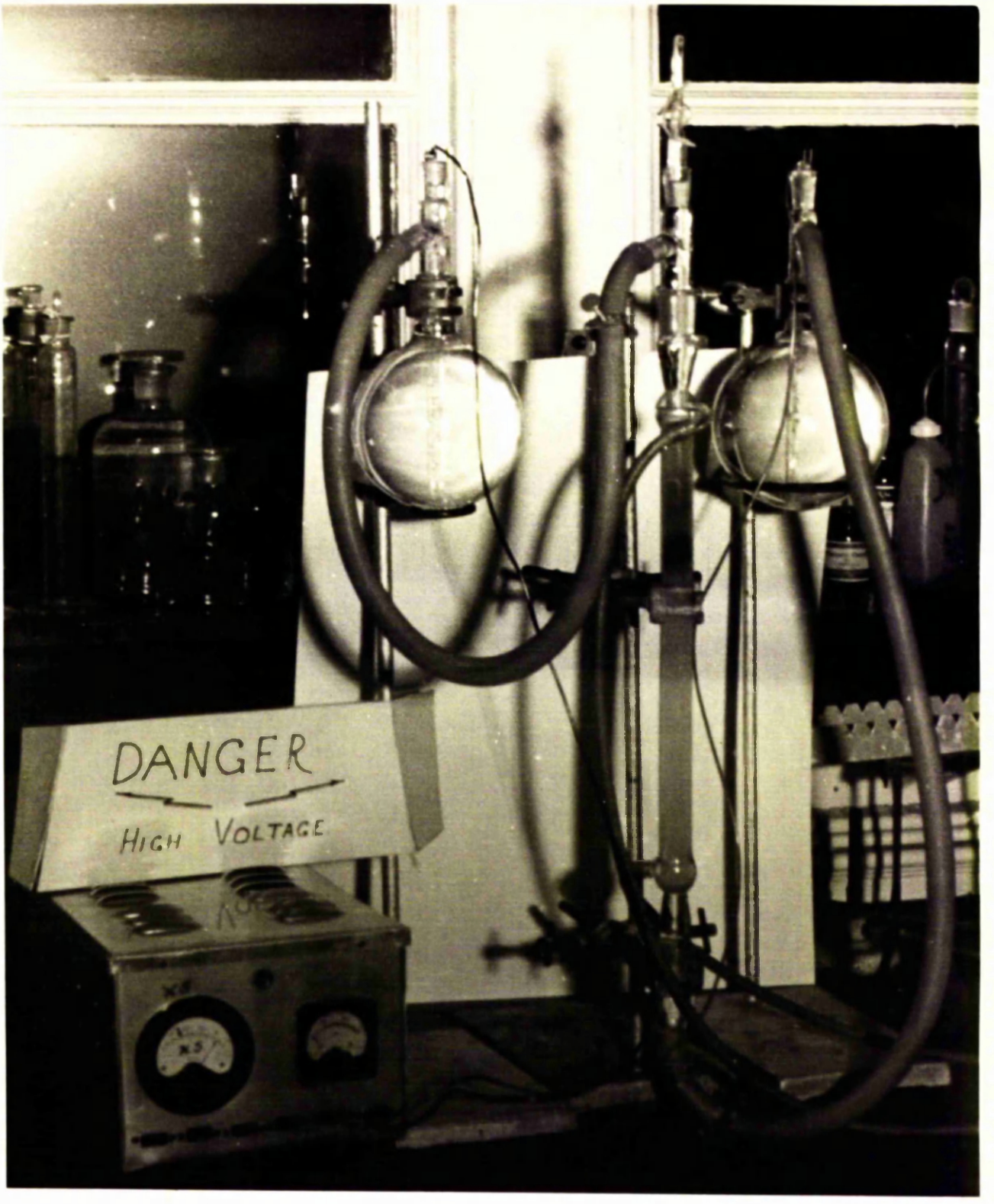
Materials. Rat serum was obtained by the procedure described previously (page 31).

Microsomes were prepared from rat liver by the method of Campbell, Greengard and Kernot (1960) - see fig.23 and page 72. Unless stated otherwise, before attempting to isolate albumin from microsomes, they were obtained in a soluble form by treating with 1% sodium deoxycholate and 0.05 M sodium pyrophosphate which was subsequently removed by passing the solution through a G 50 Sephadex column. The procedure has been described in the previous section (2) and summarised in tables 38 and 39. Frequently, the soluble microsome preparation was freeze-dried and stored.

Preliminary studies. Rat serum was examined in the analytical ultracentrifuge. Two main peaks (approximately equal in area) of S_{w20} values 4.6 and 6.8 and a small rapidly sedimenting peak were observed (fig.31).

The results of electrophoresis of rat serum on filter paper (fig.1), cellulose acetate paper (fig.30) = by the method of Kohn (1960), and starch gel (Smithies, 1955) - fig.32, were compared. The resolution obtained when cellulose acetate and starch gel are used as the supporting media is much greater than that

Fig. 33. COLUMN ELECTROPHORESIS APPARATUS



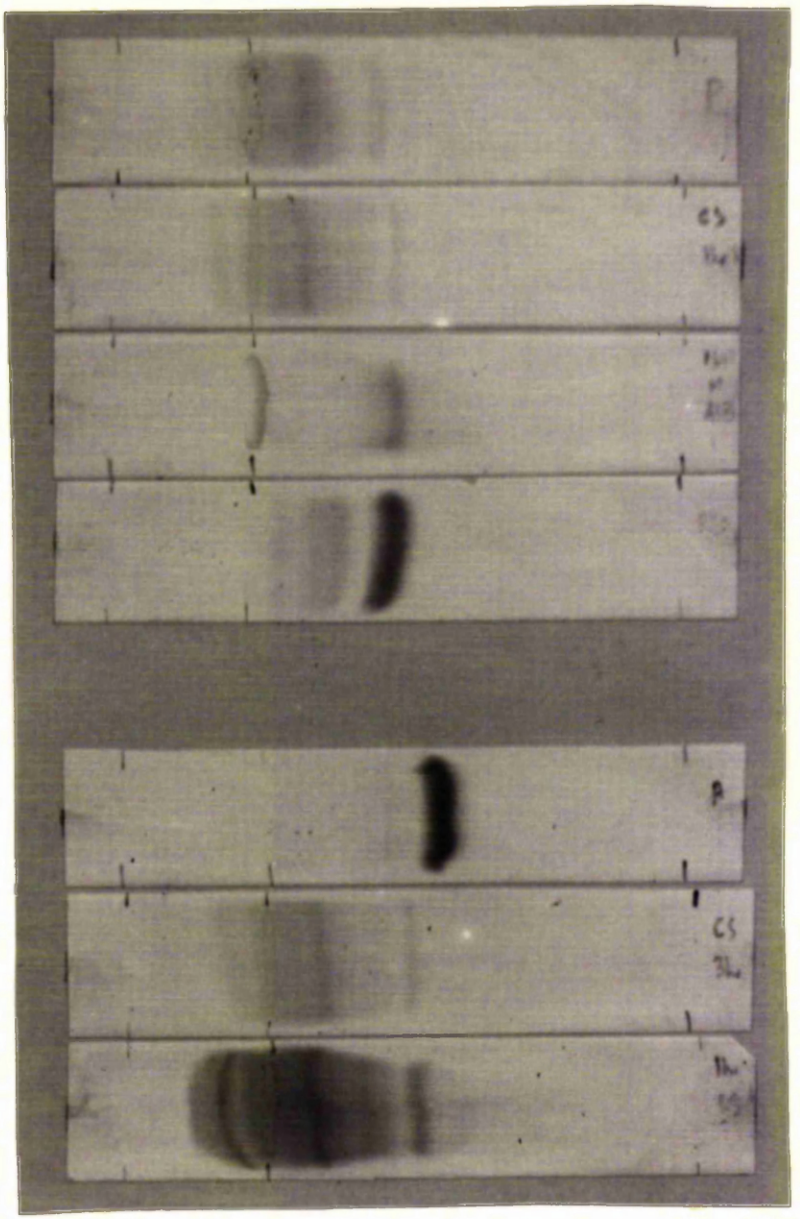
The separation of haemoglobin from cytochrome C is clearly visible.

obtained with filter paper. There is little difference in the resolution of rat serum obtained by using starch gel or cellulose acetate. For this reason, and because of the convenience and rapidity of the method of Kohn (1960) (see appendix), it was adopted as the routine method of electrophoresis to be used in the investigation of the purity of protein fractions.

1. Salt fractionation: The application of this method to the isolation of albumin from rat serum has been described (page 31) and commented on in the introduction to this section. The same procedure was applied to the soluble microsomal material. The supernatant obtained after half-saturation with ammonium sulphate was dialysed against several changes of ammonium bicarbonate buffer pH 7.4 and finally distilled water before being freeze-dried. Following this procedure, a minute quantity of white powder was occasionally obtained, which when dissolved in barbital buffer, subjected to electrophoresis on cellulose acetate and stained with Ponceau S did not indicate the presence of albumin, or indeed any protein. Ultrasonic disintegration of the soluble microsomal material was equally unsuccessful.

2. Electrophoresis: For preparative use, a simple column electrophoresis apparatus was constructed (fig.33), using laboratory "Quickfit" apparatus with either B24 or B19 cones and sockets. The electrodes were of platinum wire wound on "Perspex" supports. Two supporting media were used in the column - Sephadex G 25 medium grade and polyvinyl acetate-polyvinyl chloride copolymer obtained from I.C.I.

Fig. 34. ELECTROPHORESIS OF CELL FRACTIONS AND RAT ALBUMIN



Post-microsomal pellet

Cell sap (1)a

Microsomal albumin

Rat serum albumin

Bovine serum albumin

Cell sap (3)

Cell sap (1)b

In trial runs using 0.06 M barbital buffer pH 8.6, separation of haemoglobin from cytochrome c was good on both media (fig.33) but elution from the synthetic copolymer was more satisfactory than from Sephadex, therefore for subsequent use the polymer was chosen.

This apparatus was used in the investigation of the proteins of human bile (page 54). It was also used in the preparation of albumin, but not in the first stage from serum. Following chromatography of rat serum on DEAE-Sephadex as described later, the albumin fraction (which contained some α and β globulin) was purified by the column electrophoresis procedure in which the current was applied for 18 hours. After elution and selection of the albumin fraction, the albumin was dialysed to remove buffer and freeze-dried. The preparation when tested with rabbit anti-rat serum in immuno-electrophoresis gave a single precipition line.

The poor results of electrophoresis of dissolved microsomes on cellulose acetate paper are presented in fig.30; the possible reasons have been discussed in the previous section. Once again, treatment of the dissolved microsomes with ultrasonic waves had no effect. However, a comparison of the electrophoresis of cell sap, post-microsomal pellet (Hird, 1963), bovine serum albumin and a rat serum albumin preparation (see later) is illustrated in fig.34 and demonstrates that the procedure itself is not at fault.

3. Column Chromatography. The interesting and very useful 'Sephadex' products having recently been made available by Pharmacia (Sweden) it was decided that the possibility of using the ion-exchange form DEAE Sephadex for the preparative fractionation of the proteins of

first, rat serum and second, rat liver microsomes should be investigated. The factors in favour of DEAE Sephadex were the probability that it was a well standardised product and would not vary from batch to batch as did 'Ecteola' and the high flow rate of columns of the material in contrast to the very low flow rate quoted by Peterson and Sober (1960) for ion-exchange cellulose.

The manufacturer's advertising material indicated that it was possible to obtain fractions containing mainly γ globulins, α and β globulins and albumin by stepwise elution from DEAE Sephadex in 0.02 M phosphate buffer pH 6.6 with increasing NaCl concentration. It was therefore decided to duplicate these conditions as closely as possible and utilise a continuous concave gradient of NaCl for the fractionation, as this is stated to be the best type of gradient for this purpose (Porter, 1961).

For a column 7 cm long and 1.2 cm diameter, the manufacturers used the following procedure: after washing the applied serum with 225 ml of buffer the sodium chloride concentration was raised from 0.00 M to 0.07 M. At 400 ml the NaCl concentration was increased to 0.17 M and at 600 ml the concentration of salt was raised to 0.38 M. Peterson and Sober (1959) have devised a variable gradient device on the formula $\frac{m}{M} = \left(\frac{v}{V}\right)^{N-1}$ where M is the molarity of salt in the Nth vessel, V is the total volume of liquid in the mixing vessels, N is the number of chambers or vessels in the system and $\underline{m}$ is the molarity of the solution after a volume $\underline{v}$ has passed into the column. Using this formula it was predicted that with three, one-litre aspirator bottles, containing a total volume of 1950 ml, with

Fig. 35 a. CHROMATOGRAPHY OF RAT SERUM ON DEAE-SEPHADEX

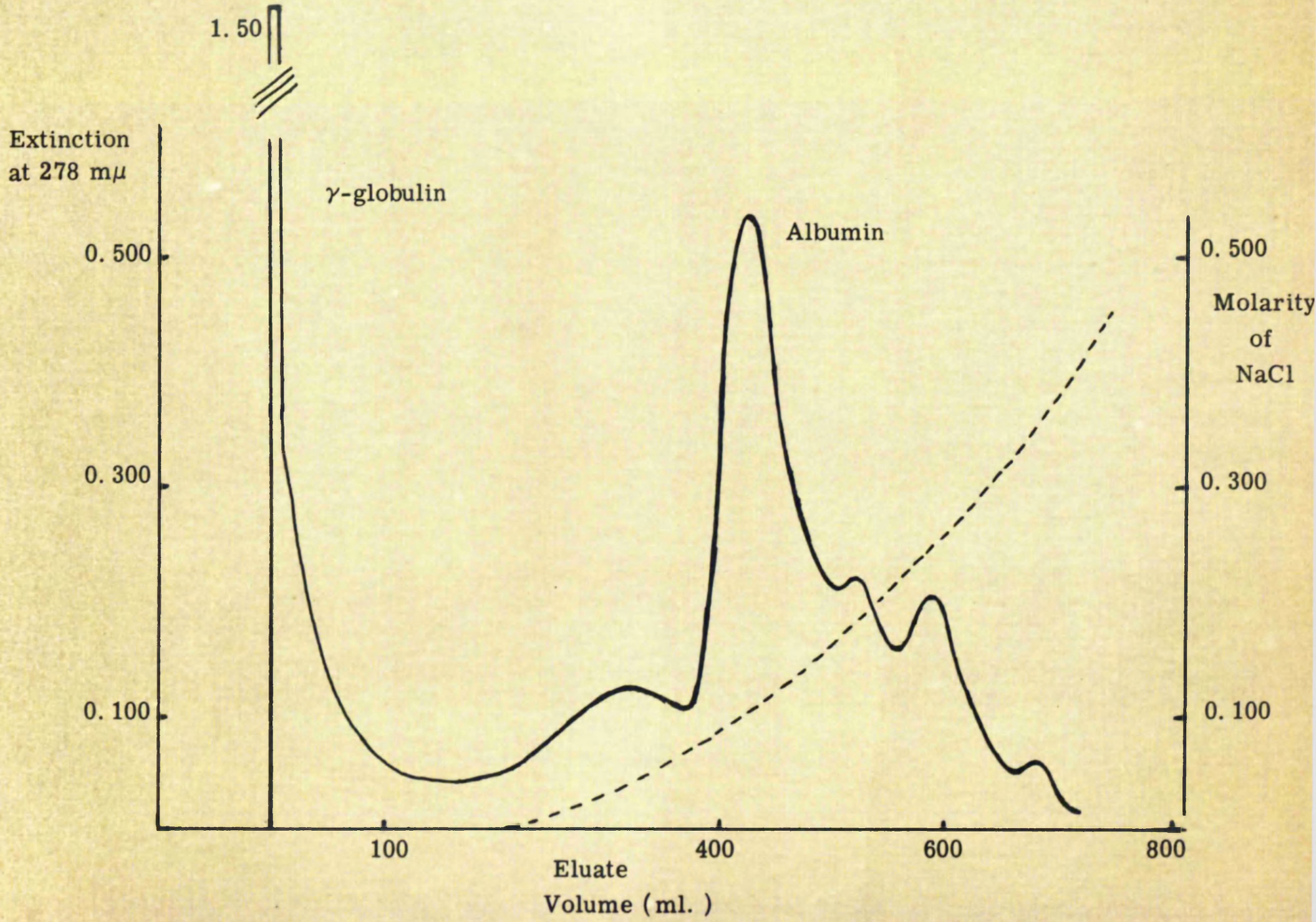
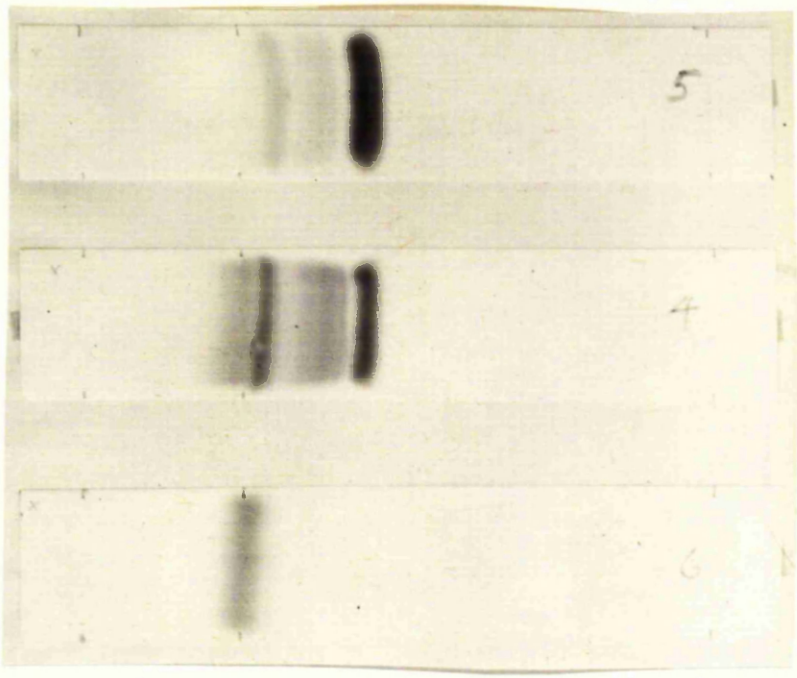


Fig. 35 (b). IDENTIFICATION OF FRACTIONS FROM COLUMN ELECTROPHORESIS



Albumin peak

Rat serum

Globulin peak

Fig. 36a. CHROMATOGRAPHY OF RAT SERUM ALBUMIN ON DEAE - SEPHADEX

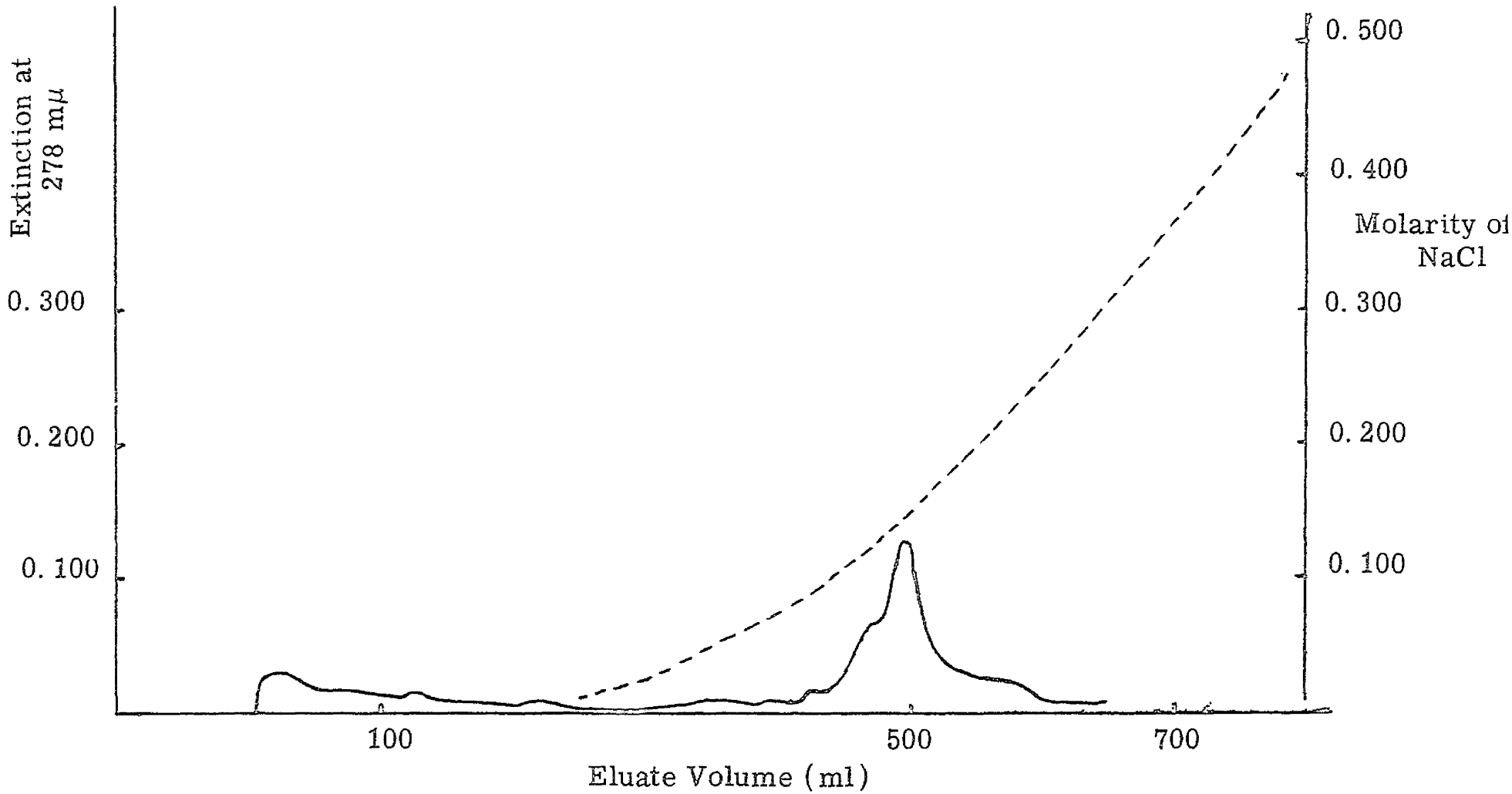
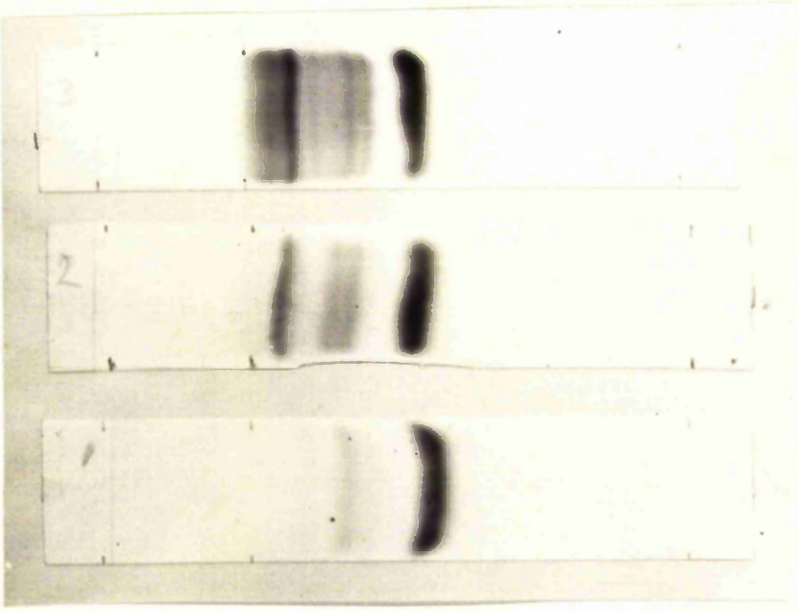


Fig. 36. PURIFICATION OF SERUM ALBUMIN ON DEAE - SEPHADEX



Rat serum

Albumin (applied to column)

Albumin peak

3.930 M NaCl in the third vessel and water in vessels one and two, a continuous concave gradient would be obtained which would closely match the required gradient. In order to obtain these conditions, three identical one-litre aspirator bottles were obtained, numbered 1, 2 and 3, and graduated at 650, 650 and 566 ml (see below). The bottles were linked by glass and plastic tubing with taps to allow filling without the contents of one vessel mixing with those of another. The outlet from vessel 1to the column was a narrow bore siphon tube with a tap to control the outflow. The vessels were stood on 3 identical magnetic stirrers at a height of about 18-24 inches above the top of the column. Before applying the gradient to a column the variation of NaCl concentration with volume of eluate was checked using a series of measuring cylinders and by titration of the NaCl in the eluate with 0.1 M $AgNO_3$ (and standard dilutions thereof) using tartrazine as indicator. In practice, the high specific gravity (1.148) of 3.930 M NaCl caused an initial rapid outflow from vessel 3. This was corrected by placing 566 ml of 3.93 M NaCl dissolved in phosphate buffer in vessel 3 and 650 ml of 0.02 M sodium phosphate buffer pH 6.6 in each vessel one and two. The gradient obtained matched the theoretical gradient closely and on checking on subsequent occasions, was highly reproducible (for example, see figs.35 and 36).

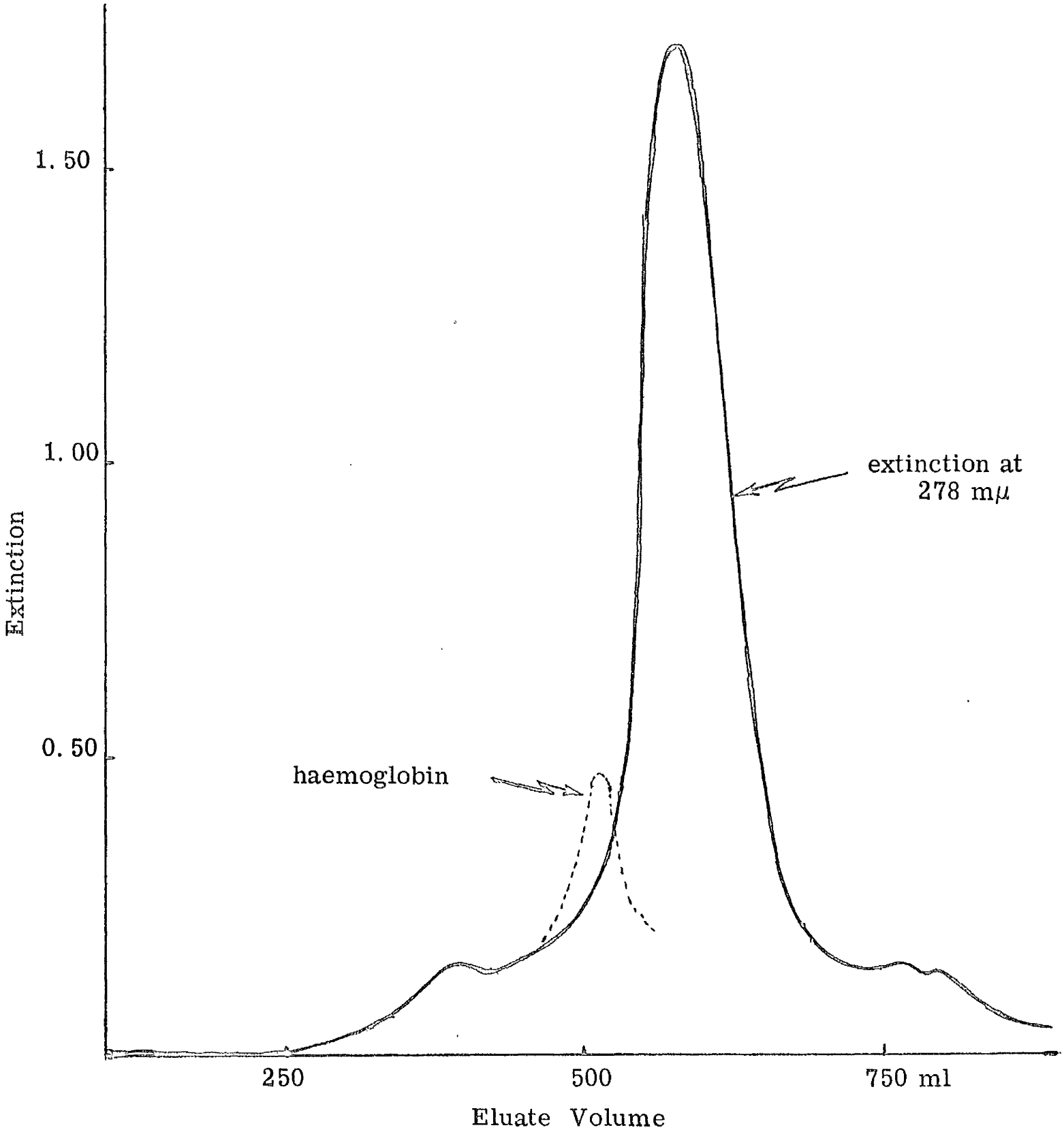
The procedure for preparing a column 7 cm long x 1.2 cm diameter of capacity approximately 200 mg protein, was as follows. Approximately 3 gm of DEAE Sephadex A 50 medium grade was weighed,

transferred to a beaker, suspended in water and the fines poured off six times with resuspension in water each time. The suspension was then poured onto a Buchner filter and with gentle suction washed with:- 100 ml water, 500 ml 0.5 N HCl, 1 litre of water, 500 ml 0.5 N NaOH, 1 litre of water, 500 ml of 0.5 M H_3PO_4 and finally 0.02 M phosphate buffer to pH 6.6 (approximately 800 ml of buffer were required). During this washing procedure, it is important always to maintain the gel under a layer of liquid, because if exposed to air, this is trapped with the later production of gas bubbles in the column.

The column consisted of a glass tube with, at the foot, only a slight taper and wide opening. A close fitting piece of sintered polythene (smooth side uppermost) was used to support the Sephadex. Alternatively, the foot of the column was sealed horizontally with glass through which a number of holes had been made and over which 2 discs of glass filter paper fitted. With either of these columns the "dead space" was negligible. In passing, it should be mentioned that it was noted by testing the separation of haemoglobin and methylene blue on 'Sephadex' G25, that the "dead space" of the small chromatography columns with an integral stopcock produced by "Quickfit", renders them totally unsuitable for use in chromatography. When the washing procedure had been completed, the column was poured in the usual way, and a "blank" run with sodium chloride elution was carried out to check that the eluate contained no material which absorbed ultraviolet light: in fact, the maximum extinction readings were 0.054 at 230 mμ and 0.025 at 260 mμ.

Rat serum was prepared for ion-exchange chromatography by passing 3 ml through a small (5 cm x 1 cm) Sephadex G 25 column to obtain the proteins in solution in 0.02 M phosphate. Following this, it was occasionally necessary to centrifuge to remove a light precipitate (possibly of γ globulin) before applying 3 ml (containing approximately 150 to 200 mg) of protein to the ion-exchange (DEAE) Sephadex column. The protein was washed with 3 applications of phosphate buffer, then connected to the gradient device and elution started. Approximately 5 ml fractions were collected in a "Locarte" fraction collector using the conductivity drop counting method. The protein content of the fraction was estimated from the extinction at 280 mμ (Beavan and Holiday, 1952). This process was considerably accelerated by using the Beckman DB spectrophotometer to which was coupled a "Transferator" (Gilson Medical Electronics) which permitted the rapid filling and emptying (with recovery of the sample) of the quartz cell in situ in the instrument. Selected fractions were pooled, dialysed against ammonium bicarbonate buffer to remove salt, lyophilised, and taken up in a small volume of 0.06 M barbital buffer pH 8.6 and subjected to electrophoresis on cellulose acetate (Kohn, 1960). Ammonium bicarbonate is volatile under these conditions (Porath, 1955; Cohn and Bollum, 1961). The results of this procedure are illustrated in fig .35. Two main peaks corresponding to albumin and γ globulin, with several smaller peaks are obtained, indicating that for fractionation of the serum proteins the method is at least as sensitive as electrophoresis on filter paper. As a result of this successful experiment, the effects

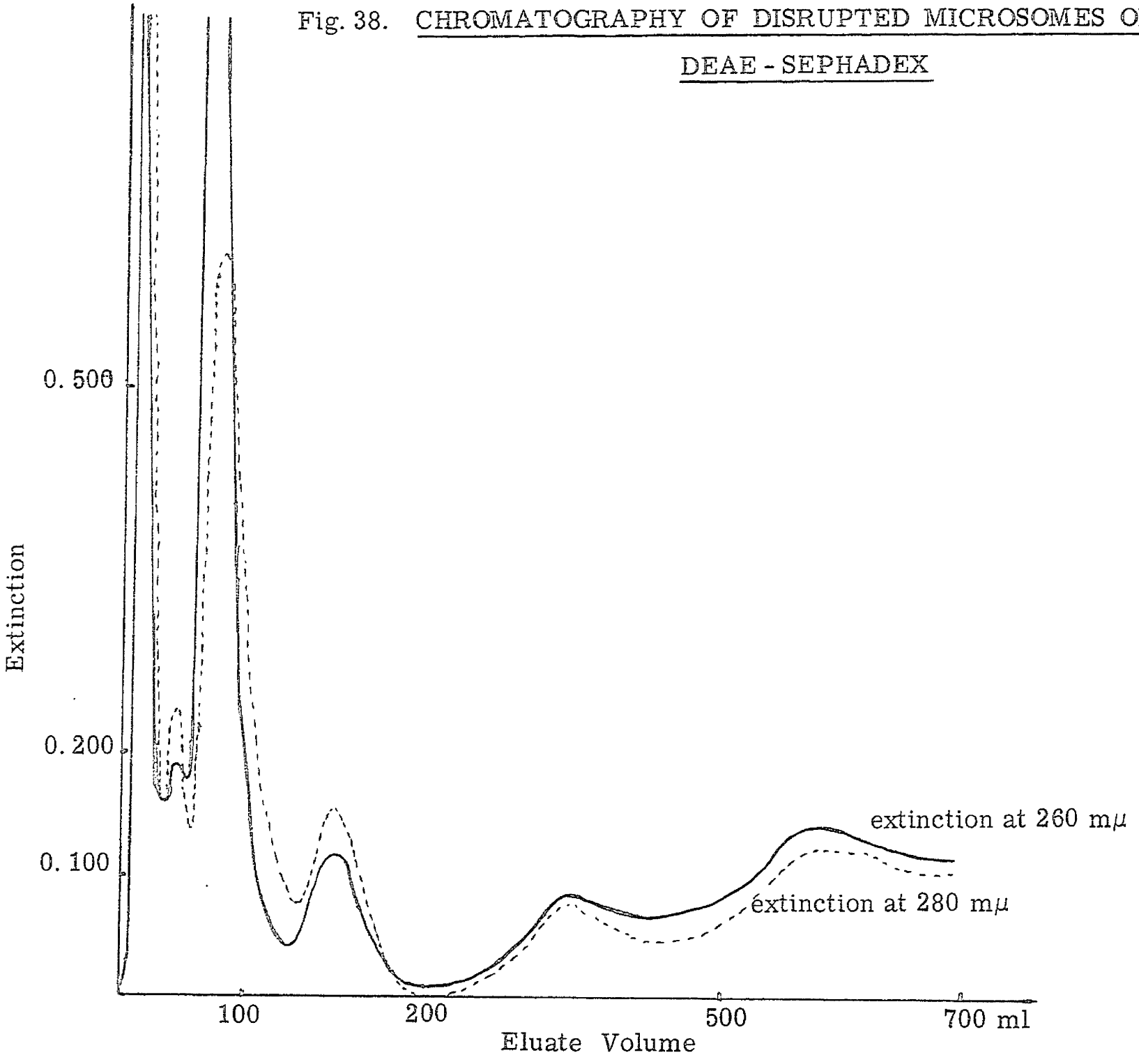
Fig. 37. PREPARATION OF RAT SERUM ALBUMIN BY CHROMATOGRAPHY ON DEAE - SEPHADEX



of pH and buffers were investigated. Retaining the sodium phosphate buffer (0.02 M) but increasing the pH to 7.2 resulted in a decrease in the first (globulin) peak and an increase in the pre- and post albumin peaks. With an 0.02 M tris-phosphate buffer pH 8.0 similar results were obtained, but in this case the post-albumin peak was small. Changing the buffer to EDTA-tris, pH 8.6 and pH 7.2 had the interesting result of completely preventing the binding of protein. Thus for the purification of albumin, the recommended buffer is 0.02 M phosphate pH 6.6. In a similar fashion, the fractionation of rat serum using C.M. Sephadex was attempted. This, however, was not satisfactory as only two large peaks were obtained.

Fig.36 illustrates the degree of purification of a crude rat serum albumin preparation which was achieved using the 7.0 x 1.2 cm column. It was found that the gradient apparatus described could be applied directly to the fractionation of approximately 1 gm of protein on a 16 x 2 cm DEAE-Sephadex column. A crude preparation of albumin obtained from slightly haemolysed serum by approximate half-saturation with ammonium sulphate was applied to such a column with the result shown in fig.37 - there is good separation from pre- and post albumin peaks and from haemoglobin, and contamination with non-albumin protein has been considerably reduced by the salt fractionation step. However, in order to obtain a sample of albumin which is pure by the criterion of immuno-electrophoresis, the albumin fraction obtained from the columns as described above must be subjected to column electrophoresis, as described previously. The satisfactory fractionation of 10 mg of

Fig. 38. CHROMATOGRAPHY OF DISRUPTED MICROSOMES ON DEAE - SEPHADEX



rat serum was achieved by using a DEAE-Sephadex column 4 cm long x 0.3 cm diameter, reducing the mixing vessels to 150 ml capacity, collecting 3 ml fractions of eluate and estimating the protein content of the eluate from the extinction at 210 mμ (Tombs, Souter and MacLagan, 1959).

In summary then, serum can be separated into several fractions by elution from DEAE-Sephadex with a gradient of sodium chloride, and a very pure sample of albumin can be obtained by approximate half-saturation of serum with ammonium sulphate followed by elution from DEAE-Sephadex with a sodium chloride gradient and a final purification by column electrophoresis.

The fractionation of the soluble material from microsomes was next attempted using the DEAE-Sephadex column procedure. In the first attempts the soluble material was dialysed for 52 hours against 0.02 M phosphate pH 6.6 in order to remove deoxycholate and sucrose, before application to the column. When the eluate from the column was obtained and examined in the spectrophotometer as previously described, only 2 fractions were detected. The first, material which had passed directly through the column, consisted mostly of RNA, as did the second fraction which did not appear till the salt concentration had reached 0.40 M. The protein could not be eluted from the column. After several repetitions of this failure, lyophilised microsome disruptate was dissolved in 0.02 M phosphate pH 7.4, applied to the column and elution carried out as before, with the results shown in fig.38. As before some RNA was not bound on the column. This was followed rapidly by a large amount of protein, then by small amounts of RNA and protein. These results

indicate that if albumin is present in microsomes it is not detectable by chromatography on columns of DEAE-Sephadex.

4. Acid-ethanol extraction: Before applying the procedure described by Charlwood (1961) and Michael (1962) to the isolation of albumin from rat liver microsomes, both methods were applied to rat serum. As neither author discusses the effect of temperature on the extraction procedure, all operations were carried out at room temperature. With each procedure, samples of albumin were obtained from serum which on electrophoretic analysis contained no globulin but which invariably contained a small quantity of material, possibly denatured protein, which did not migrate from the origin.

As the method of Charlwood (1961) is more straightforward, an attempt was made to isolate albumin from microsomes by applying the procedure directly to soluble microsomal material from which deoxycholate and sucrose had not been removed. It was soon discovered, however, that deoxycholate was extracted by the acid-ethanol and was not readily removed by dialysis. This latter observation may have been due to the pH during dialysis not rising above 7. This difficulty was not overcome by adopting the method of Michael (1962) because deoxycholate was precipitated as well as protein from the acid-methanol extract at pH 6. Removal of deoxycholate and sucrose on a Sephadex column as previously described, did not result in the preparation of albumin in reasonable yield although in occasional preparations a very faint albumin band was detectable on electrophoresis.

The method described by Campbell (1962) based on that described by Peters (1958) in which 80% ethanol buffered with phosphate buffer pH 2.3 is used, was then applied to the isolation of albumin from microsomes. Freeze-dried microsome material free from deoxycholate and sucrose was used as the starting material. A preliminary treatment with ultrasonic waves was found to be necessary for maximal yield of albumin (see Campbell, Greengard and Kernot, 1960). The amount of salt present during extraction was also found to be important so that the following procedure was adopted. Deoxycholate and sucrose were removed from the soluble microsome material by passage through Sephadex as previously described; the buffer, however, was modified to contain 0.4% NaCl. The soluble material, free from deoxycholate and sucrose was then subjected to ultrasonic waves in a "Raytheon" disintegrator for 5 mins before freeze-drying. The lyophilised material was then suspended in 0.05 M sodium phosphate buffer pH 2.4 (1 ml for the microsomal material from up to 40 gm liver), and 4 volumes of 80% ethanol pH 2.4 (adjusted by the addition of 1.0 N HCl). After shaking and centrifuging the extract was dialysed against several changes of distilled water, centrifuged to remove precipitated material and freeze-dried. The resulting white, fluffy solid contained protein and behaved as albumin on electrophoresis (fig.30). However, the preparation contained RNA, as the absorption spectrum showed a peak at 260 mμ, not at 278 mμ which is the absorption maximum of pure rat serum albumin (Peters, 1962). In addition, the total yield of material was extremely low: only rarely did it approach that claimed by Campbell and Kernot (1962) of up to 350μg from 8 gm of liver.

Discussion

The preliminary investigations of rat serum indicate that it behaves in a similar fashion to human and bovine serum on electrophoresis and in the analytical ultracentrifuge. The 's' values of albumin (4.6) and the globulin fraction (6.8) are identical with those of human and bovine serum (Cooper, 1960).

The development of a method for the preparation of a pure sample of rat serum albumin from rat serum, based on a preliminary salt fractionation step, followed by column chromatography and finally by column electrophoresis is of interest, since it avoids any factors which result in denaturation and eliminates the use of alcohol. In addition, it is rapid and convenient.

The failure to obtain a pure sample of albumin from microsomes by any of the procedures adopted, including the acid-ethanol methods, confirms the conclusion of the previous section that either the treatment of microsomes with deoxycholate and pyrophosphate does not achieve disintegration into molecules of protein, RNA and phospholipid or that the subsequent removal of deoxycholate and pyrophosphate results in random aggregation of the various types of molecule present.

Bearing in mind that the principal aim of the present experiments is the study of the effects of diet on protein synthesis, it would be best at this stage to abandon the attempt to obtain a satisfactory method for the preparation of rat serum albumin from microsomes and proceed with the study of the influence of diet on the uptake in vitro of labelled amino acids into the protein of the microsomes of rat liver, and not into a specific protein such as albumin.

This decision was influenced by several factors .

(a) The yield of albumin from microsomes using the method of Campbell and Kernot (1962) was very low.

(b) Further purification of the albumin would be difficult.

(c) If studies of the incorporation of labelled amino acids into albumin in vitro were attempted, the quantity of microsomes and hence the amounts of ATP, GTP, creatine phosphate and ^{14}C-leucine would be so large that the expense involved would permit only one or two such experiments.

(d) The sample of albumin obtained by Campbell and Kernot (1962) from microsomes using their acid-ethanol procedure when subjected to tryptic hydrolysis and electrophoresis did not give a pattern identical with that obtained from rat serum albumin.

(e) Although Peters (1962b) used an immunological method to obtain albumin from rat liver microsomes, there is evidence that portions of the albumin molecule react antigenically as albumin (Porter, 1957), and Decken (1963) has shown after careful investigation that the albumin-like protein of ribosomes which can be labelled following in vitro incubation with labelled amino acid, is not in fact albumin.

It may be possible at a later stage, following the study of the in vitro incorporation of amino acids into microsome protein, to achieve fractionation of the microsome proteins using 6 M urea or 6 M guanidinium hydrochloride following the successful fractionation on electrophoresis of the proteins of the ribosomes of E.coli into 20 components by Walter and Harris (1961).

Section 4 Studies in Protein Synthesis

The study of the rate of protein synthesis is a complex problem. Since the rate of breakdown is more readily measured (see comments of protein turnover - page 14), it is usual to maintain an experimental animal in the steady state and infer that the rate of synthesis of a protein equals its rate of breakdown. The direct measurement of the rate of synthesis of a protein in vivo is complicated by several factors among which are: (a) the choice of isotope is limited to ^{14}C, ^{35}S or tritium; (b) recycling of these isotopes may occur; (c) detection of the newly synthesised protein depends on its role and fate after synthesis; for example, plasma proteins will be rapidly found in the blood, whereas intracellular proteins will require a more or less complicated procedure of extraction and purification; (d) it is necessary to know the specific activity of the intracellular precursor amino acids.

McFarlane (1963) has overcome some of these technical difficulties by the use of 6-^{14}C-arginine. The amino acid labelled in this way gives rise to urea and is also incorporated into albumin. The specific activity of the urea corresponds to the activity of the free arginine pool in the hepatocyte and so enables the important correction to be made in the calculation of the rate of synthesis of serum albumin.

The information to be gained from such in vivo studies is limited, however, and it was thought that an exploration of protein synthesis in vitro might throw some light on the mechanism by which the response of the liver to diet is effected, despite the possible limitation that rates of synthesis in vitro might not equal the in vivo

rates of synthesis.

The initial reactions of protein synthesis and the roles of microsomes and polysomes have been discussed in the introduction to Part 2 (page 58). The present views on the mechanism of protein synthesis and its control have been admirably summarised recently by Watson (1963). It is now accepted that the amino acids linked to sRNA are assembled on polysomes (Wettstein, Staehelin and Noll, 1963; Warner, Knopf and Rich, 1963; Gierer, 1963), that the messenger RNA which links the ribosomes 'codes' for the amino acid by selecting the appropriate sRNA molecule, and that the ribosomes temporarily bind sRNA until the peptide bond is formed (Watson, 1963). As the peptide bonds are formed the ribosomes and mRNA move relative to each other so that continuous addition of amino acids to the peptide chain which remains attached to the ribosomes, takes place (Watson 1963; Staehelin, Wettstein, Oura and Noll, 1964). In this way several polypeptide chains are synthesised sequentially and simultaneously on the polysome. Thus, several polypeptide molecules are synthesised on one mRNA molecule and the size of the polysome is related to the size of the polypeptide synthesised by it. From several observations, Staehelin, Wettstein, Oura and Noll (1964) estimate that the distance between ribosomes in the polysome structure is of the order of that occupied by 90 nucleotides in the molecule of messenger RNA. This would correspond to 30 amino acids so that a 'pentasome,' that is a polysome consisting of 5 ribosomes linked by mRNA would be responsible for the synthesis of a polypeptide chain of 150 amino acids which would have a molecular weight of approximately

17,000. This is the molecular weight of a single polypeptide chain of haemoglobin and must be related to the observation that in reticulocytes the order of polysome which occurs most frequently is the 'pentasome' (Gierer, 1963; Williamson, 1963). In passing, it may be of interest that on this basis, serum albumin must be synthesised on a polysome containing 15 ribosomes.

Recently, Staehelin, Wettstein and Noll (1963) have shown that the number of polysomes containing large numbers of ribosomes is reduced by the administration of Actinomycin D. This antibiotic has been widely used in studies of the control of protein synthesis and production of mRNA (Baltimore and Franklin, 1962; Greengard, Smith and Acs, 1963). The structure of Actinomycin D is known (Reich, Goldberg and Rabinowitz, 1962) and its mode of action is believed to be due to the formation of a specific complex with DNA which prevents the synthesis of RNA and mRNA in particular on the DNA template (Reich, 1963). When Actinomycin D is administered intraperitoneally or intravenously to rats, protein and RNA synthesis in the liver is reduced to a variable extent. In the experiments of Greengard, Smith and Acs (1963), the in vivo incorporation of ^{14}C-leucine into protein was reduced by 10% and at the same time, the incorporation of ^{32}P into RNA was reduced to 60% of the control value.

Staehelin, Wettstein and Noll (1963) have shown that over a period of from 4 to 18 hours after the administration of Actinomycin D, the number of larger polysomes gradually declines while the quantity of single ribosomes increases. There exists then in the liver, in the polysome system, a mechanism for the control of both the rate and

type of protein synthesis which is mediated by 'messenger' RNA. However, the existence of polysomes free as in reticulocytes has not been demonstrated in rat liver. In the studies on polysomes in rat liver, the mitochondria-free supernatant is first treated with deoxycholate to remove the lipid-containing membrane material. Thus, the polysomes occur in the microsome fraction (see Henshaw, Bojarski and Hiatt, 1963) although attempts by Hallinan (1964) to demonstrate that polysomes are related to the rough-surfaced vesicle (RSV) fraction of the microsomes have so far not been successful.

In the methods of preparing polysomes (Wettstein, Staehelin and Noll, 1963) and ribosomes (Korner, 1961) lies the possible explanation for the claims that large polypeptides such as albumin could be synthesised by ribosomes. Ribosomes were commonly prepared from rat liver by treatment of the mitochondria-free supernatant with deoxycholate then centrifuging at 105,000g for 1 hour (Korner, 1961; Kirsch, 1962), conditions in which polysomes would also be obtained in the pellet.

In the previous discussion of the possible effects of diet on protein synthesis in rat liver (page 67), it was suggested (a) that the rate of synthesis of protein per unit of RNA or protein of microsomes did not change but that the total amount of microsomes did change in response to diet, or (b) that the actual rate of synthesis of protein did vary with the availability of amino acids. It has been confirmed in section 1 (page 69) that the amount of microsomes does vary in response to diet. It remains then to decide whether the rate of protein synthesis in relation to the RNA and

protein of microsomes is affected by diet.

For the projected in vitro studies of the effects of diet on protein synthesis, a choice of the basic system, that is microsomes, polysomes, or ribosomes had to be made. In general it is usually best to carry out preliminary investigations with a crude system, then to fractionate or refine it. The microsome system was therefore selected for investigation.

In their early investigations of the in vitro incorporation of labelled amino acids into a microsome system, Zamecnik and Keller (1954) prepared the microsome fraction in 0.25 M sucrose solution buffered with phosphate and containing potassium and magnesium. Their conditions required an ATP generating system (creatine phosphate and kinase or phosphoenol pyruvate and kinase), labelled amino acid, microsomes and a soluble, heat labile, non-dialysable fraction. Later studies in the same laboratory (Hoagland, Keller and Zamecnik, 1956; Keller and Zamecnik, 1956) showed that amino acids were activated at the carboxyl group by an enzyme which could be precipitated from the cell sap at pH 5 (known as the pH 5 enzyme), and that for the transfer of amino acids to microsome protein guanosine nucleotides were required. All subsequent in vitro microsome systems used in studies of the incorporation of amino acids into protein have been based on these findings and contain a suspension of microsomes, ATP and an ATP generating system, GTP, a pH 5 enzyme fraction and a radioactive amino acid (see Hoagland 1960; Korner, 1964). Following on the observations of Schneider (1948) that particulate fractions prepared from rat liver retained greatest

enzyme activity when the fractionation was carried out in isotonic (8.5 gm per 100 ml, approximately 0.25 M) sucrose, this has been almost universally adopted in the preparation of microsomes for protein synthesis studies. Thus, Campbell, Greengard and Kernot, (1960) utilised the same buffer mixture as Rendi and Campbell (1959) which in turn was based on the phosphate buffer pH 7.4 containing potassium bicarbonate and potassium chloride (see table 25) which had been used by Zamecnik and Keller (1954). Littlefield, Keller, Gros and Zamecnik (1955) in their studies on ribosomes also used 0.25 M sucrose, as did Allen and Schweet (1962) in their investigations of haemoglobin synthesis. Since incorporation of ^{14}C-leucine in vitro into rat serum albumin had been claimed by Campbell, Greengard and Kernot (1960) using a microsome preparation from rat liver, it was decided to follow their procedure in the study of the effects of diet on the amino acid incorporating ability of rat liver microsomes.

Experimental Methods and Results

Experimental Animals: These were young male animals (150 gm weight) of the departmental stock. The high protein and low protein diet and feeding regimen have been described previously (page 30 and tables 6,7,8 and 9). The diet was fed for 3 days prior to the experiment and the animals fasted overnight (16 hours) before sacrifice. On occasions which are specified, certain animals were offered 2 gm of casein mixed with a few mgm of sodium bicarbonate and some water 2 hours before the experiment; it was always carefully noted subsequently whether the animals had eaten and whether the stomachs were full; animals which had not eaten satisfactorily were rejected from the experiment.

Preparation of the microsome fraction

The animals were invariably killed by a sharp blow on the head, the liver rapidly removed and immersed in ice-cold sucrose buffer, to cool it and remove excess blood. After being blotted dry, the liver was rapidly weighed, then transferred to a cold beaker, 2.5 volumes of cold 0.35 M sucrose buffer added and stirred. Homogenisation was then carried out using a Potter-Elvejhem homogeniser and the microsome and cell sap fractions obtained as previously described (page 72 - see fig.23). The precipitated microsome fraction was resuspended in 0.25 M sucrose-buffer by gentle homogenisation at half speed in the Spinco tube. Centrifugation was carried out once more at 105,000g for 1 hour to yield a 'washed' microsome fraction. The resultant washed microsome pellet in an 11 ml capacity Spinco tube was gently stirred up in 0.5 ml 0.25 M

sucrose buffer of the same composition as that given in table 23, except that 0.25 M sucrose was substituted for 0.35 M sucrose and the pH was adjusted to 7.4. A further 0.5 ml sucrose-buffer was then added and resuspension completed by homogenising at half speed in the Spinco tube. At this stage, 0.1 ml samples of the suspension were removed and suitably diluted, and a 1 ml sample of the solution taken for the estimation of protein by the method of Lowry, Rosebrough, Farr and Randall (1951). A calculated volume of 0.25 M sucrose-buffer was then added to adjust the protein content of the microsome suspension to 35 mg/ml.

Preparation of the pH 5 fraction

In each experiment, in order to avoid variations which could be ascribed to the effects of diet on this fraction, the cell sap obtained from all the livers was 'pooled' and the pH 5 fraction obtained by adding cold 1 N acetic acid until pH 5 was attained. The suspension was allowed to stand for 15-20 minutes, then centrifuged for 10 minutes at 2,500 r.p.m. to yield the pH 5 precipitate. The supernatant was carefully drained off and 0.25 M sucrose-buffer added (2 ml per 14 gm liver) to resuspend and dissolve the precipitate. As before, a 0.1 ml sample was removed, diluted and the protein content estimated by the method of Lowry et al. (1951), so that the protein content of the pH 5 fraction when made up in sucrose-buffer was 20 mg per ml.

Incorporation of amino acid into microsomes

(a) Incubation procedure:

The contents of the incubation mixture used in the preliminary

Table 40

Composition of the Incubation Mixture used in in vitro Studies on Protein Synthesis (Series I)

Component	Amount present	Volume added	Concentration of solution
ATP	1 µm	50 µl	20 µm/ml
GTP	0.25 µm	50 µl	5 µm/ml
Pyruvic kinase	0.02 mg	50 µl	0.4 mg/ml
Phosphoenol pyruvic acid	10 µm	50 µl	200 µm/ml
pH 5 fraction	1 mg protein	50 µl	20 mg protein per ml
Microsome suspension	7 mg protein	200 µl	35 mg protein per ml
DL-leucine-1-^{14}C	0.72 µC	100 µl	7.2 µC/ml
0.25 M sucrose phosphate buffer	-	450 µl	-
Total volume	-	1.000 ml	-

studies of the effect of previous feeding on the in vitro uptake of labelled amino acid by rat liver microsomes are given in table 40.

The disodium salts of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) and the trisodium salt of phosphoenol pyruvic acid (PEP) were obtained from Sigma Chemical Company. After being dissolved in 0.25 M sucrose-buffer the pH was adjusted to 7.4 before finally making up to the appropriate volume. It was found that although solutions of ATP and GTP would store at -10°C without loss of activity, the solution of PEP had to be made up fresh immediately before each experiment.

Phosphoenol pyruvate kinase was obtained from Biochimica Boeringer, and the appropriate quantity dissolved in 0.25 M sucrose-buffer immediately before use. DL-leucine-1-^{14}C was obtained from the Radiochemical Centre, Amersham, and made up to the appropriate concentration in 0.25 M sucrose-buffer before use. This solution could be stored at -10°C for an indefinite period of time.

Incubation was carried out with shaking in a thermostatically controlled water bath at 37°C.

(b) Assay of radioactivity:

At the end of the incubation period, the centrifuge tubes containing the incubation mixture, or the samples therefrom (0.2 ml) were plunged into a freezing mixture of Drikold (solid CO_2) and industrial alcohol, frozen solid and stored overnight at -10°C.

For analysis, the contents of the centrifuge tube (0.2 ml) were allowed to thaw at 0°C, then protein and nucleic acid was precipitated at a concentration of 0.2 N perchloric acid (PCA) e (ice-cold) to which DL-leucine had been added. The precipitate was

centrifuged and washed twice with cold 0.2 N PCA, then the excess acid was drained off and 4 ml 0.3 N KOH added. The alkaline solution was incubated at 37°C in air for 1 hour to hydrolyse RNA, cooled, acidified to 0.2 N PCA, centrifuged, the precipitate washed twice with ice-cold 0.2 N PCA, and the residue finally carefully drained free of acid. At this stage the supernatant and washings could be combined and made up to a suitable volume for the estimation of RNA. The protein residue was dissolved in 0.3 ml, 0.3 N KOH which was kept cold in order to minimise the amount of potassium perchlorate in solution. Samples - 0.02 ml for protein estimation and 0.2 ml for ^{14}C assay - were then withdrawn, and the radioactivity assayed in a Nuclear Chicago automatic gas flow geiger counter. The results were expressed as specific activity (cpm per mg protein). Full details of the procedure and a discussion of the rationale of this method are given in the appendix and Part 3.

Results

The early experiments (Series I) were carried out using the 0.25 M sucrose-phosphate buffer (see table 23) and incubation mixture described in table 40. The radioactive assay technique used was satisfactory for up to 3 mg protein. This was demonstrated by taking a radioactive protein sample and plating out on lens paper in the planchette amounts of the solution containing 1,2 and 3 mg of protein and showing that the specific activity obtained in each case was the same.

The problem of a control for the incorporation studies was solved by taking a 0.2 ml sample from the incubation tube at zero time,

Table 41

Effects of Diet on the Incorporation of DL-Leucine-1-^{14}C into Rat Liver Microsomes in vitro

Diet	cpm/mg protein*	Mean specific activity
High protein	125	
	186	137
	100	
Low protein	174	
	240	175
	110	

*The figures are obtained from the incubation of microsomes from the livers of individual rats. The incubation period was 30 minutes.

that is before transferring the incubation mixture from the ice bath to the 37°C water bath, and also by setting up a tube for incubation in parallel with the others from which ATP, GTP, phosphoenol pyruvic acid and pyruvic kinase were omitted and 200 µl of 0.25 M sucrose-phosphate buffer had been substituted (zero energy control tube). The method of preparing the microsome protein for radioactive assay was shown to be satisfactory by the observation that the radioactivity of the zero time samples was invariably approximately equal to background. However, it was found that the radioactivity of the zero energy control increased with incubation time, so that it was necessary to express experimental results as 'net specific activity' i.e. specific activity of test sample - specific activity of zero energy control tube. The radioactivity of the zero energy control was in most cases not excessively high. However, in certain experiments high specific activities of protein in the zero energy control tubes were obtained. Attempts to nullify this effect by lipid extraction failed, owing to the fact that the solvents used extracted some protein and RNA. It was later found that the use of a Sireica teflon Potter-Elvejhem homogeniser and the adoption of a sucrose-tris buffer eliminated this difficulty (see later).

The figures of table 41 are obtained from a preliminary experiment in which the in vitro activity after 30 minutes incubation of microsomes prepared from the livers of animals fed high protein and low protein diets and fasted for 16 hours before sacrifice was compared. The microsomes prepared from the animals fed on the low protein diet were more active than those from the high protein fed

Fig. 39. TIME COURSE OF INCORPORATION OF ^{14}C-LEUCINE INTO MICROSOMAL PROTEIN

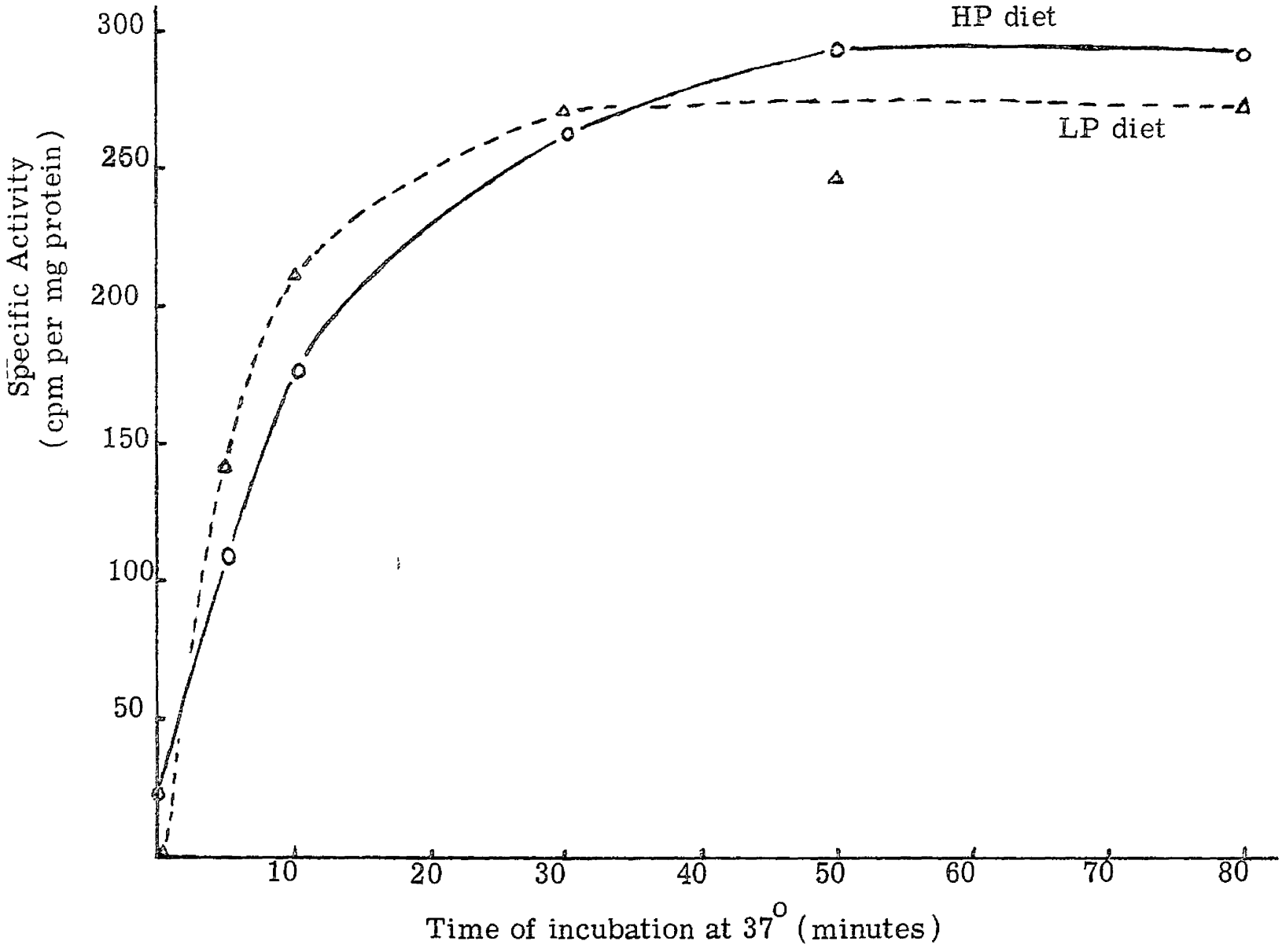
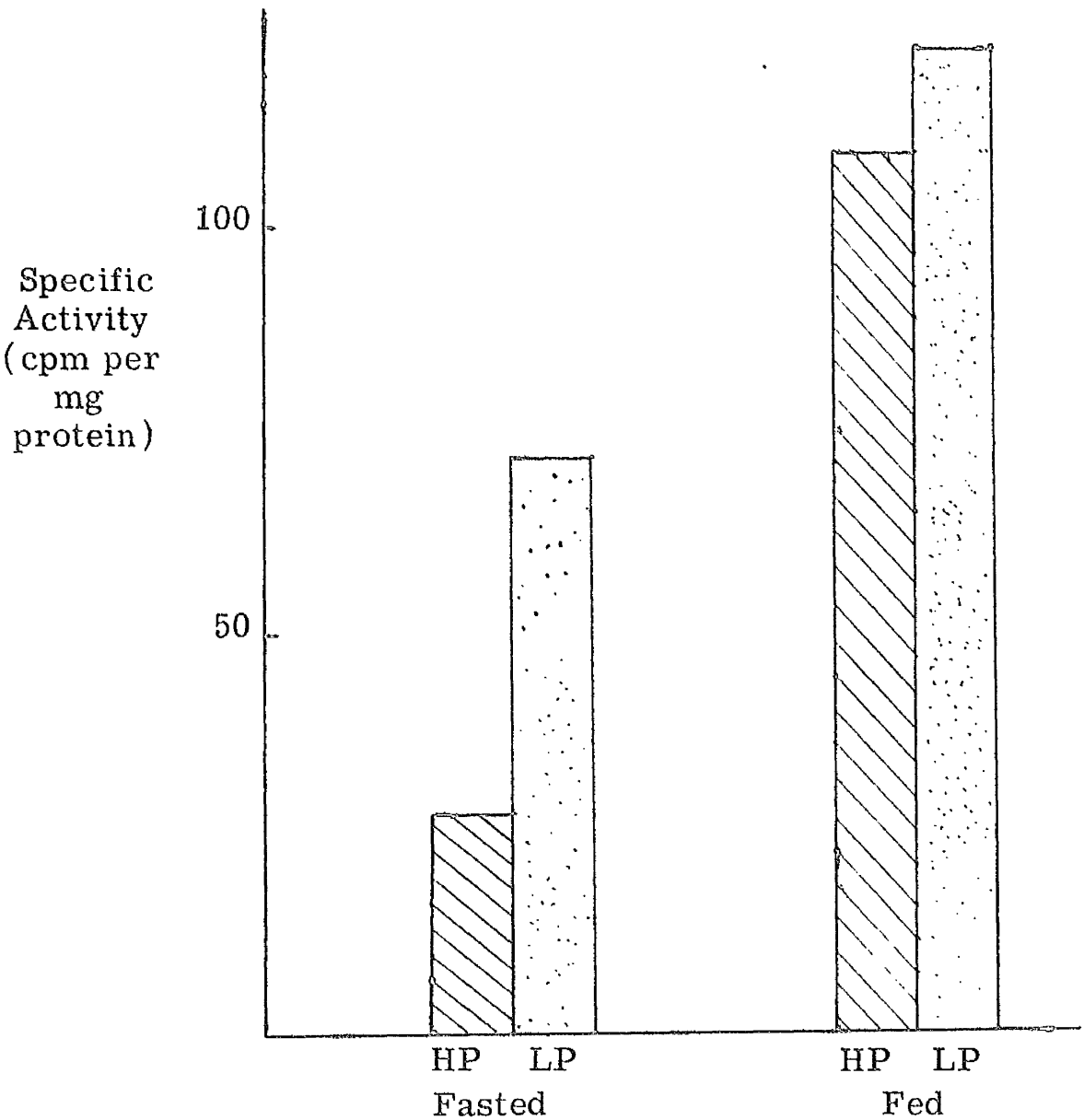


Fig. 40. THE EFFECT OF FEEDING PROTEIN ON THE ACTIVITY OF MICROSOMES

(uptake of D L leucine-1-^{14}C after 15 mins. incubation).



animals. However, the variation of these results was undesirably high, and it was considered that an investigation of the time course of the reaction would be of interest. The results of such an experiment, in which microsomes were prepared from the livers of 6 animals (3 fed HP diet, 3 LP diet and all 6 fasted 16 hours before removal of the liver), were incubated separately and 0.2 ml samples withdrawn at different times during incubation are shown in fig.39. The points on the curves correspond to the mean value of the net specific activity of each dietary group at each time interval. Here again, the activity of the microsome fraction from the LP diet animals is greater than that of the animals maintained on a high protein diet. It is obviously impossible to calculate the reaction velocities at zero time, but it is apparent that comparison of the uptake of ^{14}C-leucine by different microsome preparations should be made at times of 5-15 minutes of incubation and not at 30 minutes.

It was next decided to investigate the effects of feeding protein immediately prior to removal of the liver and preparation of microsomes for the study of their amino acid uptake capabilities. For this purpose, rats were fed 2 gm casein 2 hours before removal of the liver, preparation of the microsome fraction and incubation as before. The results of such an experiment are shown in fig.40. Once more, the activity of the microsomes from the LP fasted rats is greater than that of the HP fasted rats. The difference is not so great when casein is fed, but it is notable that feeding casein 2 hours before killing is associated with an increase in the uptake of ^{14}C-leucine by microsomes.

Table 42

Composition of Sucrose-Tris Buffers used in the Preparation of Microsomes for Studies in Protein Synthesis

	A	B
Sucrose	0.35 M	0.25 M
"Tris"*	0.04 M	0.04 M
KCl	0.025 M	0.025 M
$MgCl_2$	0.010 M	0.010 M
pH	7.8	7.4

* [2-amino-2-(hydroxymethyl)-propane-1:3-diol]

Table 43

Composition of the Incubation Mixture used in in vitro Studies on Protein Synthesis (Series II)

Component	Amount present	Volume added	Concentration of solution
ATP	2 µm	100 µl	20 µm per ml.
GTP	0.5 µm		5 µm per ml.
Creatine phosphate	14.8 µm	100 µl	148 µm/ml
Creatine kinase	0.040 mg	100 µl	0.2 mg/ml
DL-Leucine-1-^{14}C	0.075 µC	100 µl	0.75 µC/ml
pH 5 fraction	1 mg protein	100 µl	10 mg protein per ml
Microsome suspension	7 mg protein	500 µl	14 mg protein per ml
Total volume		1.000 ml	

Fig. 41 PRELIMINARY INVESTIGATION OF THE MICROSOME INCUBATION SYSTEM PREPARED IN 'TRIS' BUFFER

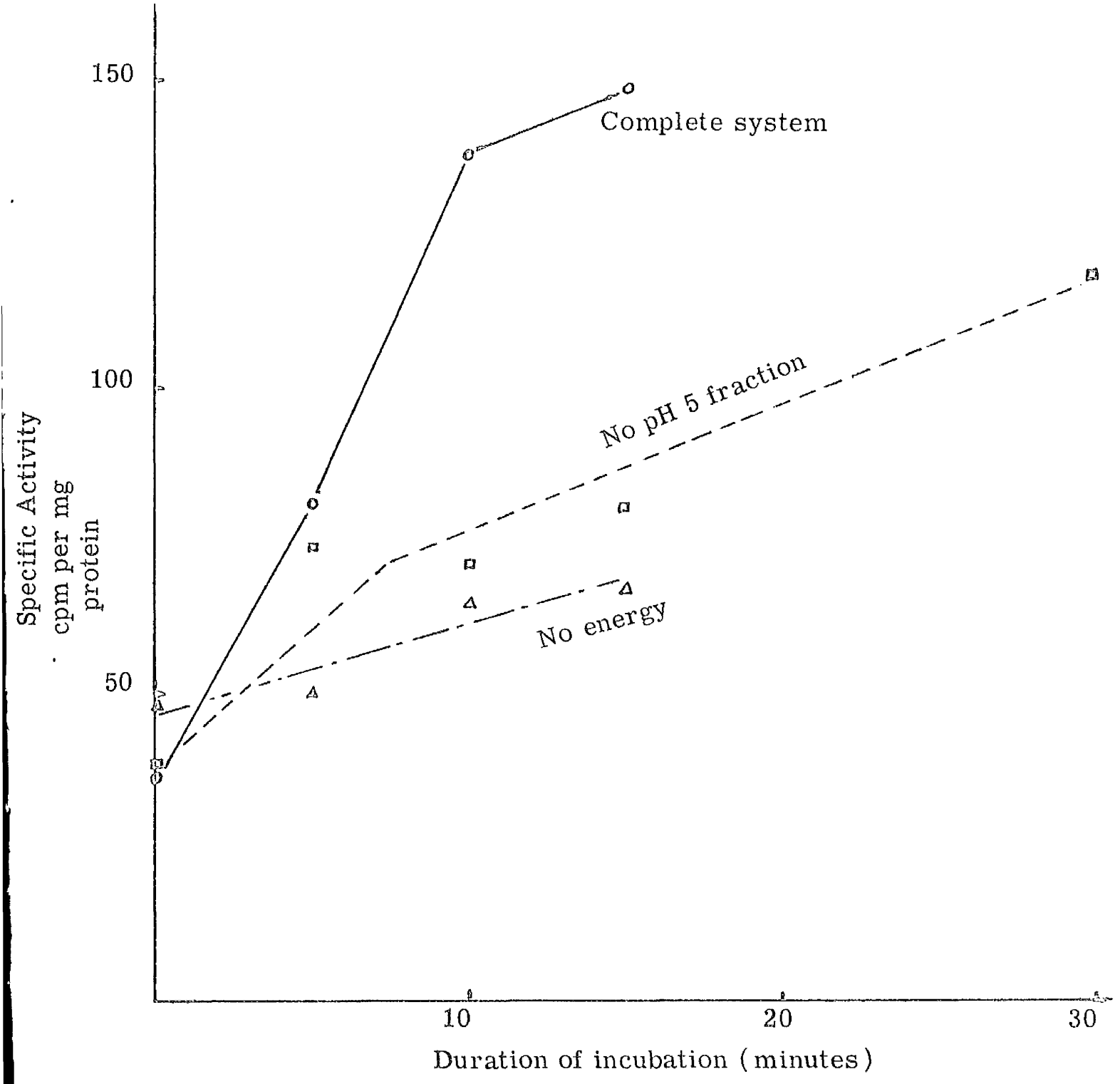
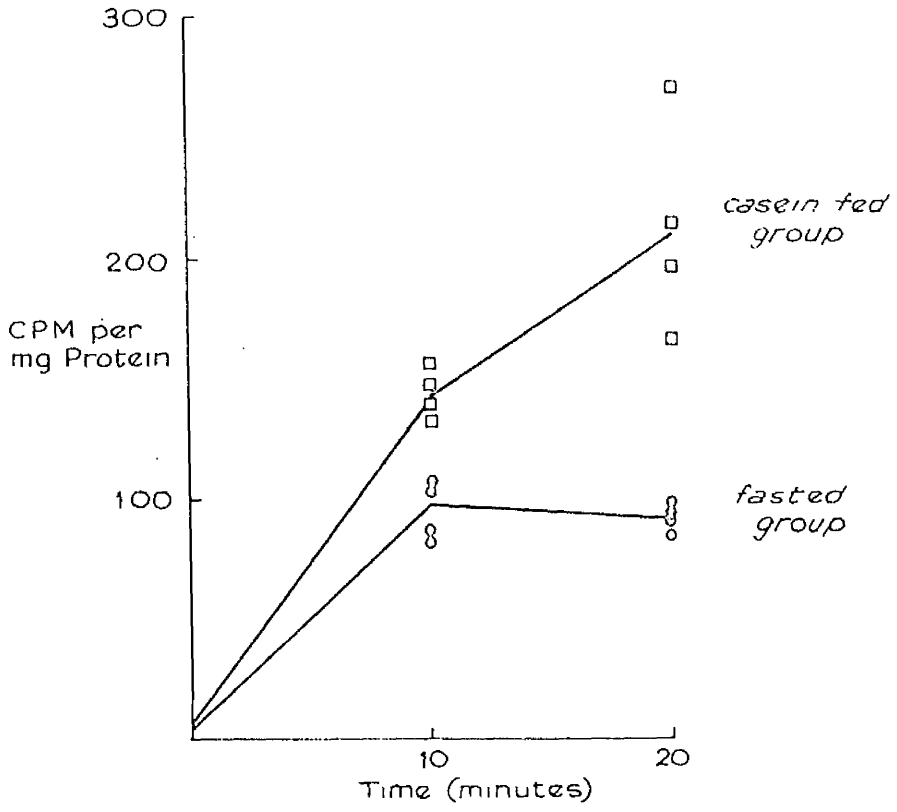


Fig. 42 . EFFECTS OF FEEDING PROTEIN ON ACTIVITY OF MICROSOMES

Uptake of ^{14}C D-L Leucine by washed microsome fraction from rat liver. Animals were maintained on protein-free diet prior to experiment pH 5 preparation was "pooled" from all livers



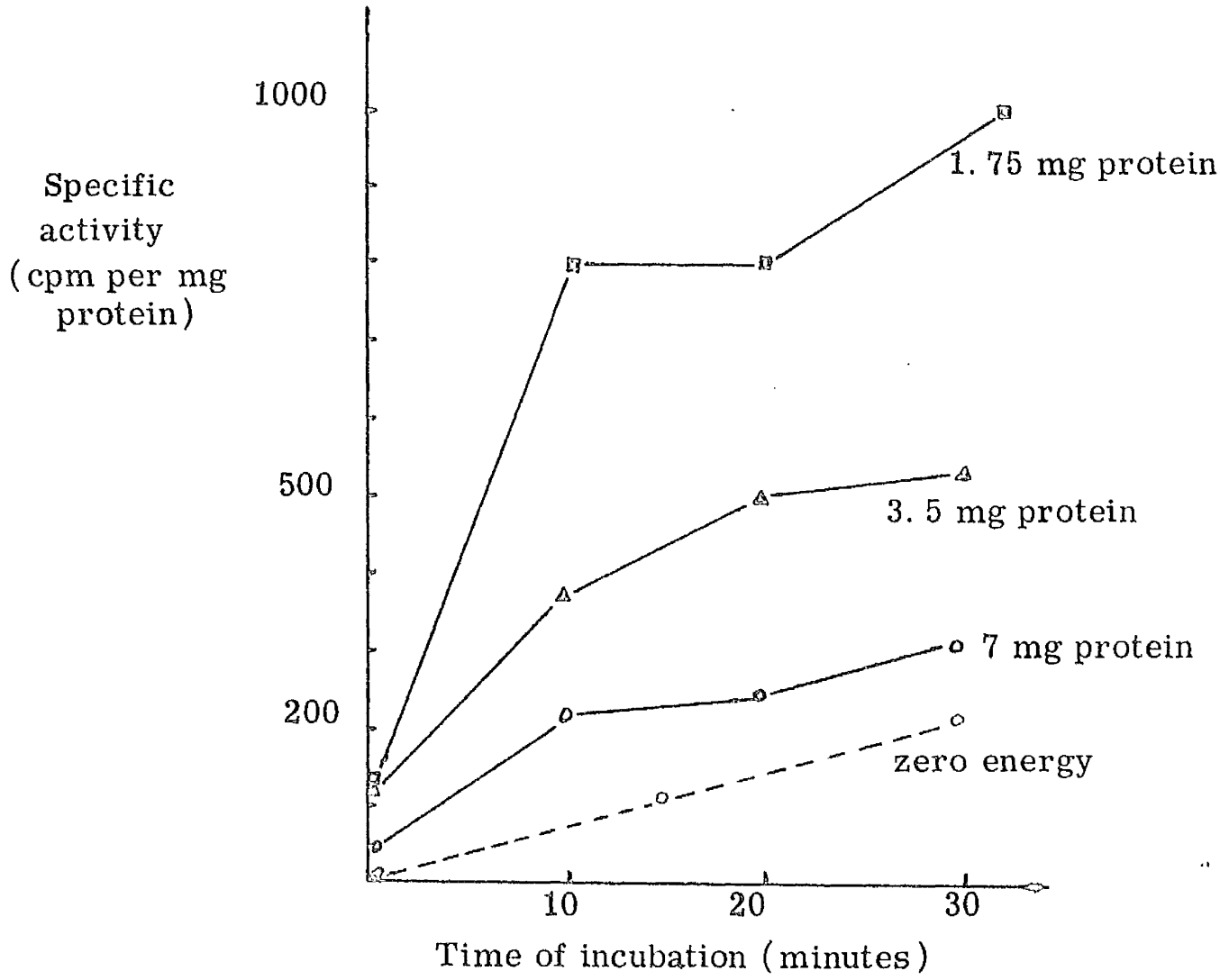
Attempts to confirm these results were disappointing and it was concluded that it would be profitable to establish firmly the observation that feeding casein 2 hours before removal of the liver led to a stimulation of the uptake of ^{14}C-leucine by the microsome fraction. Because of the more variable results obtained with the animals fed the HP diet, attention was confined to animals deprived of protein. Difficulties were also encountered due to high zero energy incorporation of leucine by the microsomes, and because on storing the sucrose-phosphate buffer at 0^{o}C, a precipitate of magnesium phosphate formed.

For these reasons it was decided to change to sucrose-tris buffers (table 42) and to use teflon Potter-Elvejhem homogenisers made by 'Sirieca,' U.S.A. The composition of the incubation medium was essentially unchanged except for the change in buffer, but the constituents were added in different volumes (table 43) in order to save time and to improve the uniformity of the microsome suspension. A preliminary experiment using these modified conditions showed that the uptake of ^{14}C-leucine was satisfactory and that the omission of the pH 5 fraction and energy had the expected effects of markedly reducing the uptake of leucine (fig.41). The uptake of ^{14}C-leucine in vitro by the microsome fraction prepared from animals maintained on the protein-free diet for 3 days was then investigated. The results (fig.42) show that there is a clear difference in ^{14}C-leucine uptake by the 2 groups of animals: the uptake by those fed casein 2 hours before killing was greater than that of the animals which had been fasted overnight. This result was reproducible.

Since the pH 5 fraction was prepared from both the fed and fasted animals the observed stimulation of ^{14}C-leucine uptake by the microsomes must be due to a change in the microsome fraction. If this change or stimulation were due to increased production of mRNA, then the administration of Actinomycin D to the animal before feeding should prevent the increase in activity.

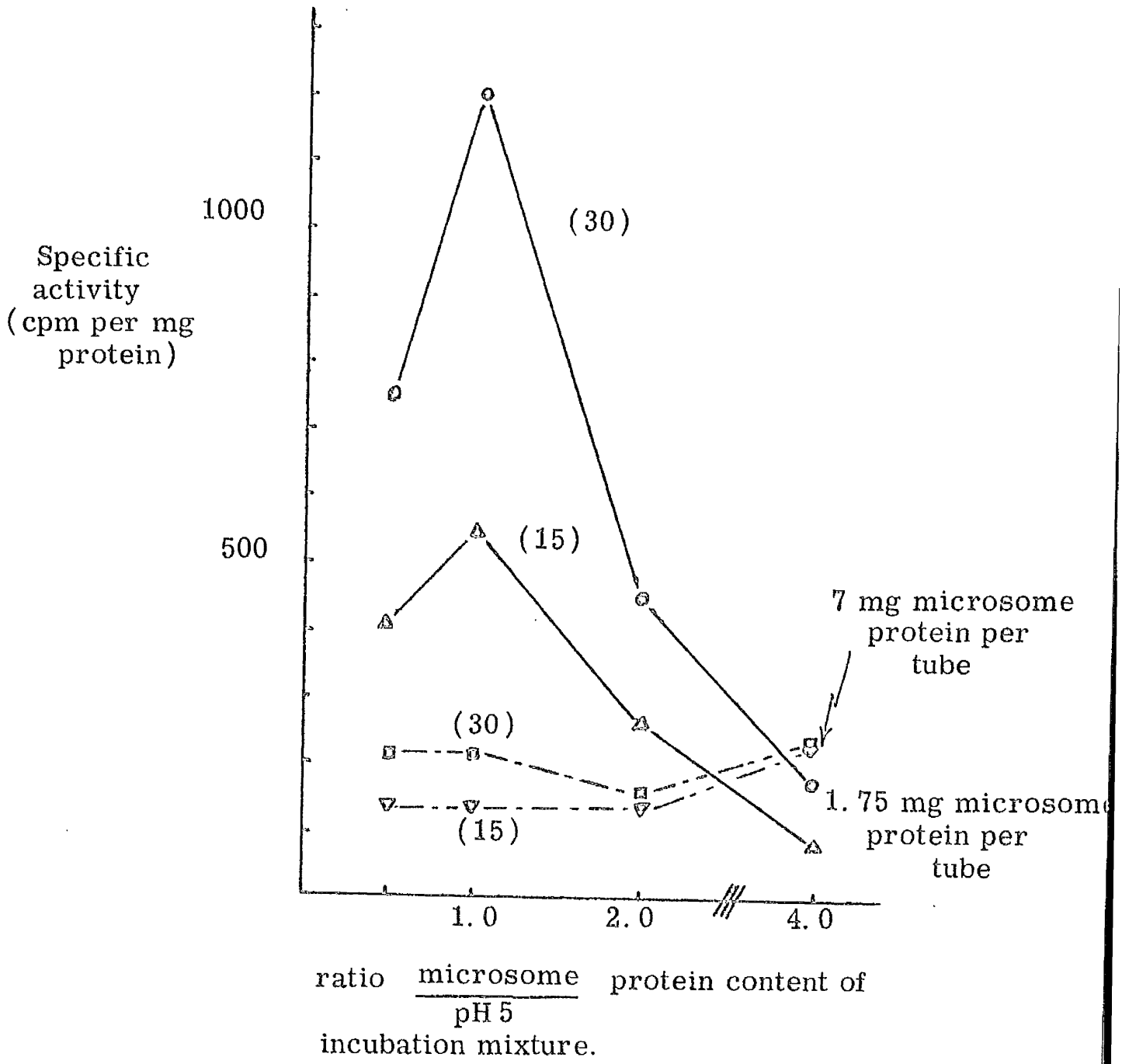
To test this hypothesis, 12 rats were given the LP diet for 3 days; at zero time 6 animals were given Actinomycin D (0.07 mg in 0.9% NaCl per 100 gm body weight), 1 hour later, 3 of the six were fed 2 gm casein. Two hours after feeding the animals were killed, the livers removed and the microsome fraction prepared as before. (This procedure was based on that successfully adopted by Greengard, Smith and Acs (1963)). In this experiment, for the first time L- [U-^{14}C] leucine was used instead of D-L leucine-1-^{14}C in the incubation mixture and protein was estimated by the modification by Miller (1959) of the method of Lowry et al. (1951). As a result, the specific activity of the microsome protein obtained after incubation was much higher than before, but the experiment was unsatisfactory due to the wide scatter of the results, although Actinomycin D reduced the activity of the protein-fed group to equal that of the fasted group. Since Actinomycin D is a very scarce antibiotic, it was decided to examine the conditions of incubation, in particular the ratio of microsomal to pH 5 protein before repeating the experiment. When the amount of pH 5 protein (1 mg per tube) in the incubation mixture was constant and the other additions, energy and L-leucine etc. were the same as before, reducing

Fig. 43. THE EFFECT OF VARIATION OF MICROSOMAL PROTEIN CONTENT ON THE UPTAKE OF ^{14}C-LEUCINE



Each point is the mean of duplicate determinations. The protein content indicated refers to the total quantity of microsomal protein in the incubation mixture.

Fig. 44. EFFECT OF VARIATION OF MICROSOME AND pH 5 PROTEIN ON THE UPTAKE OF ^{14}C-LEUCINE



Figures in brackets refer to incubation time.

the amount of microsome protein present caused an increase in the specific activity of the protein after incubation (fig.43). The relation between the specific activity obtained and the amount of microsome protein present in the incubation mixture was not linear. When the amounts of both microsome protein and pH 5 protein are varied but the amount of energy and L-[^{14}C-U] leucine is constant, a sharp optimum of net specific activity of protein (corrected for pH 5 fraction protein) after incubation was obtained (fig.44). The maximal incorporation occurred when the ratio of microsome protein to pH 5 protein was 1:1 and the amount of microsome protein was 1.75 mg per tube. No such optimum was observed when the incubation tubes contained 7 mg of microsome protein. Thus when conditions were optimal for maximal final specific activity of microsome protein, that is when the amount of microsome protein in the incubation mixture was small and equal to the amount of pH 5 fraction protein, the specific activity of the microsome protein after incubation will vary in a very sensitive fashion with the amount of microsome protein added to the incubation mixture.

These conditions are not ideal for testing the response to diet of the microsomes in vitro, as slight errors in the estimation of protein and dilution of the microsomes to the appropriate protein content could easily mask differences due to diet. Subsequent experiments using the new concentrations of microsomes and pH 5 fraction showed that the effect of diet was demonstrable but was not so striking as before. However, since it has been established that differences in diet can produce differences in the uptake of labelled

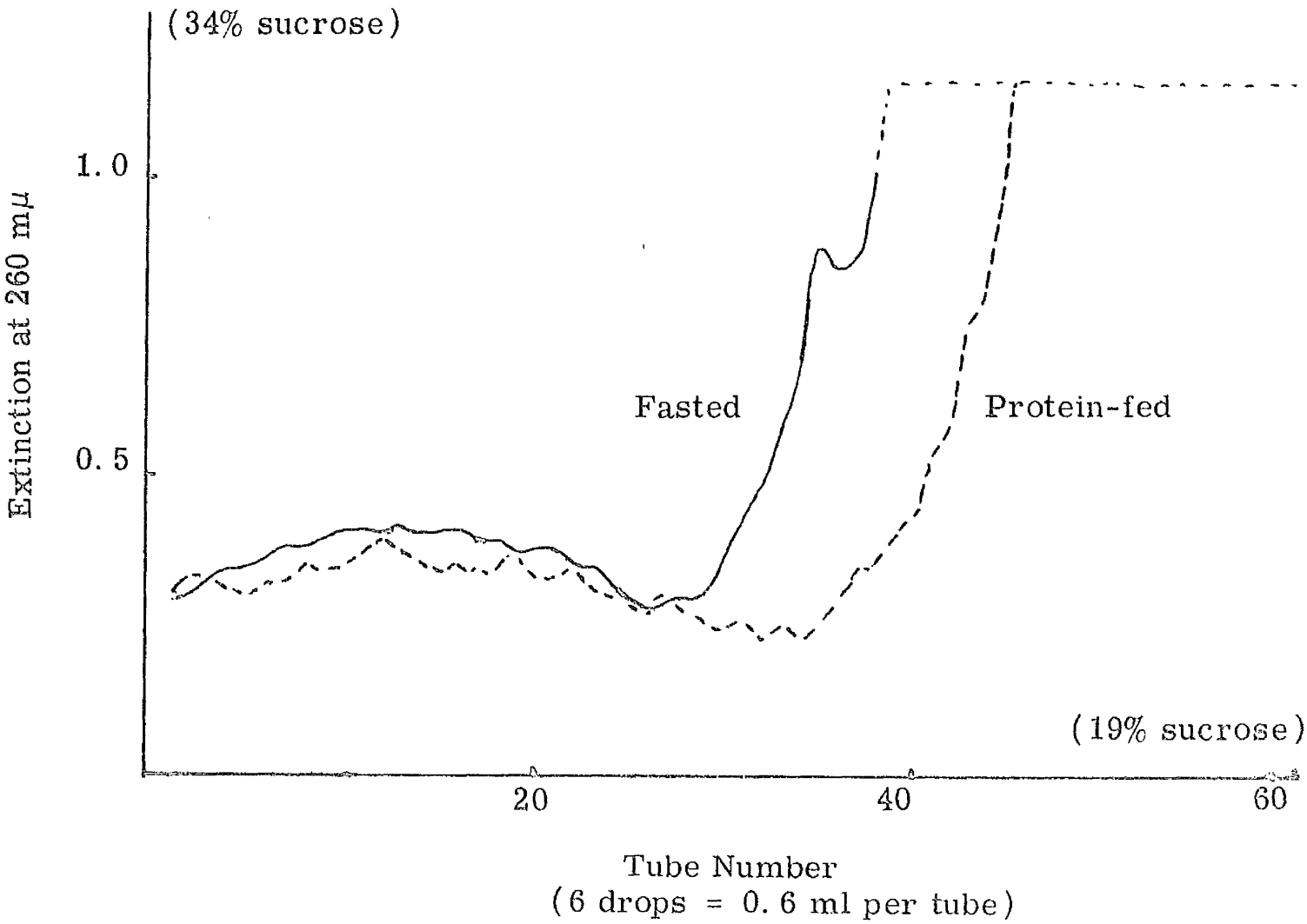
amino acids by the microsome fraction of rat liver, it was decided to investigate the effects of diet on the polysomes of rat liver.

The effects of diet on the polysomes preparation from rat liver

Experimental Methods: Liver was homogenised in sucrose-buffer and freed of nuclei, cell debris and mitochondria as before. To 5 ml of the mitochondria-free supernatant 0.75 ml of 10% sodium deoxycholate was added to dissolve the membrane fraction of the microsomes. A linear sucrose gradient (17-30% sucrose) was prepared in a 3 x 1 inch Spinco cellulose centrifuge tube by running 12% sucrose into an equal volume of 30% sucrose in an identical container, mixing by bubbling a stream of nitrogen through the second vessel (with the concentrated sucrose) and simultaneously slowly running off 25 ml of the solution into the centrifuge tube. In this way, highly reproducible linear sucrose gradients were produced. After adding deoxycholate to the mitochondria-free supernatant, 0.4 ml of the clear mixture was carefully layered on the sucrose gradient from a pipette. The tubes were then centrifuged in the Spinco SW 25 swing-out rotor at 53,500g (average) for 2½ hours. After centrifugation, the tubes were placed in a specially constructed perspex holder, pierced with a hypodermic needle and the apparatus placed over a Locarte fraction collector fitted with a photo-electric drop counting device so that the drops were counted as they formed and fell into the test tubes. Fractions containing 6 drops were collected. By using a dye and making up the solution to a given volume it was readily shown that despite the change in the viscosity of sucrose during this procedure, the size of the

Fig. 45. THE EFFECT OF PROTEIN FEEDING ON THE POLYSOME FRACTION OF RAT LIVER

(Protein-fed animals were given casein 2 hours before removal of the liver).



The sucrose gradient was linear (34 - 19% sucrose)

drops did not change significantly. The extinction at 260 mμ of the small volume (approximately 0.6 ml) in each test tube was estimated in a Unicam SP500 spectrophotometer using a 1 cm light path and the appropriate micro-cell attachment.

Results

The extinction at 260 mμ of the eluate collected from the sucrose density gradient was plotted against tube number to obtain a graph of which fig.45 is a typical example. In each experiment the deoxycholate treated mitochondria-free preparation ("polysome-fraction") from an animal fed protein and a fasted animal was compared. Although the area corresponding to the free ribosomes was not definitely identified, it is apparent that in fasted animals there is a greater amount of free ribosomes and smaller polysomes than in animals which had been fed protein immediately before removal of the liver.

Discussion

There are many possible criticisms of the approach described in this section to the study of dietary effects on protein synthesis in vitro. The flaws lie in the inadequate determination of the optimal conditions for in vitro incorporation of amino acids into microsomal protein. When the change from the undesirable sucrose-phosphate buffer was made, there were available several incubation media, for example, those of Rendi and Hultin (1960) and Korner (1961). The medium selected was simple and the results obtained in its use, similar to those obtained when the sucrose-phosphate buffer had been used.

The difficulties in the determination of the optimal

concentrations of each constituent of the incubation medium are however, considerable. It will be obvious, for example, that there is the possibility of interaction of components, as Korner (1961) and Von Der Decken (1961) have shown for magnesium and ATP, and as has been demonstrated here for pH 5 fraction and microsomes. With so many factors involved, there is the final difficulty of presenting the results in a meaningful form, for example, the requirement for magnesium is related to the amount of ATP and probably RNA present, so that a three-dimensional graph would be required for the selection of the correct conditions. Allen and Schweet (1962) have investigated the conditions required for maximal synthesis of haemoglobin by ribosomes prepared from reticulocytes, and among other findings, have shown that the incorporation of isotope increases in a linear fashion with the amount of ribosomes present in the mixture. In the experiments described in this section, this finding did not apply to the microsome system from rat liver. From these few comments it will be obvious that a working compromise was essential in order to obtain any information on the effects of diet on the amino acid incorporating activity of the microsomes of rat liver. It was, therefore, decided to adopt the simplest buffer and quantities of ATP etc. that would give satisfactory uptake of radioactive amino acid by the microsome preparation.

Another factor which led to difficulties was the occasional high uptake of amino acids by the zero energy control samples. When care was taken to avoid excessive homogenisation and all steps

were carried out at 0°C, this hazard was avoided. The explanation of the phenomenon may be related to the presence of lipids from the microsomal membrane in the incubation medium. Hendler (1958, 1961 and 1962) has suggested that lipid-amino acid complexes are involved in protein synthesis. Haining, Fukui and Axelrod (1960) showed that amino acids could be firmly bound by the non-phosphatide lipids of microsomes and that the process was not energy-dependent. The spontaneous (i.e. non-energy dependent) incorporation of amino acids into purified lipoproteins has also been observed by Mokrasch (1962). Thus although the practice of setting up a control tube from which ATP and an energy generating source have been omitted, does not appear to be common, there is considerable evidence that it is necessary. Attempts to reduce the incorporation of labelled amino acid in the zero energy control samples by extraction with lipid solvents failed because of the considerable amounts of protein and RNA which could be extracted by the lipid solvents (see Downie, 1963 and Part 3).

Although it was not demonstrated in the experiments described in this section, it is probable that under the conditions used, ^{14}C-leucine would have been incorporated into albumin. Peters (1959 and 1962) and Campbell (1960) have presented evidence that albumin is synthesised in vivo in the microsome fraction prepared from the livers of chicks and rats. These conclusions have been confirmed by a number of workers. For example, Ogata, Hirokawa and Omuri (1960), Lindgrel and Webster (1961), Von Der Decken and Campbell (1961) and Morgan, Perlman and Hultin (1962) have shown

that when microsomes from rat liver are incubated in the presence of ATP, GTP and an energy source such as creatine phosphate, pH 5 fraction and a radioactive amino acid, incorporation of radioactivity into serum albumin can be demonstrated either by adding a "carrier" albumin and isolating the albumin by acid-ethanol extraction procedure or by using an immunological method. The problem of obtaining albumin from microsomes in a sufficiently pure form and suitable quantity have been discussed previously. It can readily be computed from the data of Peters (1962a) that the maximum quantity of albumin which would be present in the incubation mixture used in the final series of experiments would be of the order of 90 µg per incubation tube. Quantitative isolation of this small amount, which would be necessary for the study of the effects of diet on albumin synthesis, would present considerable technical problems.

Despite occasional difficulties due to poor reproducibility of results with the incubation system used, the effects of diet on the microsome system in vitro seem to be well established. When animals are maintained on a protein-free diet which is adequate in calories, feeding protein results in increased capacity for protein synthesis by the microsomes. The interesting observation that when animals had been fasted for 16 hours, those previously fed a high protein diet had less amino acid incorporating ability than those previously deprived of protein, may be explained in two ways. Animals fed a high protein diet have more protein in the microsome fraction than those deprived of protein, and since the greater part of the protein is probably not concerned with the uptake of amino acids, it

will lead to dilution of the specific activity of the fraction. The second explanation for which there is more direct evidence is that during fasting, there is breakdown of microsomes to inactive smaller fragments, which may still be precipitated by centrifuging at 105,000g for 1 hour. Since the degree of breakdown of the microsome fraction from the HP-fed animals will be relatively greater than that of the fraction from animals deprived of protein, once more the specific activity of the microsomal protein obtained from the HP animals will be less after in vitro incubation with labelled amino acid, than that of the LP-maintained animals. Clark (1957) when investigating the uptake of ^{32}P by microsomes of rat liver, found that after fasting, the specific activity of RNA was reduced owing to dilution of the activity of precursor "pools" with breakdown products from RNA. MacLean (1963) found that the yield of post-microsomal pellet material was greater in animals which had been fed a high protein diet, then fasted, than in those animals which had been fasted after being deprived of protein. Finally in the present series of experiments on the polysome fraction, animals which had been fasted showed a greater amount of free-ribosome material than those which had been fed.

All the results obtained in this section are explicable by the hypothesis that the effects of diet are mediated by messenger RNA in the following way. The absence of amino acids in the animals deprived of protein lead to decreased production of messenger RNA so that the existing polysome and microsome structures slowly break down to inactive fragments. On the other hand, feeding protein and thus

supplying amino acids to the liver causes an increased production of messenger RNA, so that there is an increase in the amount of protein synthesised.

PART 3

THE ESTIMATION OF TISSUE CONSTITUENTS

The Estimation of Tissue Constituents

The analysis of the major components of a tissue is usually understood to include the estimation of protein, lipid, carbohydrate, nucleic acids (RNA and DNA) and perhaps minerals. The methods of estimating each of these major components with the exception of carbohydrate will be reviewed in turn and a procedure for the estimation of the protein, lipid and nucleic acid content of a tissue will be outlined. This investigation was undertaken because each analytical method applied during the experimental work described in Part 1 gave some reason for dissatisfaction.

The Estimation of Protein

The determination of protein was fully reviewed 15 years ago (Kirk, 1947), and in some aspects of the subject there is surprisingly little to be added or modified. Methods can be roughly classified as: (a) direct; (b) chemical; (c) physical and (d) subsidiary or related procedures (fig.46).

Direct methods

There are 2 direct methods of estimating protein: the gravimetric method and the estimation of protein volume, neither of which is popular.

The preparation of tissue protein for direct weighing is so tedious and fraught with the possibility of error (Kirk, 1947) that the method has been rarely adopted since the investigation by Addis, Poo, Lew and Yuen (1936). The main difficulty is, of course, in obtaining the tissue protein free from nucleic acid, lipid, carbohydrate and water. In the estimation of serum proteins, however, this difficulty is not so great, as was demonstrated by Fleury, Courtois and Eberhard (1951), who following a discussion of the disadvantages of thermal coagulation, utilised boiling ethanol in the preliminary stage of preparation of the proteins for weighing. Hoch and Vallee (1953) using TCA in the preliminary precipitation of the protein for weighing, obtained good agreement with methods based on N content or the UV absorption of the protein.

Determination of protein mass by estimation of the volume of a protein precipitate has been occasionally used. Standardisation of the conditions of precipitation and centrifugation is so

Fig.46 Some of the Available Methods of Estimation of Proteins

A. Direct
1. Gravimetric
2. Determination of protein volume

B. Chemical
1. By analysis of elements
C., N., Fe, I, S.
2. By analysis of groups
a. Amino acids: tyrosine, tryptophan, arginine { xanthoproteic, Folin-Ciocalteau, Sakaguchi reaction }
b. Amide-N determination.
c. Amino groups. Ninhydrin, Formol titration
d. Peptide bond. Biuret, Cu-Folin-phenol, Protein-bound Cu
3. Miscellaneous

a. Immunological; b. isotope dilution; c. dye binding, including the "protein error of indicators;" d. reaction with cationic detergents; e. copper-manganese reaction; f. reaction with Iodine; g. Djermilloss method.

C. Physical
1. ultraviolet spectrophotometry
2. turbidimetry
3. specific gravity
4. refractive index
5. monolayer films
6. surface tension

unsatisfactory as to render the method not truly quantitative (Kirk, 1947). In an investigation of the efficiency of TCA as a protein precipitant which was undertaken in this laboratory, it was observed that the volume occupied by the same mass of protein decreased as the concentration of acid used in precipitation was increased; this was also noted by Miller and Van Slyke (1922). In a comparison of several methods of CSF protein estimation it has been shown that while a biuret method gave reliable results, the measurement of the volume of the protein precipitated in a graduated tube gave unreliable results (Burtin, Doutriaux and Pocidalo, 1958).

Chemical methods

There are numerous chemical methods of estimating protein. They can be roughly classified as methods based on the analysis of elements, such as N, C, in the molecule, or analysis depending on certain groups, such as the peptide bond or the side chain of tyrosine and in addition, certain properties of the intact protein molecule such as the binding of dyes or its antigenic properties have been utilised in protein estimation (fig.46).

In all chemical procedures of analysis, a common hazard occurs in the expression of the results. For example, N analysis may be carried out and the result stated as "x mg of protein-N", or as "y mg protein", and since "y" = "x" x f, in which the factor *f* is the reciprocal of the percentage of nitrogen in the protein, it is obvious that the N content of the protein must be accurately known or the second form of expressing the result is both inaccurate and meaningless. The difference in specific extinction value of

different proteins in certain colorimetric reactions such as, for example, the biuret method, leads to similar difficulties both in choice of standard and expression of results. Ideally the standard should be a pure sample of the protein under investigation, but this may be impossible to obtain and if so, a sample of serum albumin is frequently used, with concomitant dubiety about the absolute value of results. In practice in this laboratory, good agreement (that is within ± 5 or 10%) has been obtained in comparisons of protein estimation of mixed tissue proteins based on N determination (micro-Kjeldahl or Nessler) and on the Biuret copper-Folin-Ciocalteau methods (see below).

Methods based on the analysis of elementary constituents

1. Methods based on carbon analysis, although having some advantages over N determination (Kirk, 1947) have fallen into disuse.

2. Protein estimation via N determination finds such widespread use and is of such importance that Nitrogen analysis will be discussed later.

3. Other elements may be used in the determination of certain proteins, for example, Fe in haemoglobin and Iodine in thyroglobulin (see Dawes, 1963).

Methods based on the determination of certain chemical groups

The analysis of sulphur-containing amino acids compared with the sulphur content of the protein has been used in protein molecular weight determination (Dawes, 1963). However, the amino acid groups more commonly used in protein estimation have been the benzene ring - xanthoproteic reaction - (Kirk, 1947; Buriana, 1958,); the

phenol group of tyrosine - reaction with the Folin-Ciocalteau reagent, (Kirk 1947; Garner 1952); and the arginine side chain - Sakaguchi reaction- (Albanese, Irby and Saur, 1946).

Amino and amide groups have also found occasional use in protein estimation. Amide nitrogen determination has been reported as accurate, but has no advantages over the micro-Kjeldahl method (Karunina and Shilovitch, 1953). Free amino groups may be determined by formol titration or ninhydrin reaction. The reaction with ninhydrin, although widely used in the determination of free amino acids and ammonia is not used in the determination of protein or peptides.

The formol-titration method of estimating protein continues to find occasional use (Karunina and Shilovitch, 1953; Dudenkov, 1959). It is difficult to understand why this method is still used for protein analysis following its condemnation, for this purpose, by Kirk (1947), its obvious flaws when applied to proteins, (one example being the large number of possible different reactive groups in the molecule which must render the selection of suitable end-points very difficult), and the availability of more convenient and reliable methods.

The above-mentioned methods have been included for completeness; some find now only occasional use (xanthoproteic, Folin-Ciocalteau) and others, such as the formol titration, it is inadvisable to use for the estimation of protein.

Methods based on the copper-chelating properties of peptide bonds

Three methods of estimation of proteins are based on the chelating properties of copper ions and polypeptides. In one method, the amount of copper bound to the protein is determined directly (Westley and Lambeth, 1960); in another, the colour of the copper-protein complex in alkaline solution is estimated colorimetrically (the biuret method - Gornall, Bardawill and David, 1949) and in the third, the copper complex catalyses electron transfer and reduction of the Folin-Ciocalteau reagent to produce a blue colour (Lowry, Rosebrough, Farr and Randall, 1951).

Historically, the biuret method was the first of the copper-protein-complex methods to be introduced. It was probably first used for the estimation of protein in urine (Riegler, 1914; Kirk, 1947). For some time a reagent containing only copper sulphate and a fairly high concentration of sodium hydroxide (Kingsley, 1939) was popular (Kirk, 1947). However, this reagent has been criticised because of instability (Weichselbaum, 1946; Gornall et al., 1949) which is probably due to the tendency of copper to precipitate in alkaline solution (Gurd and Wilcox, 1956). Several methods of stabilising the reagent have been tried (see Kirk, 1947; Gornall et al., 1949); the addition of ethylene glycol (Mehl, 1945), citrate (Henry, Sobel and Berkman, 1957) or tartrate (Gornall et al., 1949) to the reagent or increasing the concentration of sodium hydroxide (Kibrick, 1949).

Reagents containing copper sulphate, sodium hydroxide and sodium potassium tartrate as stabilising agent, following the

Table 44

Final Concentrations of Reagents in Biuret Reaction Mixture

	$CuSO_4 5H_2O$	Sodium potassium Tartrate	NaOH
Weichselbaum	0.03 M*	0.075	0.1
+G B D	0.005 M	0.016	0.60
Strickland et al.	0.01 M	0.08 M	0.1 M
Westley	0.002 M	0.008 M	0.11 M
Lowry	0.0003 M	0.0003 M	0.08 M
Benedict	approx. 0.03 M	citrate	carbonate

* Large excess $CuSO_4$ required.

+ Only critical investigation of relative reagent concentrations. (Gornall et al., 1949).

initial use by Weichselbaum (1946) and investigation by Gornall et al. (1949), have found widespread use in the biuret method (Dustin, 1950; Jayle, Boussier and Badin, 1951 for example), in the direct method (Westley and Lambeth, 1960), and in the copper-Folin-phenol method (Lowry et al., 1951). Although tartrate has found general use as stabiliser in different circumstances, there appears to be lack of general agreement on the relative concentrations of the individual ingredients of the biuret reagent (see table 44).

Benedict's qualitative glucose reagent which utilises sodium carbonate and citrate as stabilising agents has been suggested for use in a quantitative biuret procedure (Henry et al., 1957; Hussain, Shah and Chaudhuri, 1961), although Gornall et al. (1949) report that citrate is ineffective for this purpose. Ammonium ions interfere to some extent in the biuret reaction (Gornall et al., 1949), but despite this ammonium hydroxide has been incorporated in a quantitative biuret reagent which is said to be stable and give almost immediate formation of the biuret complex (Levin and Brauer, 1951).

In selecting a method or reagent for use it should be remembered that most quantitative biuret reagents have been developed for use with serum or C.S.F. proteins (Kingsley, 1939; Gornall et al., 1949; Levin et al., 1951) and limitations may be found in applying any reagent to other systems. The reagent of Gornall et al. (1949) is the only one which has been derived following a published investigation of the optimal concentrations of its constituents - copper sulphate, sodium hydroxide and sodium potassium tartrate, and in 1950, following an investigation of the

biuret method applied to protein estimation, was concluded to be the best (Dustin, 1950).

The stabilising action of tartrate in the strongly alkaline biuret reagent is due to the formation of a copper-tartrate complex (Strickland, Freeman and Gurule, 1961). Physical-chemical data support the concept that copper forms a four-ligand 'square' planar complex with suitable groups, and that the chelate complex is much more stable than the complex formed with amino acids or other simple molecules (Gurd and Wilcox, 1956). Tartrate presumably 'competes' to a slight extent with polypeptides for copper because increasing the tartrate content of the biuret reagent reduces the colour intensity that the reagent gives with protein (Gornall et al., 1949).

Agreement is general that, at the high pH (13) of the biuret reagent almost all the copper is complexed to the peptide groups (Mehl, Pacovska and Winzler, 1949; Gurd and Wilcox, 1956; Strickland et al., 1961), and to at least two or more basic nitrogen atoms (Strickland et al., 1961). There is evidence that at pH 4.5 copper complexes with carboxyl groups, at pH 6.5 with imidazole groups, and at pH 9.6 with the more basic nitrogen groups (Gurd and Wilcox, 1956). There is also evidence for additional binding of copper, in the biuret reaction, to non-peptide nitrogen (Mehl et al., 1949) and it has been calculated that the copper-histidine complex may account for about 1.5% of the total light absorption of the biuret-protein complex (Strickland et al., 1961). If the peptide groups only were involved in the complex it would be expected that

the wavelength of maximal absorption and the specific extinction coefficient of different proteins would be the same. In fact, this is not so (Mehl et al., 1949; Strickland et al., 1961), although for many proteins the wavelength of maximal absorption of the biuret complex is 545 mμ (Strickland et al., 1961). Dustin (1950) reached the conclusion that the copper to protein ratio determined the colour of the biuret-protein complex: the optimal ratio (using the reagent of Gornall et al., 1949) was 1:1.5 (see table 44); if greater, a blue colour was obtained, and if less, pink. No change in the wavelength of maximal absorption of the complex was observed, so that the effect was probably related to the absorption of the 'blank' or uncomplexed copper at 545 mμ.

The stoichiometry of the biuret reaction is, however, of some interest. It has been suggested that the ratio of copper to peptide nitrogen is 1:4 (Mehl et al., 1949), but more recently it has been shown to be 1:6, except in serum globulin, with which it is 1:5 (Strickland et al., 1961). A model structure of the copper-protein complex in which the ratio of copper to peptide nitrogen is 1:6 and in which the four ligand planar copper chelate is present has been devised by Strickland et al. (1961).

Sources of interference in the biuret reaction are few. Although copper forms complexes with many amino acids, the protein chelate complex is much more stable (Gurd and Wilcox, 1956) and chromogenic (Strickland et al., 1961). Free histidine could lead to errors (Strickland et al., 1961), as could bases such as ethylene-diamine (Gurd and Wilcox, 1956) or chelating agents such as EDTA

(Strickland et al., 1961), but these substances are rarely present in biological materials in sufficient concentration to be a hazard or care may be taken to exclude them. Sulphydryl groups may present a more serious problem as copper forms a very stable mercaptide with a large and variable absorptivity so that proteins containing a relatively large amount of cysteine, such as fibrinogen, cannot be estimated accurately by the biuret method (Strickland et al., 1961). High concentrations of neutral salts may interfere in the biuret reaction, usually to only a slight degree which may be simply corrected by the use of standards and blanks containing the same concentration of the salt (Rosenthal and Kawakami, 1956). Copper phosphate is only sparingly soluble (Stiff, 1949) so that it is best to avoid the use of phosphate buffers in the preparation of proteins for the biuret reaction. In the application of the biuret method to the estimation of serum proteins, the development of turbidity following the addition of the clear reagent to the clear serum has been reported (Kingsley, 1942; Henry et al., 1957). Both these groups of investigators agree that this is best eliminated by ether extraction as it is probably due to lipid (Levin and Brauer, 1951). Finally, the preliminary treatment of the protein may alter its behaviour with the biuret reagent. Denaturation, acid precipitation, or alcoholic fractionation may bring about alteration of the molecular configuration and thus, slightly altered absorption of the biuret complex. (This may account for the difference in absorption between serum proteins and heat-coagulated serum proteins observed by Juillan and Bats-Maillet (1958) and which was attributed by them to "biuretogenic" substances). However,

this difficulty may be readily overcome by the use of a similarly treated protein standard.

The hazards in the use of the biuret reaction in the estimation of protein are thus of a minor order of importance and it is scarcely surprising that a reviewer states: "In my experience, the biuret reaction in its modern form, has proved to be the most reliable absorptionmetric method for direct determinations of protein in clinical work" (Salt, 1953), and that the method has been found suitable for the determination, in addition to serum protein, of the protein of CSF and urine (Kibrick, 1949), wheat protein (Pickney, 1949), the proteins of fish muscle (Dyer, French and Snow, 1950), and the protein of milk and whey (Sangiorgi and Cariello, 1959).

The direct determination of protein-bound copper using the sensitive diethyl dithiocarbamate copper reagent has been utilised in the estimation of protein (Stiff, 1949; Nielsen, 1958; Westley and Lambeth, 1960). The copper reagent is liable to turbidity or precipitation which is prevented by the presence of protein (Stiff, 1949; Westley and Lambeth, 1960). As a result of this, there are two approaches to the determination of protein-bound copper. In the first (Stiff, 1949) sparingly soluble copper phosphate is added to an alkaline solution of the protein and after mixing, allowing time for complexing of copper to protein, and centrifugation to remove the uncomplexed copper (as copper phosphate) diethyldithiocarbamate is added to determine the protein-bound copper. Since there is protein present when the copper reagent is added, turbidity is avoided. A disadvantage of this method is

the possibility of obtaining blanks of high extinction values (see Westley and Lambeth, 1960) due to the slight solubility of copper phosphate (Stiff, 1949). This difficulty has been overcome by Westley and Lambeth (1960) who adopted the following procedure. An alkaline copper tartrate biuret reagent (see table 44) is mixed with the protein solution. After a few minutes, a suspension of a cation-exchange resin (Dowex-1 chloride form) is added; this removes the excess or non protein-bound copper. The resin is removed by centrifugation and the copper reagent added. With this procedure blanks are low and since the diethyldithiocarbamate reagent is made up in a very dilute protein solution, turbidity of the reagent is avoided. The method of Nielsen (1958) utilises precipitation of non-complexed copper as the phosphate and so is liable to high blanks, and precipitation of copper diethyldithiocarbamate is said to occur (Westley and Lambeth, 1960). The rationale of the method of Westley and Lambeth (1960) seems good but the method does not appear to have become popular, possibly because it is too recent to have had sufficient trial. However, in this laboratory, the method was found to be inaccurate. Following a preliminary investigation in which it was established that the estimation of copper by the diethyldithiocarbamate reagent was very accurate and reproducible, it was concluded that there was some variability in the reaction of the copper, protein and ion exchange resin which resulted in excessively large experimental error.

The final method (that of Lowry et al., 1951) which utilises the ability of copper to complex with protein in alkaline solution is

highly sensitive (25-500 µg protein/ml), reliable and popular, although the reaction on which it is based is complicated (Chou and Goldstein, 1960). The method is colorimetric and the procedure requires two stages. In the first, the protein is mixed with an alkaline copper tartrate solution and the second consists of the addition of diluted Folin-Ciocalteau phosphomolybdic-phosphotungstic acid reagent which is reduced by the copper-protein complex to give a blue coloured product with a wavelength of maximum absorption at 750 mµ (constant for all proteins and peptides), (Chou and Goldstein, 1960). This reaction was investigated by Chou and Goldstein (1960) who confirmed that there is some variation in the chromogenicity of different proteins (Lowry et al., 1951), that a protein hydrolysate gives only a fraction of the colour of the intact protein, and that the chromogenicity of single amino acids (mainly tyrosine, tryptophan and cysteine) is additive. Dipeptides give some colour in the reaction but tripeptides are highly chromogenic and it is concluded that there are large numbers of different chromogenic sequences in proteins. The preliminary formation of the copper protein complex is, of course, essential for the high sensitivity of the method as it increases the chromogenicity of proteins with the Folin-phenol reagent from 3 to 15 times.

In carrying out the reaction, care must be taken to retain the pH at 10 and to mix immediately on addition of the Folin reagent otherwise optimal colour is not obtained. Several substances may interfere in the reaction (Zondag and Boetzelaer, 1960) but they do not constitute a serious hazard as they are not normally present in

biological materials. In this laboratory, it has been found that "Tris," EDTA and phosphate should be avoided. An additional slight disadvantage is the non-linearity of the colour-protein concentration relation. The advantages of the method are its great sensitivity (10-100 µg protein or less) and reliability.

The copper-Folin-phenol reagent method of estimating protein has found fairly wide use, for example, in the determination of CSF protein (Daughaday, Lowry, Rosebrough and Field, 1952; Waldman, Krause and Borman, 1953; Rieder, 1958), serum protein (Debro, Tarver and Korner, 1957), and fibrinogen (Reiner and Cheung, 1959). There is a modification using citrate instead of tartrate in the copper solution (Rieder, 1959) and another which was devised for application to large numbers of samples, in which the time required for the estimation is reduced by half (Miller, 1959).

A further modification of the biuret method has been suggested by Ellman (1962), in which the sensitivity is claimed to be 10-200 µg protein. In the modified procedure, the biuret complex is formed in the usual way, but the extinction is estimated at 263 mµ. It is claimed that protein can be determined in the presence of RNA and DNA using this procedure. In a preliminary investigation of the method in this laboratory, it was found that the reagent blank had such a high extinction (0.600-0.700 at 263 mµ) that it was difficult to apply the method with accuracy.

Apart from those chemical methods previously discussed, numerous others exist (see fig.46). The antigenic properties of proteins have been utilised for their estimation. This has been

mainly applied to plasma proteins (Goodman, Ramsey, Simpson, Remp, Basinski and Brennan, 1957; Peters, 1962). Plasma albumin has also been estimated by an isotope dilution technique (Lubran and Moss, 1957). All proteins have the property of binding dyes, although different proteins do so to different extents (Wolstenholme and Miller, 1956). This has been utilised both qualitatively and quantitatively in the electrophoretic investigations of plasma proteins (Smith, 1960). The binding of such dyes as Orange G and Amido Black has been used in the determination of the proteins of milk (Shiga, Shimizu, and Hamada, 1959) and tissues (Bunyan, 1959). In this latter case, the method was stated to be inaccurate, which was to be expected following the discussions of the quantitative electrophoretic methods of estimating serum proteins (Wolstenholme and Miller, 1956). Although most of the dyes utilised absorb in the visible range, a fluorescent procedure has been described (Betheil, 1960). An extension of the property of proteins of binding dyes is the "protein error" in measuring pH by an indicator. This too, may be utilised in the estimation of protein (Ketomaa and Ruosteenoja, 1952; Scheurlen, 1959).

Investigation of the interactions of proteins and detergents has led to a method of estimation of protein based on the ability of cationic detergents (mostly quaternary ammonium compounds or "invert soaps") to form precipitable cationanion complexes with protein (Chinard, 1948; Abelin and Pfister, 1951). Reproducible results have been reported, but the method is not recommended for plasma or urine (Kimbel and Amon, 1953). Other methods of

estimating protein which have appeared in the literature are: the copper-manganese reaction (Bitter, 1952), the reaction of protein with iodine (Furi and Singh, 1951) and the method of Djermillo (Babin and Muserskii, 1954) in which the preliminary stage is fusion with sodium acetate.

Physical methods

Of the many physical procedures which could be adopted for the estimation of protein, only a few in fact have found application and only two of these (ultraviolet spectrophotometry and nepholometry) could be said to be used with any frequency. The absorption peak at 280 mμ possessed by most proteins has been known and utilised for some years as a method of protein estimation (Waddell, 1956). The method is not without snags (Beavan and Holiday, 1952): since the absorption at 280 mμ is mainly due to the amino acids containing aromatic rings and the amount of these in various proteins differs, the method is usually applicable only to pure proteins. The examination of the separation of protein fractions in column chromatography (Petersen and Sober, 1960) is probably the most frequent application of the method. More recent investigations indicate that a very sensitive method of protein estimation (Tombs, Souter and MacLagan, 1959) is possible, utilising the wavelength of maximum absorption of proteins at 200 mμ which is due to the peptide bond (Goldfarb and Saidel, 1951; Tombs and MacLagen, 1962). Nucleic acids, since they also absorb considerably in the regions 200 and 280 mμ are potent sources of interference and because their separation from protein cannot readily be effected

without the denaturation of the protein (Fleck and Munro, 1963) with resultant alteration in the ultraviolet absorbing properties of the protein (Beavan and Holiday, 1952), ultraviolet spectrophotometry is not used in the direct estimation of tissue proteins.

Nephelometric, turbidimetric, or light-scattering methods of protein estimation are becoming less popular. Although the accuracy of the method is not high (Kirk, 1947; Bauer and Angelstein, 1952) it has found such diverse applications as the estimation of plasma protein (Knüchel, 1951; Discombe, 1959) and wheat flour protein (Feinstein and Hart, 1959). Microbiologists find the method convenient for bacterial counts.

Measurement of specific gravity has been used as a method of protein estimation (Kirk, 1947); mainly for the determination of plasma proteins (Phillips, Van Slyke, Dole, Emerson, Hamilton and Archibald, 1943). The method is rapid and intrinsically capable of high accuracy (Kirk, 1947). It is, however, in the correlation of specific gravity with protein content that errors may arise (Adams and Ballou, 1946).

Apart from the "Schlieren" techniques applied in the analytical ultracentrifuge and electrophoresis, the effect of protein concentration on refractive index is little used as a method of protein estimation. Refractive index measurement is less sensitive than specific gravity (Kirk, 1947; Salt, 1953), and as with the specific gravity methods, applications have been mainly to the determination of plasma proteins (Kirk, 1947; Bugyi, 1956).

The area of a monolayer film of a protein has been used

in an analytical technique (Heinemann and Parker, 1951), as has the surface tension of a protein solution (Gunton and Barton, 1947). Neither of these methods warrants serious discussion here as they are apparently little used and require specialised equipment.

Summary

The choice of a method of protein estimation may be governed by a large number of factors, some of which apply only in certain cases. However, in general the characteristics of reliability, reproducibility, accuracy, sensitivity and convenience are determinative. Applying these criteria to the problem of the selection of a method of protein estimation immediately eliminates a large number of methods, leaving nitrogen determination as the standard procedure (Salt, 1953), and the biuret method and the related and more sensitive copper-Folin-phenol method of Lowry et al., (1951) as suitable subsidiary methods which have been adequately investigated. Ultraviolet spectrophotometry, immunological and isotope dilution methods, with adequate standards, may also be suitable in special circumstances.

Addendum

There has been some dispute as to whether it is more accurate in colorimetric analyses to read the absorption of the unknown against a distilled water blank or against a reagent or control blank (see for example, Strickland et al., 1961).

Considering the most general case:-

(spectrophotometer readings unless stated to the contrary are considered to be against distilled water or other solvent with zero absorption at the appropriate wavelength).

When A_t = total absorption of reaction mixture containing the unknown:

A_u = absorption due to colour complex of unknown;

A_r = absorption due to reagents alone (Spectral characteristics different from the colour complex of the unknown);

A_c = absorption due to impurities etc. (spectral characteristics may be similar to the unknown colour complex). This is considered to be constant within a run but may be variable from run to run.

then, $A_t = A_u + A_r + A_c$

If E is the specific extinction of the unknown, the concentration of which in the reaction mixture is U, then as U increases, A_r must decrease due to the consumption of the reagents in the reaction in which the colour complex is produced, so that if the absorption of the reagent blank is B,

then, $A_r = B - kU$, where k is a constant.

thus, $A_t = E.U + B - kU + A_c$ or,

$$A_t = (E - k)U + B + A_c.$$

Since B, A_c, E and k are constants, this is the equation of a straight line gradient (E - k) and intercept B + A_c and

$$U = \frac{A_t - B - A_c}{E - k}.$$

In some colorimetric procedures, the reagents are colourless so that k and B are zero and $U = \frac{A_t - A_c}{E}$. In this case, reading the absorption of the unknown (A_t) against the control blank (A_c), gives ($A_t - A_c$) (= A_u) directly, and is a valid procedure, saves time and does not affect the final accuracy of the procedure.

Other methods utilise reagents which have some absorption at the wavelength of reading the absorption of the unknown (for example, the biuret method).

In a similar way to the above, reading against the reagent blank (B) gives the straight line

$$U = \frac{\text{spectrophotometer reading } (= A_t - B) - A_c}{E - k},$$

so that to derive U it is necessary to subtract the control blank ($= A_c$) before dividing by (E - k).

In practice, the control blank is usually only very slightly larger than B so that the absorption of the control blank ($= A_c$) approximately equals (B) and negligible error is incurred by reading against the control blank.

Thus, the empirical finding of straight-line calibration curves in such reactions as the orcinol and biuret reactions, and the validity of the normal procedure of calibration (that is, reading the absorption of the unknown against the control blank to obtain a

calibration curve through the origin with simplification of derivation of results) is justified and there is no increase in experimental error.

The determination of organic nitrogen

Introduction

The quantitative study of the nitrogen content of food, excreta and body tissues and fluids has been of interest for more than a century. It was early recognised that the nitrogenous compounds of the body derived their nitrogen from protein-nitrogen (Liebig, 1842). In later analytical studies on proteins, the nitrogen content has been used as a reference standard.

There are two principal methods of estimation of organic nitrogen, the practical aspects of which have been recently reviewed by Ingram (1962). In one, the Dumas procedure, complete combustion of the material yields nitrogen gas which is then determined directly by volume. The other procedure, that of Kjeldahl, involves treatment of the substance with hot, concentrated sulphuric acid which converts the organic nitrogen to ammonium sulphate, the ammonia of which may then be determined by a variety of methods.

The Dumas method

The Dumas procedure is used frequently by organic chemists as a standard method of micro-analysis. Earlier forms of the Dumas method were somewhat slow for general purposes. For example, one procedure required seven hours by one man to perform ten analyses (Kirsten and Greenbaum, 1955). This expenditure of effort can be readily understood, since the method requires heating of the sample in a stream of CO_2, over a nickel catalyst at 1000°C, followed by a

variety of stages required to oxidise the carbon present to CO_2. Finally, water is removed, CO_2 absorbed in KOH and the nitrogen determined directly by volume (Parks, Bastin, Agazzi and Brooks, 1954). The method appears to be of uniform applicability as nitrates etc. can be analysed without serious modification of the procedure. Minor modifications are common (Childs, Meyers, Johnston and Mitulski, 1956; Charlton, 1957) and the time required for analysis has been reduced (Shelberg, 1951; Shah, Pansare and Mulay, 1956; Kirsten, 1957; Otter, 1958). The method has been automated (Sternglanz and Kollig, 1962) and in this way the time for a single analysis reduced to 6-15 minutes (Dorfman, Oeckinghaus, Anderson and Robertson, 1962). Despite this, the method apparently is not popular with biochemists, possibly because the special apparatus, high temperatures and technical skill required compare unfavourably with the general convenience of the Kjeldahl method, and notably with the ease with which large numbers of samples can be handled. However, following an evaluation of 6 methods of estimating the N content of nitroguanidine Fauth and Staloup (1958) stated that greater accuracy was obtained with the Dumas method (98.51 - 100.65% recovery) than with the Kjeldahl method (98.09 - 99.50% recovery).

The most recent development of the Dumas method is to follow combustion of the sample with gas chromatographic determination of nitrogen (Parsons, Pennington and Walker, 1963; Stewart, Foster and Beard, 1963) or to include analysis of carbon and hydrogen as well as nitrogen (Nightingale and Walker, 1962; Mozumi and Kirsten, 1963).

The Kjeldahl method

The Kjeldahl method, or one of its many modifications is widely employed. The method consists of three successive operations; (a) digestion of the sample with sulphuric acid; (b) distillation of the ammonia produced by digestion and its trapping in a form suitable for estimation (this step is occasionally omitted), and (c) the estimation of ammonia.

Quantitative conversion of the organic nitrogen in the sample to ammonia is a critical stage in the Kjeldahl procedure and the conditions and reagents utilised in digestion have been frequently investigated and reviewed (Kirk, 1947, 1950; Bradstreet, 1954). The detailed chemistry of the process of digestion is not known, but it is obvious that in general, treatment of organic substances with hot concentrated sulphuric acid leads to the removal of molecules of water with consequent charring, pyrolytic decomposition of the material and the achieving of a fine balance in oxidation and reduction, since amino groups are oxidised to ammonia which is not oxidised in turn to gaseous nitrogen (see Kirk, 1950; Bradstreet, 1954).

Factors in digestion

1. Catalysts

In order to accelerate the process of digestion many catalysts have been recommended but the evidence, however, has long favoured mercury as the most reliable (see review by Kirk, 1947; Patel and Sreenivasan, 1948; Miller, Plazin and Van Slyke, 1948) and this continues to be supported (Kirk, 1950; Perrin, 1953; Joint Committee of AOAC, 1955; Rozental, 1958). Indeed there are

no reported losses of ammonia nitrogen attributable to the use of mercury as a catalyst; this does not hold true for any other catalyst, the use of which has been seriously advocated. Mercury may be used in the form of metallic mercury (Hiller et al., 1948; Lake, McCutchan, Van Meter and Neel, 1951), HgO (McKenzie and Wallace, 1954), or $HgSO_4$ (Belcher, 1956); the optimal amount appears to be in the range 33-43 mg HgO per ml concentrated H_2SO_4 (Lake et al., 1951; McKenzie and Wallace, 1954).

Selenium continues to be used (for example, Adams and Spaulding, 1955; Paul, 1958), although Patel and Sreenivasan (1948) after investigation had concluded, "selenium cannot be recommended as a general reagent for Kjeldahl determinations," since they had observed a variable loss of nitrogen which was attributable to the use of this catalyst. This observation has been frequently confirmed (Hiller, Plazin and Van Slyke, 1948; Perrin, 1953).

Copper, also has been found to be inferior to mercury as a general catalyst (Joint Committee of AOAC, 1955; Morgan, Lackey and Gilchreas, 1957) and few other catalysts have received serious consideration or sufficient use to permit adequate assessment.

2. Other additions to the digestion mixture

From time to time, other substances, which are not catalysts, have been added to the sulphuric acid in order to accelerate digestion. Examples are, potassium phosphate or phosphoric acid, sodium sulphate and potassium sulphate. The main effect is to raise the boiling point of the sulphuric acid mixture and thus raise the temperature at which digestion occurs. Potassium sulphate has no known

disadvantages, but sodium sulphate somewhat readily leads to solidification of the digest (McKenzie and Wallace, 1954), and potassium phosphate or phosphoric acid etches glassware (Miller et al., 1948) and may also cause an excessive increase in the temperature of digestion (Perrin, 1953).

3. Temperature of digestion

There is now considerable evidence that the temperature of digestion is very important. In normal methods of digestion (i.e. open-tube) 20% loss of nitrogen has been shown to occur at 440°C (McKenzie and Wallace, 1954), and in sealed-tube digestion Greenbaum, Schaffer and Kirk (1952) demonstrated destruction of ammonia at temperatures in excess of 450°C (see also Grünbaum, Kirk, Green and Koch, 1955). In an investigation of the effects of digestion temperature on Kjeldahl analyses, Lake et al (1951) showed that the safe upper limit of temperature in normal digestion was 410°C, and the safe lower limit 370°C. Perrin (1953) confirmed these values, as did McKenzie and Wallace (1954). These last concluded that the optimal digestion temperature was 390°C. With the refractory compounds (pyridine, nicotinic acid and amino acids such as tryptophan) used in these studies, complete conversion of organic nitrogen to ammonia does not occur if the temperature is less than 370°C. There is agreement that the best means of controlling the temperature of digestion is by varying the amount of potassium sulphate in the digestion mixture (Lake et al., 1951; Perrin, 1953; McKenzie and Wallace, 1954).

Although there are some discrepancies in the results obtained by various workers in the relation between temperature of digestion

and the amount of K_2SO_4 in the digestion mixture when the quantity of K_2SO_4 present is used as criterion, there is general agreement that from 0.67-1.00 gm K_2SO_4 per ml concentrated H_2SO_4 gives optimal digestion. The ratio 0.67:1 is the minimum desirable, in that below this there is danger of incomplete conversion of the organic nitrogen to ammonia, and a ratio greater than 1:1 may lead to loss due to pyrolytic breakdown of ammonia. Bradstreet (1957) in an investigation of the acid requirements in Kjeldahl digestion showed that if the salt:acid ratio was high enough to cause solidification of the digest on cooling, then nitrogen loss was probable. Solidification took place when the salt:acid ratio was greater than 1:1; in his experience, the optimum ratio for digestion was about 0.8:1. Loss of nitrogen has been observed at lower temperatures in the presence of selenium (Baker, 1953) and the oxidising agent potassium permanganate (Beet, 1955).

4. Time of digestion

With the use of the recommended amounts of a mercury catalyst and potassium sulphate (40 mg HgO and approximately 0.75 gm K_2SO_4 per ml concentrated H_2SO_4 and 1.5 ml H_2SO_4 for up to 2 mg nitrogen or not more than 30 mg material), and consequently a digestion temperature of from 370° to 400°C, clearing of the digest may occur after 5 minutes digestion and completion in 20 minutes (McKenzie and Wallace, 1954). Using these components and quantities digestion times required with the macro-Kjeldahl procedure should not exceed one hour (Lake et al., 1951) and with the micro procedure, 30 minutes digestion should be adequate (Perrin, 1953). Prolonged

digestion should be avoided since loss of acid occurs with consequent gradual increase in digestion temperature which in turn may lead to loss of nitrogen (Bradstreet, 1957).

5. Use of oxidising agents

Since the Kjeldahl digestion achieves a fine balance of oxidation and reduction, the use of added oxidising agents must be, at least theoretically, hazardous. Kirk (1950) suggests that occasional judicious use might be possible, particularly when much organic carbon is present, as this will be preferentially oxidised before ammonia. This may be the explanation of the success claimed by Beet (1955) for the use of potassium permanganate in the estimation of the nitrogen content of coal by the Kjeldahl procedure. That oxidising agents may lead to loss of nitrogen and should not be used without careful investigation is, however, evident from the reviews of Kirk (1947 and 1950) and Bradstreet (1954), and from investigations such as that of Wicks and Firminger (1942) in which loss of nitrogen due to the use of perchloric acid was demonstrated. The action of hot perchloric acid on ammonia has been investigated recently by Moore and Diehl (1962). Their conclusion was that thermal decomposition of perchloric acid to chlorine took place, the chlorine then formed hypochlorous acid which in turn reacted with ammonia to yield gaseous nitrogen. The production of gaseous nitrogen by this mechanism was increased by the use of mixtures of perchloric and sulphuric acids. The possible exception to the opinion that the use of oxidising agents is hazardous is hydrogen peroxide with which no loss of nitrogen has been reported (see Kirk, 1947 and 1950). McKenzie and Wallace (1954) state that

its use with sulphuric acid alone did not lead to nitrogen loss, but had no advantage over the mercury, potassium sulphate, sulphuric acid mixture; they suggest that the low temperature of decomposition of hydrogen peroxide (160°C) and the practice of cooling the digest before its addition may be important factors in its successful use. If the use of an oxidising agent is considered necessary the evidence thus favours the careful use of hydrogen peroxide (which should of course be nitrogen-free).

6. Alternative digestion procedures

Since oxidized forms of nitrogen (such as nitrates) are rarely present in material of biological origin, the use of hydrogenation (Gelman and Korshun, 1950) or reducing agents such as thiosulphate (Bradstreet, 1957) and Devarda's alloy (Kuck, Kingsley, Kinsey, Sheehan and Swigert, 1950) need not be considered here. The interesting method of Rémy and Pitiot (1951), in which heating an organic substance with calcium oxide and sodium hydroxide to effect reduction of nitrogenous compounds to ammonia is utilised, should be mentioned.

The previous discussion has been concerned with the more usual method of digestion in an open tube or (micro-) Kjeldahl flask. Digestion may also be carried out in a sealed tube, and, if the temperature is maintained about 400°C, no additives apart from concentrated sulphuric acid are required (see Greenbaum et al., 1952; Grunbaum et al., 1955).

7. Distillation and titration of ammonia

Following digestion, the next stage in the determination of

nitrogen is the estimation of the ammonia content of the digest. When mercury is used as catalyst it is, however, first necessary to decompose the mercury-ammonium complex which forms in the digestion mixture (Hiller et al., 1948). This may be achieved by addition of zinc dust or sodium thiosulphate (Hiller et al., 1948), and while both may give equally good results (Polley, 1954; McKenzie and Wallace, 1954), Hiller et al. observed that sodium thiosulphate was liable to give rise to acid fumes in the distillate. This observation has been confirmed in this laboratory, so that the use of zinc dust is recommended.

Ammonia in the digest may be estimated directly or by making the digest alkaline then utilising steam-distillation, aeration or diffusion of the ammonia into a suitable acid solution in which the ammonia may be readily determined. The common practice is steam-distillation of the ammonia into acid. The apparatus described by Markham (1942) and the procedure recommended by Ma and Zuazaga (1942), in which only two minutes distillation is required for quantitative recoveries of up to 1 mg of nitrogen, have been found reliable in this laboratory. Other forms of distillation apparatus have been described (Silverstein and Perthel, 1950; Steyermark, Alber, Aluise, Huffman, Kuck, Moran and Willets, 1951; Hyde, 1951; Scandrett, 1953; Schöniger and Haack, 1956), including one designed for ultramicro steam-distillation (Tourtellotte, Parker, Alving and DeJong, 1958).

The ammonia distilled over is usually trapped in standard acid and then estimated by back-titration. However, in the

micro range boric acid has been frequently used to trap the ammonia (Ma and Zuazaga, 1942). There have been some reports of incomplete recovery with the use of boric acid (Hiller et al., 1948; Joint Committee of AOAC, 1955). It has been suggested that this is due to heating of the boric acid by the distillate but that if the temperature in the receiver is not allowed to rise above 50°C no loss occurs (Wingo, Davis and Anderson, 1950).

The occasional reports of unreliability of boric acid may be related to the observation that boric acid in concentrated solution exhibits stronger acid properties than in dilute solutions (Stetten, 1951). The highest accuracy and most reliable results have been claimed when the ammonia is collected in a standard acid, usually sulphuric, and standard alkali (NaOH) used for titration (Hiller et al., 1948; Joint Committee of AOAC, 1955). (It has been suggested that for maximal precision these should be independently standardised - Joint Committee of AOAC, 1955). Potassium hydrogen iodate has also been used to collect the ammonia (McKenzie and Wallace, 1954), the subsequent determination of which was by the usual acidimetric method. Iodometric methods (e.g. Ballantine and Gregg, 1947) on the distillate have not proved popular although they have been advocated for direct determination of ammonia in the digest without prior distillation (Harvey, 1951; Dixon, 1955). Other direct methods have made use of the formol titration (Adams and Spaulding, 1955) and a back-titration of sodium hypochlorite with arsenious oxide (Belcher, 1956).

In the titrimetric methods of determination of ammonia

in the distillate, various indicators have been used. For example, Ma and Zuazaga (1942) used methyl red and bromocresol green in the ratio of 1:5, and an interesting two-step indicator, which gives a "warning" of the approach of the end-point by a green to blue colour change and at the end-point (pH 4.6) changes from blue through grey to yellow, has been described by Sher (1955).

8. Other methods of ammonia estimation

Aeration methods of determining ammonia in protein digests do not seem to be favoured, and their greatest sensitivity is in the micro range, thus presenting no advantage over steam-distillation. Diffusion methods, on the other hand are popular, especially for the determination of extremely small amounts of nitrogen of the order of micrograms (Seligson and Seligson, 1951; Bool and Shen, 1954; Short, 1954; Vallentyne, 1955). The best known diffusion method is that of Conway (1947) in which a microtitration method is used in the estimation of ammonia, although the sensitivity of colorimetric methods renders them very convenient in this range.

Colorimetric methods of ammonia estimation

There are three generally used colorimetric methods of ammonia estimation:

(a) the Nessler procedure; (b) methods based on the reaction with ninhydrin or chemicals closely related to it; (c) methods based on the reaction with phenol and hypochlorite with formation of indophenol. Each of the methods has been adapted for direct estimation of ammonia in the digest.

(a) The Nessler procedure

The Nessler method is the oldest and probably most frequently

used of the colorimetric methods. The colour obtained - yellow - brown - is due to the formation of a mercury-iodine-ammonia compound which is only sparingly soluble and in the usual form of the procedure, is said to be present as a colloid (Nichols and Willets, 1934; Wicks, 1941). Thus in carrying out the estimation, careful control of the following conditions is required: pH; temperature; ionic strength; method and rate of formation of the complex. In practice, this necessitates avoidance of variation in:
1. the preparation of the reagent; 2. the condition of the reagent at the time of Nesslerisation; 3. conditions obtaining during digestion (especially the concentration of salt present);
4. temperature during addition of the reagents; 5. pH before and after Nesslerisation; 6. rate of addition of the reagents and rate of mixing; 7. time of reading the extinction (see Thompson and Morrison, 1951).

In this laboratory the Nessler reagent recommended by Paul (1958) has been found to be stable and to give reproducible results over a long period with pure solutions of ammonium sulphate in water. Numerous other satisfactory reagents have been described (for example, Polley, 1954; Lang, 1958). Some other forms of the Nessler reagent alter with storage, and their condition must be checked before use (Thompson and Morrison, 1951).

If direct estimation of ammonia in the digest is attempted, conditions must be strictly controlled. In particular, digestion must be complete, suitable blanks must be prepared and the addition of salts limited, since the presence of sodium sulphate in an ammonia

solution is known to cause a colour increment (Thompson and Morrison, 1951). In one direct method, barium nitrate has been added to remove this interfering sulphate (Herbain, 1953). The presence of calcium, magnesium, or phosphate affects results (Miller and Miller, 1948). The use of zinc dust to break down the mercury-ammonium complex has been found a convenient preliminary in a direct method using Nessler's reagent (Polley, 1954); without this step, irregular results can be obtained.

Throughout the Nessler procedure, pH is an important factor. If the ammonia solution is alkaline or if alkali is added to the solution before the Nessler reagent, turbidity will result (Thompson and Morrison, 1951), while if the solution remains acid, colour development is poor or the reagent may be precipitated. It is thus advisable to neutralise acid digests containing ammonium salts before the addition of Nessler's reagent (Thompson and Morrison, 1951). The concentration of alkali during colour development is also important; if too low, colour development is poor and mercuric iodide may precipitate, and if too high, turbidity results (Thompson and Morrison, 1951).

The temperature at which the colour is developed is another important factor which must be carefully controlled (Miller and Miller, 1948; Toal and Daniel, 1950; Thompson and Morrison, 1951). For the reagent used by Thompson and Morrison (1951) the optimum temperature was 20°C and if temperatures in excess of 30°C were used, the solution became turbid.

Rapid and thorough mixing of the reagents during addition

to the ammonia solution is essential (Toal and Daniel, 1950) and the rate of addition of the reagents is also critical (Miller and Miller, 1948). Thompson and Morrison (1951) state that the rate of mixing during the addition of sodium hydroxide is extremely important, as turbidity can result from local high concentrations of alkali.

Following addition of the reagents and thorough mixing, colour development is very rapid for two minutes and increases slowly thereafter (Miller and Miller, 1948). According to Thompson and Morrison (1951), there is a continuous gradual increase in colour which is minimal between 20 and 30 minutes after addition of the reagents; after 50 minutes turbidity may occur. With larger quantities of nitrogen of the order of 100 µg, the rate of colour development is greater, so that unless extinctions are read at a fixed time, the errors with large amounts of nitrogen will be disproportionally greater than with smaller amounts.

In view of the number of variables, it is surprising that accurate and reproducible results with the Nessler method have been claimed frequently (for example, Paul, 1958; Lang, 1958; Burok, 1960), and agreement with the Kjeldahl method obtained (Kalmikov, 1953), although others disagree (Boissonos and Haselbach, 1953). Even more surprising is the claim that direct Nesslerisation of protein digests is an accurate procedure (Johnson, 1941; Anderson, 1952; Lang, 1958). It must be noted, however, that certain reagents (that of Paul, 1958 for example) give highly reproducible results with solutions of pure ammonium salts, but when used directly with

Kjeldahl digests reproducible results may or may not be obtained. In the light of the evidence cited above, it is essential, when using the Nessler procedure, to check the reliability by carrying out the estimation simultaneously on standards of pure ammonium salt solutions and also a protein of known nitrogen content with each run; failure to do this gives results of indeterminate reliability. There is also little doubt that the distillation or diffusion of ammonia from the digest before its estimation by the Nessler method should give more reliable results than those obtained following direct estimation. Reliable results should be obtainable if digestion is carried out with sulphuric acid alone in a sealed tube and the digest carefully neutralised before the estimation of ammonia (see Grumbaum et al., 1955).

(b) Methods based on the ninhydrin reaction

Good results in the estimation of ammonia with the ninhydrin reagent have been claimed (Jacobs, 1959 and 1962). If the digest is diluted with a citrate buffer, the method may be used directly without distillation (Boissonas and Haselbach, 1953; Fels and Veatch, 1959; Jacobs, 1959). Citrate buffer is essential in order to obtain a linear relationship between ammonia concentration and extinction (Fels and Veatch, 1959).

(c) Methods based on indophenol formation

The indophenol of sodium phenate method of Russell (1944) for the estimation of ammonia has been modified (Exley, 1956) for the direct estimation of ammonia in Kjeldahl digests of protein. The sensitivity of the method has been increased by the introduction

of sodium nitroprusside as catalyst (Lubochinsky and Zalta, 1954). The colour development depends on the concentration of chlorine (Scheurer and Smith, 1955) and when the chlorine water (hypochlorite) is used fresh and the ammonia is buffered in boric acid, good reproducibility is obtained (Bolleter, Bushman, and Tidwell, 1961). Copper, zinc and iron salts and bromide ions interfere, but other salts do not (Bolleter et al., 1961).

The method is rapid, as only 3 minutes heating at 100°C is required (Bolleter et al., 1961; Chaney and Marbach, 1962), and appears to be gaining in popularity, although for the determination of protein nitrogen, it would seem to be safest to apply it to acid-trapped ammonia after distillation from the digest. However, Mann (1963) has developed a procedure for the analysis of 1-15 ug N as NH_3 in which, after digestion with H_2SO_4, K_2SO_4, HgO, digestion mixture and careful neutralisation of the digest with alkali, the NH_3 is estimated as indophenol.

Summary

1. The micro-Kjeldahl method is for biochemists the most popular method of nitrogen estimation.

2. In the micro-Kjeldahl method, digestion should be with mercury (as catalyst), sulphuric acid and sufficient potassium sulphate to raise the boiling point during digestion to about 390°C. Alternatively, digestion may be carried out at 440°-450°C with sulphuric acid alone in a sealed tube.

3. The estimation of ammonia is best carried out after separation from the digest.

4. In the range (0.2 - 1 mg N) ammonia is best determined by titration. When greater sensitivity is required, there is a choice of three colorimetric methods, of which it is difficult to select the most reliable.

Nitrogen Estimation - Experimental Section

A. In the range 0.5-2 mg nitrogen

It was decided to investigate the accuracy of the micro-Kjeldahl procedure for the estimation of nitrogen in the range 0.25-2.00 mg N.

Since the procedure consists of: 1. digestion; 2. distillation; 3. estimation of ammonia, in that order, it is obvious that an investigation of the accuracy of the method should be carried out in the reverse sequence.

1. The estimation of ammonia

For the quantities of nitrogen to be determined in this investigation, a titrimetric method of estimating ammonia is suitable, and, for reasons discussed in the introduction to this section, 0.01 N H_2SO_4 was selected as the NH_3-trapping agent, and titration was with 0.01 N NaOH.

The end-point in this titration in the presence of ammonia was determined, using the "EIL" pH meter to be in the pH range 4.8-6.8. Some suitable indicators are therefore, methyl red (pK 5.3), bromocresol purple (pK 6.3), chlorophenol red (pK 5.8), and De Wesselow's indicator - a mixture of methyl red and methylene blue with a presumed pK of 5.3. The results of titration of accurately standardised acid and alkali using these indicators indicated that the most accurate and easy to use indicator was De Wesselow's. With each indicator apart from De Wesselow's, slow fading of the colour occurs at the end-point particularly in the presence of ammonia. This leads to occasional high titrations. The error introduced

Table 45

Recovery of Ammonia in the Distillation Stage of the micro-Kjeldahl Procedure

mg N added	mg N recovered	% recovery
0.25	0.267	106.8*
0.50	0.491	98.2
0.75	0.748	99.7
1.00	1.002	100.2
1.25	1.256	100.5
1.50	1.491	99.4
2.00	1.976	98.8

*eliminating this figure (see text for reasons)

Mean recovery = 99.5%

s = 0.86%

by this fading of colour is greater than the error due to the choice of indicator. Thus, of the indicators tried, the only satisfactory indicator for the titration of H_2SO_4 with NaOH in the presence of NH_3 is De Wesselow's.

When the titration of 0.01 N acid and alkali using De Wesselow's indicator was compared with the titration of 1 N acid and alkali from which the more dilute reagents had been prepared, the agreement was within $\pm$ 0.4%.

2. The distillation of ammonia

Samples of a solution of "Analar" $(NH_4)_2SO_4$ in water, were pipetted directly into the Markham apparatus, then followed by alkali and steam-distilled into 10 or 20 ml of 0.01 N H_2SO_4, which was then titrated with 0.01 N NaOH; in this way, the recovery of ammonia was investigated.

In order to obtain full recovery of nitrogen 3 minutes distillation was required, although 2 minutes distillation usually resulted in 97% accuracy.

When distillation was carried out for 3 minutes, the recovery of nitrogen was virtually 100% (table 45), with a slightly restricted range for the method of from 0.5 to 1.5 mg N. Although frequent runs were carried out using 0.25 mg N, the error in this region was always unacceptably large.

3. Digestion

When the digestion mixture contains mercury, it is essential to decompose the mercury ammonium complex before distillation. This may be achieved by the addition of thiosulphate or zinc dust.

Table 46

The Effects of Duration of Digestion on the Recovery of N in the micro-Kjeldahl Procedure

Material	Recovery after a) 30 min. digestion	b) 60 min.	c.120 min.	Theoretical recovery %
1. Standard ammonium sulphate solution	0.990 1.001	0.999 1.001	0.998 -	99.8
2. Albumin solution	0.710 0.723	0.710 -	0.710 0.708	-
3. Tryptophan	0.840 0.859	0.837 0.868	0.868 0.868	97.1
4. Lysine	0.924 0.936	0.942 0.960	0.945 0.933	98.2*

* These materials were obtained directly from a BDH reagent bottle and were stated to contain not less than 98.5% N.

Although Naismith (1955) found thiosulphate satisfactory, in our hands, erratic results were occasionally obtained with it. These were found to be related to the emission of acid fumes which occurs as soon as warming of the mixture takes place. These fumes come through the condenser immediately before the first drop of distillate but nevertheless, in routine estimations cannot be separated from the ammonia. Accordingly, zinc dust was utilised in the present investigation and was found to be satisfactory; the possible difficulty of removing the zinc amalgam from the apparatus being readily overcome by dissolving it in nitric acid.

With a digestion mixture containing 1.5 ml concentrated H_2SO_4, 1.2 gm K_2SO_4 and 40 mg HgO, the time required for complete digestion of organic nitrogen to ammonia was investigated using solutions of ammonium sulphate, albumin, tryptophan and lysine. 30 minutes digestion was found to be adequate and up to 2 hours digestion did not lead to loss of nitrogen (table 46). It was also apparent (table 46) that the recovery of nitrogen with the complete method was satisfactory.

Summary and Conclusions

Following a survey of the literature, it was concluded that in the micro-Kjeldahl procedure for the estimation of nitrogen -

(a) digestion of 0.5-2 mg N should be carried out using 1.5 ml concentrated H_2SO_4, 1.2 gm K_2SO_4, 40 mg HgO.

(b) the ammonia released during digestion should be steam-distilled into standard acid (0.01 N H_2SO_4) then titrated with standard alkali (0.01 N NaOH).

Each stage of this procedure was carefully investigated and the optimum conditions at each stage determined.

The final procedure adopted for the estimation of organic nitrogen as a result of this investigation is described in detail in the appendix.

D. The Estimation of Quantities of Nitrogen Smaller than that Possible with the Micro-Kjeldahl Method

The Nessler method of estimating ammonia was selected for investigation as it is the most widely used procedure. The method was applied to the estimation of 5-30 µg nitrogen and the experimental procedure is described in detail below.

The Nessler method for the estimation of nitrogen

Procedure: from Paul (1950).

Sensitivity: a. 50-150 µg N per sample (see note 1).
b. or 5-30 µg N as NH_3.

Principle: Compounds containing N are digested, the N being converted to NH_4^+ (as sulphate). The NH_3 is then estimated colorimetrically without distillation (see note 2).

Reagents: N.B. use pyrex test tubes, graduated at 10 ml.

1. Selenium dioxide 1% in 50% (v.v.)
Sulphuric acid (N free-Analar) (for digestion)

2. Nessler reagent (modified)

a) KI 4G
HgI_2 4G
} in 25 ml water

b) add to this solution gum acacia - 3.5 gm. in 750 ml water. Make up to 1 litre.

3. NaOH 2 N (8% w/v.)

Standards: Stock standard 471 mg. $(NH_4)_2SO_4$ in 100 ml water (or BDH 100 µg N/ml). 1 ml of stock standard in 100 ml gives a solution containing 10 µg N/ml.

Method: (Digestion procedure for organic N compounds) Add 0.5 ml of the selenium in sulphuric acid solution to the sample containing N, place an "anti-bump" rod in the tube and boil till the water is evaporated and the acid concentrated. Digest until the solution is clear, cool and make up to 10 ml with water.

To 2 ml of this solution, add 2 ml Nessler's reagent then 3 ml of alkali. Mix and allow to stand for 10 minutes and read, within 20 minutes, at 490 mµ.

Notes: 1. Sensitivity

a) 50-150 µg N refer to N for digestion, this amount is made up to 10 ml and 2 ml taken for colorimetry, to which the figures in b) refer. The range 5-30 µg N also referring to N for digestion, is obtained by scaling down the procedure by using 0.1 ml acid for digestion, making up to 2 ml, then adding the reagents.

Note: 2. The colour complex is colloidal and precipitates on standing. It is also affected by larger quantities of NH_3, the presence of organic solvents or more than small quantities of acid.

The Nessler reagent is not stable for more than a few weeks.

Alternative Digestion Procedure

Method

To material containing 5-30 µg N, add 0.1 ml digestion mixture (HgO, K_2SO_4, H_2SO_4 as for the micro-Kjeldahl procedure diluted in water). Heat for about 1 hour in 95°C oven to remove excess water then at approximately 200°C in a sand bath till only coloured concentrated acid remains; (time required is about 1-2 hours). Then digest completely (i.e. till colourless) on an electric rack at position 3 - (time required, about ½ hour). The mixture is then cooled and 1.95 ml H_2O added. 2 ml Nessler's reagent are added followed by 3 ml alkali. The extinction at 490 mµ after 10 minutes is noted.

(for undigested NH_4^+ standards - take NH_4^+ in 2 ml and add Nessler's reagent as above).

Results

1. Preliminary

Using Analar $(NH_4)_2SO_4$ several calibration curves were obtained. Reagents made up at different times give the same extinction values for the same amount of ammonia. That turbidity occurs when the Nessler reagent is added after the alkali, was confirmed. The

method is sensitive (5-30 µg ammonia-nitrogen), reproducible, and reliable for solutions of ammonia.

2. Estimation of organic nitrogen as ammonia after digestion

Several standard ammonia and albumin solutions were digested using the selenium-containing digestion mixture. Nesslerisation was carried out directly on the cooled digest. On only one occasion was good agreement among 10 samples obtained. On every other occasion, either the extinction after Nesslerisation of the digested standard ammonia solution was not in agreement with the extinction of an undigested standard - the differences were usually of the order of 10% - or there was a 10% random variation in the extinction of solutions which contained the same amount of organic nitrogen. These variations were not due to the blank or to the presence of the acid used in digestion.

Attempts to use mercury as a catalyst followed by direct Nesslerisation were equally unsuccessful.

It should be noted that these difficulties with the Nessler method would not have been observed if careful standardisation had been omitted, and that the results are in agreement with others who have investigated the Nessler method (see introduction to this section). It may be that those who claim that the Nessler method is satisfactory for the direct Nesslerisation of digests of organic nitrogen without distillation or aeration have not been sufficiently critical.

Modifications of the Nessler Procedure

Following these results it was decided to try to remove the ammonia from the digest and to trap it in a dilute acid solution

prior to Nesslerisation. This would avoid interference due to salts, and a very low pH. Three methods were tried:-

1. steam distillation; 2. the Conway micro-diffusion method; 3. diffusion under reduced pressure.

1. Steam distillation

With micro-Quickfit, a steam distillation apparatus was constructed. In use, the accuracy was never better than 10% and the apparatus was not suitable for large numbers of samples.

One particular difficulty encountered was the adjusting of the amount of K_2SO_4 in the scaled-down digestion mixture so that solidification of the digest did not take place on cooling. Digestion was carried out on electric racks, and with the small quantities of concentrated acid present (about 0.1-0.2 ml) and the possibility of poor contact of the bottom of the digestion tube with the heating element, it was liable to be erratic.

It was then decided to attempt to digest the smaller quantity of nitrogen (5-30 µg) with the normal amount of digestion mixture used in the micro-Kjeldahl method, followed by steam distillation in the Markham apparatus and collection in a small volume of dilute acid, a sample of which could then be removed for Nesslerisation. This technique was an improvement. However, the extinction of the blank remained high and the experimental error was again of the order of 10%.

The high extinction values of the blank was shown to be due to nitrogen in each component of the digestion mixture. The high blank could be eliminated by scaling down the quantities of digestion

reagents in proportion to the amount of nitrogen to be digested. This, however, immediately led back to the difficulty of erratic digestion with a very small quantity of acid used.

Although these results were promising, it was decided to abandon the investigation at this stage since it appeared that special apparatus, considerable time and expense would be required to develop the method to obtain satisfactory analytical accuracy.

2. The Conway diffusion method

With the apparatus available, although a fairly satisfactory calibration curve for the procedure could be obtained, it proved impossible to obtain agreement between a non-diffused and a diffused standard ammonium sulphate solution.

3. Diffusion under reduced pressure

This technique, with the apparatus available, was time-consuming and not suitable for large numbers of samples.

Summary

No satisfactory method for the analysis of large numbers of samples for organic nitrogen content in the range 5-30 µg nitrogen was found.

With the possibility of estimating protein by a direct colorimetric method in the range of approximately 100 µg of protein, it was decided not to proceed further with attempts to estimate accurately small amounts of organic nitrogen.

The Estimation of DNA

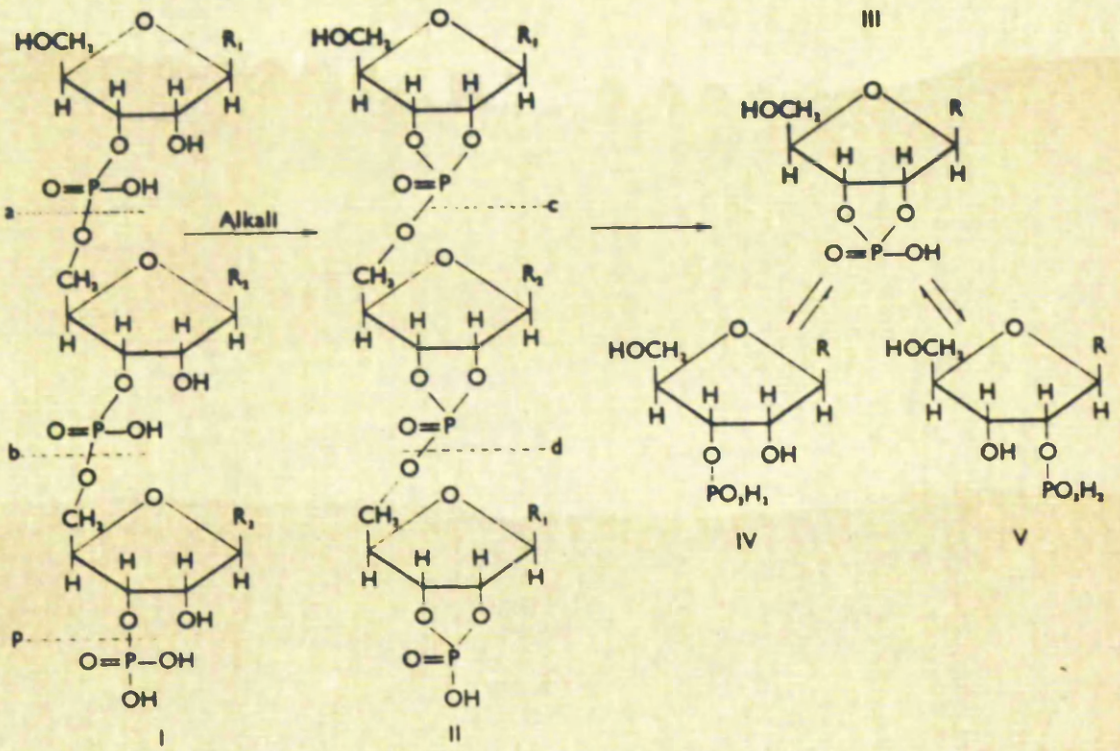
Introduction

The two most commonly used procedures for the estimation of nucleic acids in tissues are those of Schneider (1945) and of Schmidt and Thannhauser (1945); they are discussed critically in a recent review of nucleic acid analytical procedures (Hutchison and Munro, 1961). An additional method (Ogur and Rosen, 1950) has been introduced but gives incomplete separation of RNA and DNA (Holden, 1952).

In the Schneider procedure, hot acid is used to extract the nucleic acids from the tissue. The subsequent estimation of the nucleic acids is superficially simple; the orcinol reaction for RNA and the diphenylamine or indole reaction for DNA (see Hutchison and Munro, 1961). The orcinol method for RNA may, however, be inaccurate due to interference from DNA (Schneider, 1945), glycogen and protein degradation products (see below). The choice of acid for extraction is complicated in that the use of TCA eliminates the possibility of ultraviolet spectrophotometry and possibly the Ceriotti (1952) method for DNA (Ceriotti, 1955), while PCA gives some destruction of DNA (Hutchison, Downie and Munro, 1962); with neither acid are the optimal extraction conditions known (Hutchison, Downie and Munro, 1962).

Alkali at 37°C is used to separate RNA from DNA in the Schmidt-Thannhauser procedure; in this, RNA is hydrolysed to acid-soluble nucleotides while DNA remains chemically intact, largely undegraded and acid-precipitable (Hutchison and Munro, 1961).

Fig. 47. ALKALINE HYDROLYSIS OF RNA



Hydrolysis of RNA by alkali; R represents a purine or pyrimidine base. (Reproduced from Davidson, J. N., "The Biochemistry of the Nucleic Acids," Fourth Edition, Methuen & Co. Ltd., London)

Criticism (Drasher, 1953) of this separation of RNA and DNA has never been substantiated (Hutchison and Munro, 1961) and the chemical mechanism of the reaction is understood. RNA is hydrolysed by alkali via the cyclic 2'-3' phosphate to nucleotide 2' or 3' phosphates (fig.47), and in suitable conditions nearly quantitative (95-97%) hydrolysis to mononucleotides occurs (Hutchison and Munro, 1961) so that electrophoresis (Davidson and Smellie, 1952) to determine base ratios or check accuracy and purity is possible. Unfortunately, substances other than RNA become acid-soluble as a result of alkaline digestion. The phosphorus content of the acid-soluble fraction is usually greater than the RNA-P (Davidson and Smellie, 1952). Secondly, carbohydrates other than ribose have been reported in the RNA fraction causing interference in the orcinol reaction (see Hutchison and Munro, 1961). Finally, the presence of protein or protein degradation products (Hutchison, Crosbie, Mendes, McIndoe, Childs and Davidson, 1956) can cause errors in the ultraviolet absorption measurements of RNA. Whereas the non-RNA phosphorus and non-RNA orcinol-reacting material may have various origins, the error in the ultraviolet absorption measurement of RNA should be limited to protein and peptides and thus this method appears to offer the best prospect of exclusion of sources of error.

Apart from separation of contaminant peptides by electrophoresis (Davidson and Smellie, 1952) or ion-exchange chromatography (De Decken-Grensen and De Decken, 1959), two methods of evading the interference in ultraviolet absorption due to peptide

material have been proposed. Scott, Fraccastoro and Taft (1956) altered the conditions of digestion to 1 N alkali at 22°C for one hour (from the usual 1 N alkali at 37°C for 18 hours) and noted that the ultraviolet absorption spectrum of the acid-soluble fraction closely resembled that of pure hydrolysed RNA, and that extraction of RNA was complete. However, the question of whether the hyperchromic effect was fully developed in this short time was not investigated, nor was it shown conclusively that peptide material was absent from the RNA fraction. In advocating the second alternative Tsanev and Markov (1960) state that unsatisfactory results were obtained with the method of Scott et al. (1956), but give no evidence; they used instead the longer digestion time and previous conditions (1 N alkali, 37°C, 15 hours) and introduced a two-wavelength method of measuring RNA in the presence of protein-degradation products. This method can also be criticised: yeast RNA was used as a standard for all tissues; deamination of cytidine could occur with their conditions of digestion; their peptide material prepared from the RNA fraction is unlikely to provide an ultraviolet spectrum representative of the protein degradation products usually present (due to the excess pre-treatment with hot acid in its preparation).

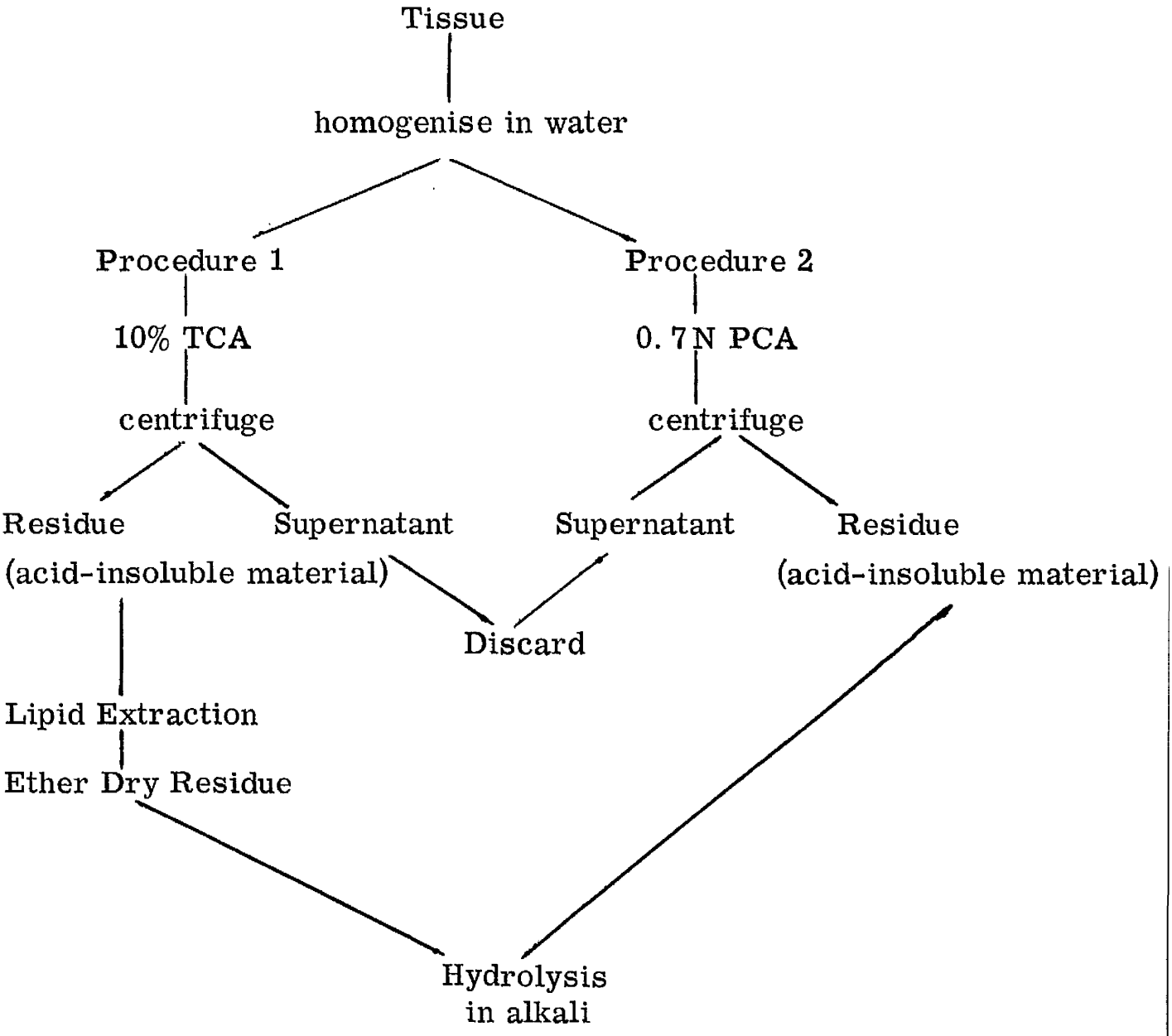
The following investigation was undertaken to explore the possibility of obtaining a suitable method of estimating the RNA of rat liver.

Methods

Livers (fresh or deep frozen) from adult male albino rats were homogenised for 5 minutes in 19 volumes of ice-cold water in

Fig. 48.

PREPARATION OF TISSUE FOR EXTRACTION OF RNA



a "Neloo" blendor, and 5 ml samples (equivalent to 250 mg wet weight of original tissue) transferred to centrifuge tubes for subjection to a standard Schmidt-Thannhauser procedure (see fig.48) for removal of acid-soluble compounds and lipids, or to a modified procedure in which extraction of lipids was omitted; in the latter PCA was used in the preliminary protein precipitation stage to allow subsequent ultraviolet absorption measurements.

Standard procedure: 0.5 volume of cold 30% TCA was added to 5 ml of homogenate, the precipitate centrifuged down and washed twice with cold 10% TCA. Lipids were then extracted from the precipitate using: cold acetone, then ethanol, ethanol-chloroform (3:1) twice, ethanol-ether (3:1), and ether at room temperature. The tissue residue was finally dried at room temperature before digestion in alkali.

Modified procedure: 0.5 volume of cold 2.1 N PCA was added to the sample of homogenate and after 15 minutes standing, the precipitate was separated centrifugally and washed twice with cold 0.7 N PCA. The PCA was carefully drained off before the addition of alkali for digestion.

Alkaline digestion: The extracted samples were digested in an incubator at 37°C with 1 N NaOH or 0.3 N KOH. The alkali must be free from ultraviolet absorbing contaminants. 3 ml of alkali was added to the tissue residue in the standard procedure and 4 ml in the modified procedure, in order to allow for acid remaining in the precipitate. At the end of incubation the samples were chilled in ice, then neutralised with 10 N PCA and acidified with 1 volume

Table 47

Analytical Data of Rat Liver RNA and "Acid-Soluble Polypeptide" Material

RNA

Nitrogen 11.4 ± 0.1% (micro-Kjeldahl)

Phosphorus 7.2 ± 0.4% (Griswold)

6.8 ± 0.2% (indirect, from U.V. data)

Protein Nil, (Lowry et al., 1951)

Optical Properties (obtained after hydrolysis in 0.3 N KOH at 37°C for 18 hours).

All data at pH 1, in $^{N}/_{10}$ FCA

- $\lambda_{max.}$ - 260 mμ; $E^{1\%}_{1cm}$ at $\lambda_{max.}$ - 213 ($\epsilon_{1cm}{}^{P}$ at $\lambda_{max.}$ - 10110)
- $\lambda_{min.}$ - 232 mμ; $E^{1\%}_{1cm}$ at $\lambda_{min.}$ - 69.4
- $E^{1\%}_{1cm}$ at 275 mμ - 179; $E^{1\%}_{1cm}$ at 280 mμ - 159

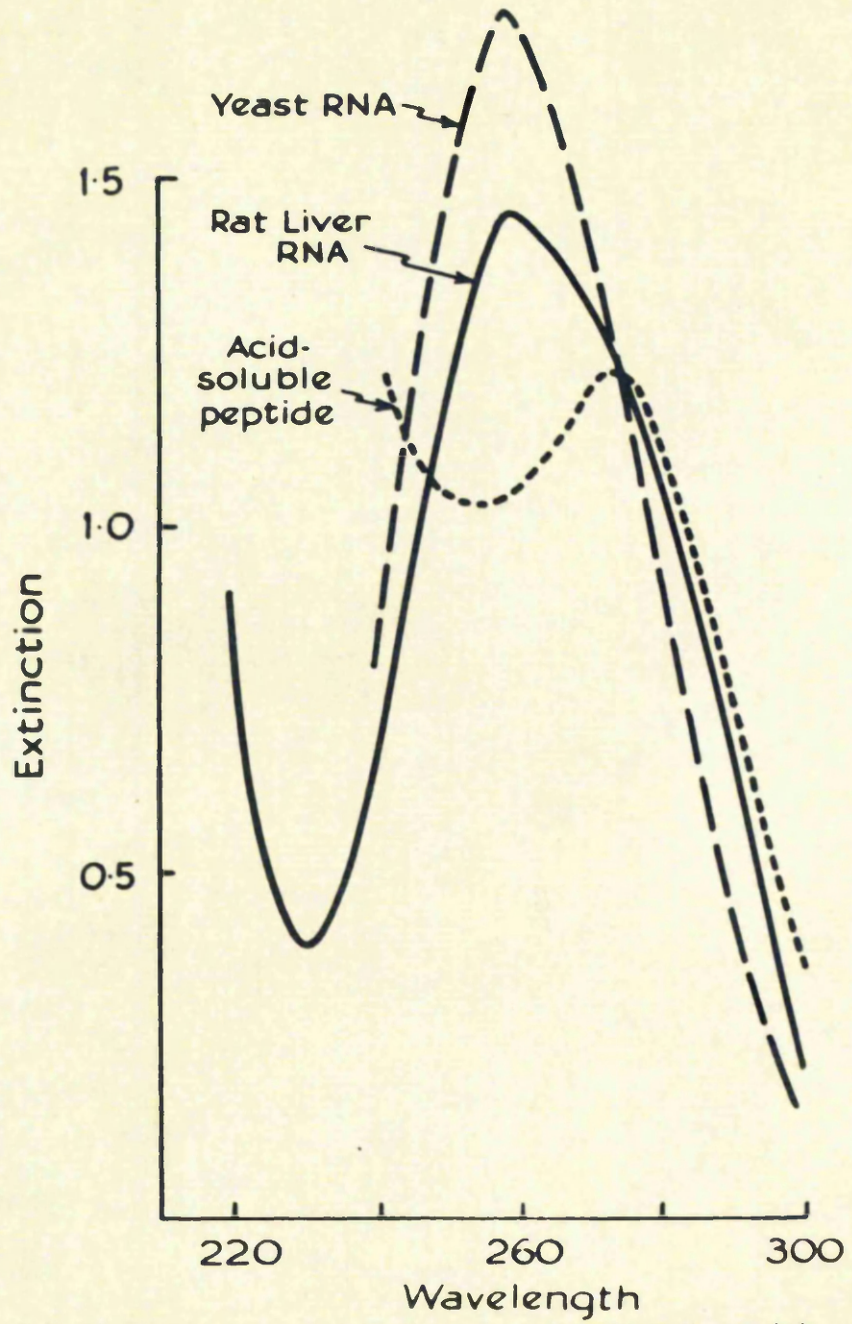
Acid-soluble polypeptide (890 mg obtained from 30 G rat liver)

Nitrogen 11.9%; Phosphorus 0.08%; Glucose 3%

Optical Properties (at pH 1 in $^{N}/_{10}$ FCA)

- $\lambda_{max.}$ - 275 mμ; $E^{1\%}_{1cm}$ at $\lambda_{max.}$ - 8.77
- $\lambda_{min.}$ - 255 mμ; $E^{1\%}_{1cm}$ at 260 mμ - 7.75
- $E^{1\%}_{1cm}$ at 232 mμ - 24.3

Fig. 49. ABSORPTION SPECTRA OF HYDROLYSED LIVER AND YEAST RNA AND ACID-SOLUBLE POLYPEPTIDE



Ultraviolet absorption spectra in 0. 1 N PCA of (1) pure rat liver RNA after hydrolysis in 0. 3 N K0H for 18 hr. at 37°, (2) commercial yeast RNA after hydrolysis under similar conditions and (3) polypeptide material appearing in the Schmidt-Thannhauser RNA fraction.

cold 1 N PCA. After centrifugation the precipitate was washed twice with cold 0.5 N PCA and combined supernatant and washings made up to 100 ml in a final concentration of 0.1 N PCA, to constitute the RNA fraction. The residue, dissolved in 1 N alkali and made up to 50 ml in a final concentration of alkali of 0.1 N is the DNA fraction.

RNA of rat liver: This was prepared by the phenol method of Kirby (1956). The yield was approximately 50%. Polysaccharride was extracted using methyl cellosolve which had been redistilled over KOH under reduced pressure in the presence of nitrogen to give a product with extinction at 260 mμ below 0.100.

Acid-soluble polypeptide material: This was prepared by subjecting 30 gm of rat liver to the standard Schmidt-Thannhauser procedure, followed by incubation in 1 N NaOH at 37°C for 18 hours. The incubation mixture was then cooled, acidified with PCA etc. in the usual way to obtain the RNA fraction, which was subsequently dialysed against several changes of distilled water in the cold for 5 days. Following dialysis the solution was lyophilysed to yield 890 mg of a white powder.

The RNA and acid-soluble peptide material were analysed - for results see below and table 47 - the absorption spectra in 0.1 N PCA are shown in fig.49.

Chemical analysis: Protein was estimated by the copper-Folin phenol method of Lowry, Rosebrough, Farr and Randall (1951). Nitrogen estimation was by the micro-Kjeldahl procedure (Ma and Zuazaga, 1942) using metallic mercury as catalyst (Miller, Plazer

and Van Slyke, 1948). The estimation of Phosphorus was by the method of Griswold, Humöller and McIntyre (1951). The glucose oxidase method of Hugget and Nixon (1957) was used in the estimation of Glucose. The method of Ceriotti (1952) was used in the estimation of DNA. The pentose of RNA was estimated by the method of Kerr and Seraidarian (1945), using a 30 minute heating period (Albaum and Umbreit, 1947).

Results

Acid-soluble polypeptide material

That this water-soluble white substance consisted mainly of polypeptide was shown by the ultraviolet absorption spectrum (fig.49), positive biuret reaction and nitrogen content (table 47). The phosphorus content was negligible but 3% glucose (in combined form - probably as polysaccharride) was present.

Rat liver RNA

The absence of protein, the nitrogen and phosphorus content (table 47), and the ultraviolet absorption spectrum all indicate that this preparation was pure. This was further confirmed as follows:- A solution of the RNA was incubated at 37°C for 18 hours with 0.3 N KOH in order to hydrolyse it to nucleotides. (1 N alkali was avoided in case of deamination of cytidylic acid). Following neutralising with PCA and centrifuging to remove the precipitate of potassium perchlorate, aliquots were treated;

(a) 0.1 ml samples containing the nucleotides from about 100 µg RNA were applied to Whatman No.1 paper and subjected to two-dimensional chromatography by the method of Lipschitz and Chargaff (1960). Four

Fig. 50. CHROMATOGRAPHY OF HYDROLYSED PURE RAT LIVER RNA

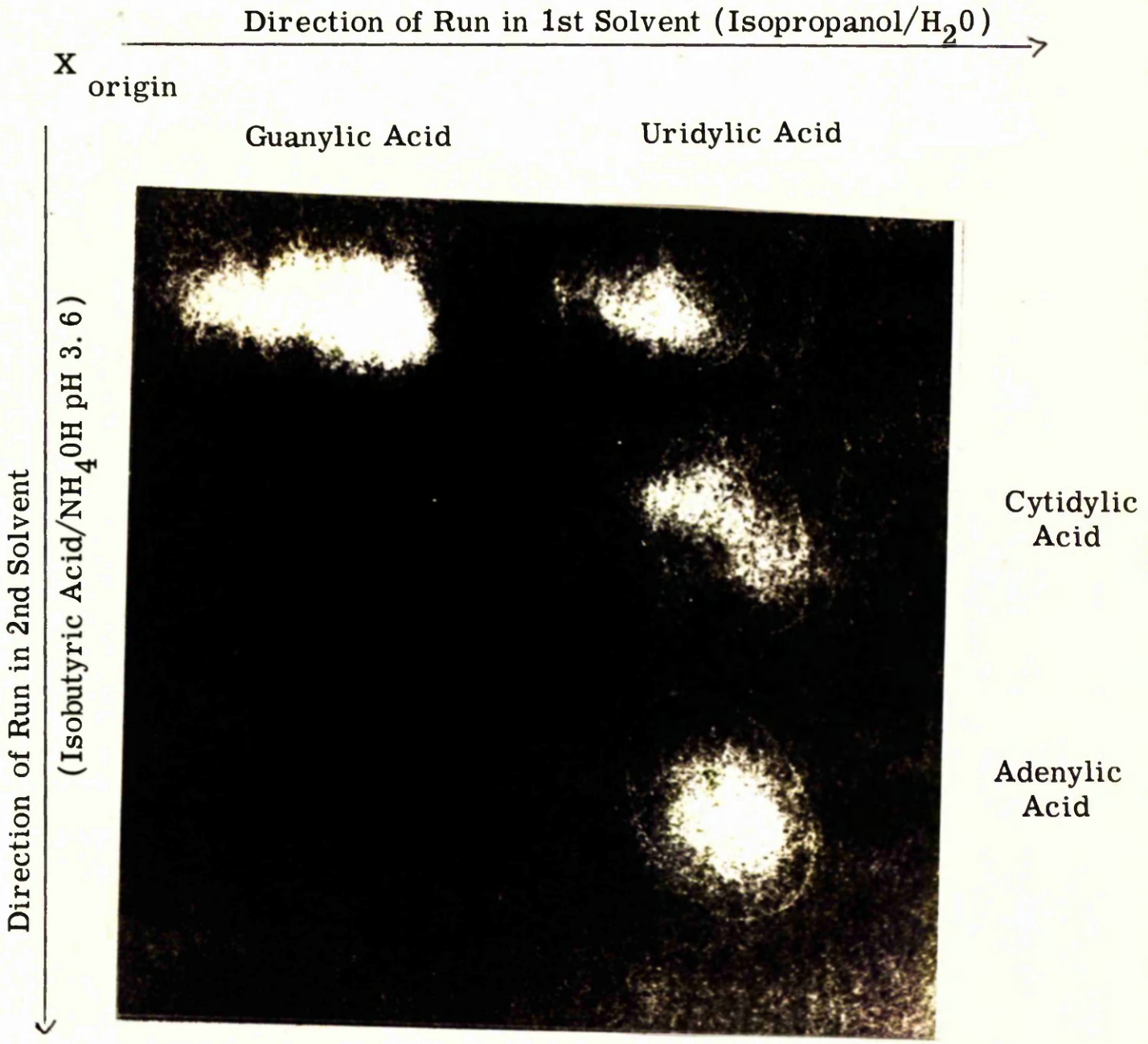


Fig. 51. ELECTROPHORESIS OF PURE HYDROLISED RAT LIVER RNA.

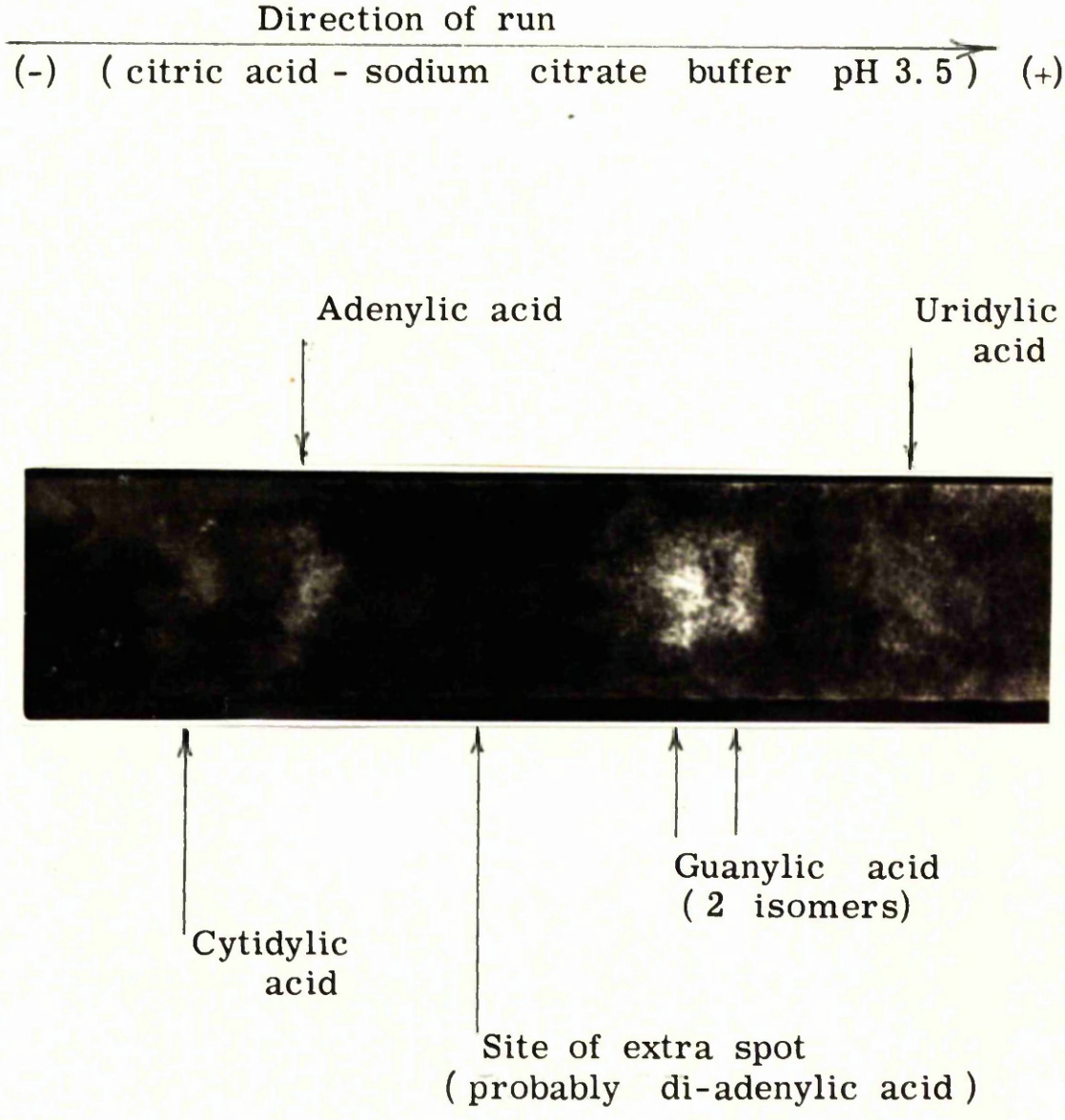


Fig. 52. **ABSORPTION SPECTRA OF NUCLEOTIDES OBTAINED AFTER PAPER ELECTROPHORESIS OF HYDROLYSED RAT LIVER RNA**

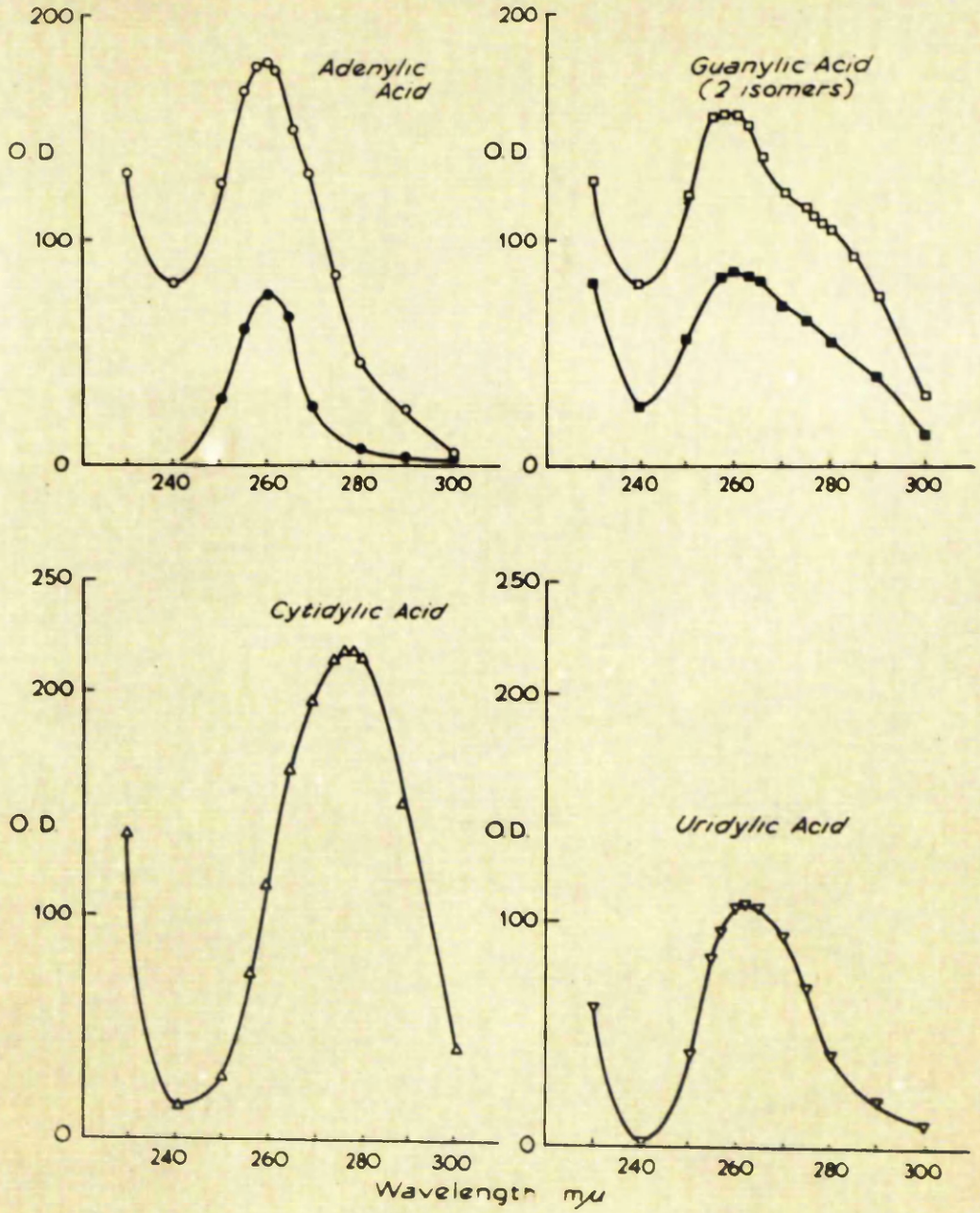


Table 40

Data on Purified Rat Liver RNA obtained by Paper Electrophoresis

(see text)

Nucleotide	E_{260}	ϵ_{260}	µM/ml	P µg/ml	Relative Base ratios
Adenylic acid	0.163	14.2	11.5	0.3	10
Guanylic acid	0.242	11.8	20.5	0.6	18
*Cytidylic acid	0.115	6.8	16.9	0.4	15
Uridylic acid	0.109	9.9	9.9	0.3	10
Di adenylic acid	0.075	14	5.4	0.1	

*Cytidylic acid E_{279} - 0.218, ϵ_{279} 12.9 giving 16.9 µM/ml

Total E_{260} - 0.700 Total µM/ml 65.3 Total P /ml 1.7

Total E_{280} 0.482

Hydrolysed RNA applied: E_{260} = 0.664; E_{280} = 0.496 Total P - 1.85 ug/ml

∴ recovery by U.V. absorption = 105%-97% i.e. 101 ± 4%

recovery by P analysis = 92%

$\frac{E_{260}}{E_{280}}$ of RNA = 1.34

"Theoretical" $\frac{E_{260}}{E_{280}}$ of RNA of above base ratios = 1.38

spots only, corresponding to the four common nucleotides - adenylic, guanylic, cytidylic and uridylic acids - were obtained (see fig.50).

(b) The same quantity of RNA (containing about 10 µg RNA-P) was applied to a strip of Whatman No.1 paper 72 cm by 5 cm and subjected to electrophoresis after the method of Davidson and Smellie (1952). On completion of the run, the strip was dried, and photographed, then the bands were eluted with 0.1 N FOA. Accurate ultraviolet spectra of the bands were obtained by comparison with appropriate blanks obtained from a blank strip run in parallel. In this way, the bands were identified and quantitative recovery checked (see figs.51 and 52). No peptide material was detectable. Recovery of ultraviolet absorbing material was 101± 4% and recovery of phosphorus 92± 10% (see table 48). It is apparent that the accuracy of the phosphorus method is poor in comparison with estimates based on ultraviolet spectrophotometry, so that in all calculations the phosphorus content of the RNA is taken as 6.8%; that is the figure based on the known phosphorus content of the constituent nucleotides obtained in this experiment, and not the figure obtained by direct phosphorus analysis.

The effects of alkali on pure rat liver RNA

Before applying an ultraviolet spectrophotometric method to the estimation of RNA in the Schmidt-Thannhauser procedure, certain effects of alkali on RNA require to be elucidated. These are:-

1. Under what conditions of exposure to alkali does RNA become subsequently acid-soluble?

2. At what stage does complete hydrolysis to nucleotides occur?

3. At what stage does the "hyperchromic effect" attain its maximum?

4. Could deamination of cytidine be a source of inaccuracy in the estimation of RNA (in the presence of protein) by a two-wavelength method?

Using the pure rat liver RNA described above, the effects of incubation with alkali (0.3 N KOH and 1 N NaOH) on:-

a. Solubility, b. Dialysability, c. Extinction at 260, 275 and 280 mμ were studied. An aqueous solution of the RNA was prepared. Samples were pipetted into measuring cylinders and an equal volume of either 0.6 N KOH or 2 N NaOH added. The mixtures were incubated for various intervals of time from 15 minutes to 24 hours in an air oven, then chilled. PCA was then added so that when made up to volume the RNA hydrolysate would be in 0.1 N PCA. After noting the extinction at 260, 275 and 280 mμ, the solutions were transferred to "Visking" dialysis tubing for dialysis first against running water, then several changes of distilled water for 4 days, after which the extinction of the solution was again noted.

Solubilisation of RNA

a. While carrying out the incubation procedure outlined above, it was observed that the zero-incubation time specimens (i.e. ones to which alkali was added then immediately followed by PCA) remained cloudy while all the others were clear. Since a blank showed that potassium perchlorate did not precipitate under these conditions, and the precipitate was also observed in the zero-time tubes to which NaOH had been added, it was concluded that the precipitate was RNA.

Fig. 53. THE EFFECTS OF ALKALINE HYDROLYSIS ON PURE RAT LIVER RNA

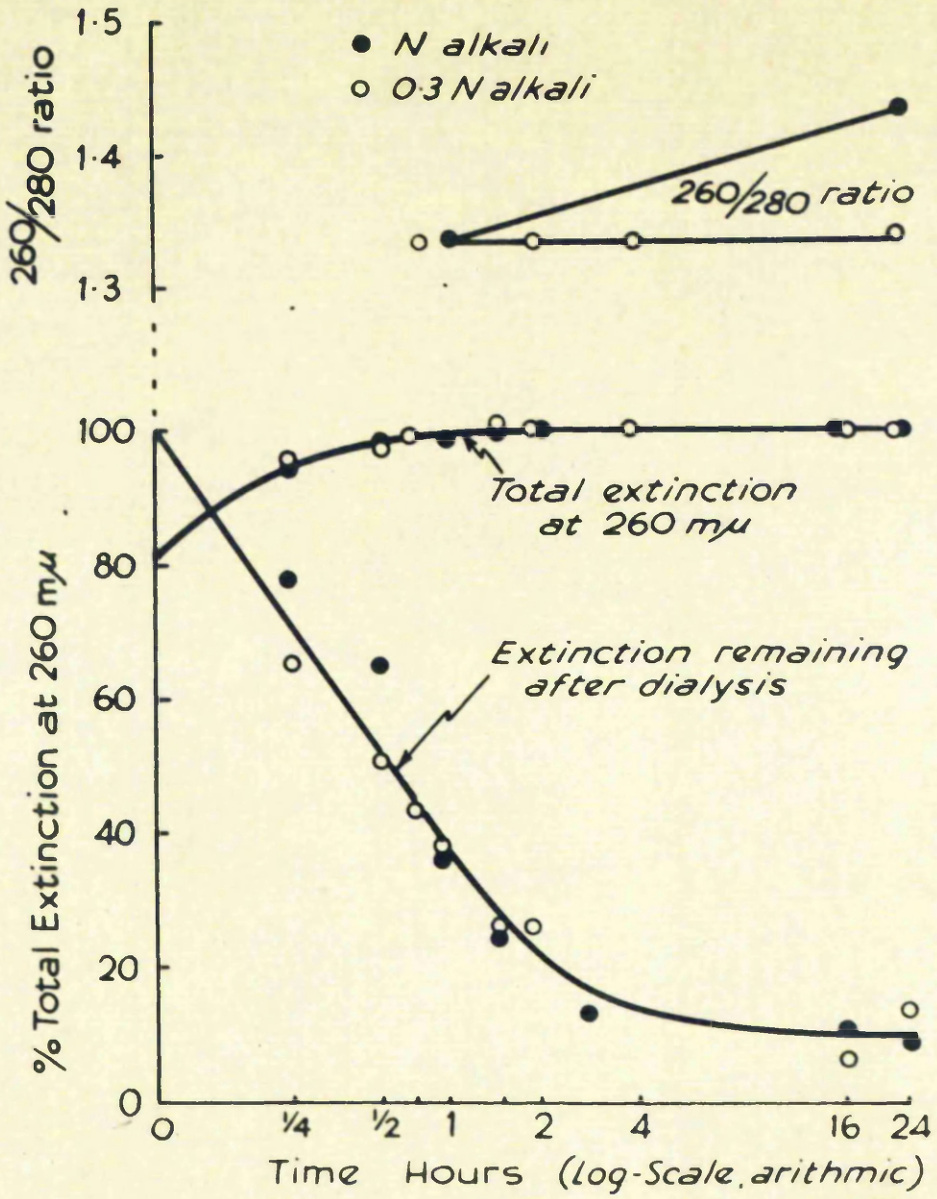


Table 49

The Effects of Alkaline Incubation on the Extinction of RNA at 275 and 280 mμ

Time of incubation (hours)	Incubation in N NaOH at 37°C		Incubation in 0.3 N KOH at 37°C		
	I	II	III	IV	$\frac{II - IV}{IV}$ as %
	$\frac{260}{275}$	$\frac{260}{280}$	$\frac{260}{275}$	$\frac{260}{280}$	
$\frac{3}{4}$	-	-	1.192	1.328	Within Experimental Error
1	1.188	1.336	-	-	
2	1.192	-	1.195	1.338	
18	1.227	1.372	1.185	1.332	2.8 ± 0.5
24	1.244	1.423	1.180	1.341	6.6 ± 0.5
		Mean:	1.188 ±0.007	1.335 ±0.007	

Note: in columns I, II, III, IV the figures $\frac{260}{275}$, $\frac{260}{280}$ refer to the ratios of the extinction at 260 mμ over the extinction at 275 mμ or 280 mμ.

Summary (N NaOH at 37°C) 18 hours incubation

increase in extinction at 260 mμ — 0.1% (within experimental error)

decrease in extinction at 280 mμ — 2.6 ± 0.5%

increase in ratio of extinctions at $\frac{260}{280}$ — 2.8 ± 0.5%

24 hours incubation

decrease in extinction at 280 mμ — 5.5 ± 2%

increase in ratio of extinctions at $\frac{260}{280}$ — 6.6 ± 1%

(experimental error > 1%; < 2%)

No cloudiness was observed in the "15-minute" tubes so that RNA was rendered acid-soluble by only 15 minutes exposure to alkali. (No attempt was made to estimate more accurately the actual minimal solubilisation time).

(b) By dialysis of the acid-soluble fractions an indication of the rate of production of dialysable products was obtained from the plot of the extinction at 260 mμ remaining after dialysis expressed as a percentage of the sample's original extinction at 260 mμ. Up to 2 hours of incubation produces a rapid decrease in non-dialysable end-products; thereafter, the rate falls gradually (fig.53). The method, obviously crude, reveals no differences in treatment with 0.3 N KOH or 1 N NaOH.

Variation of extinction with time of incubation

(c) The plot of extinction at 260 mμ against time shows that the hyperchromic effect is complete after $\frac{3}{4}$ hour incubation in both 0.3 N KOH and 1 N NaOH (fig.53).

The changes in extinction at 275 and 280 mμ with time of incubation and concentration of alkali are shown in table 49. The changes are expressed in the form of ratios, using the extinction at 260 mμ as a reference point because at the relevant incubation times it was constant, would reduce variation, and allow the comparison of different concentrations of RNA. It is obvious that for incubation times between $\frac{3}{4}$ and 24 hours, when 0.3 N KOH is used, there is no difference in the spectra over the range 260-280 mμ. However, when 1 N NaOH is used the spectra change with time: the extinction at 275 and 280 mμ decreases with increase in incubation time

Table 50

The Effect of Deamination of Cytidylic Acid on Extinction of a Hydrolysate of RNA in 0.1 N PCA

% deamination of cytidylic acid	% increase in E_{260}	% decrease in E_{280}	% increase in $\frac{E_{260}}{E_{280}}$ ratio
5	0.4	1.8	2.6
10	0.8	3.6	4.8
15	1.2	5.4	7.1
20	1.6	7.3	9.6
25	2.1	9.0	12.3
30	2.4	10.6	14.6
40	3.3	14.6	21

Note: These figures were obtained by calculation from spectral data (Beavan, Holiday and Johnson, 1955) assuming the base ratios of rat liver RNA to be:

Adenine	Guanine	Cytidine	Uracil
10	18	14	10

Table 51

Deamination of Cytidine during Alkaline Hydrolysis of Rat Liver RNA

Hydrolysing Agent	Time of Hydrolysis	% Deamination of Cytidine
0.3 N KOH	1 - 24 hours	none
N NaOH	1 hour	none
N NaOH	16- 18 hours	6 ± 3
N NaOH	24 hours	14 ± 3

(table 49 and fig.53).

There are various reports in the literature (Davidson and Smellie, 1952; Marrian, Spicer, Balis and Brown, 1951) which state that when RNA is incubated in 1 N alkali at 37°C for 18 hours a significant degree of deamination of cytidylic acid (with production of uridylic acid) occurs. Cytidylic acid has a wavelength of maximum absorption at 279 mμ and uridylic at 262 mμ, so that conversion of cytidylic acid to uridylic acid during alkaline incubation would result in a decrease in extinction of the RNA hydrolysate at 280 mμ and an increase at 260 mμ. However, in order to test this adequately, the quantitative aspects must be examined, and in order to do this the data shown in table 50 was calculated. Comparison of the data of tables 49 and 50, indicates that if the experimental error is about 1-2%, then in order to detect alterations in extinction at 260 mμ due to deamination of cytidylic acid, the extent of deamination must be greater than 20-25%. In fact, no change in extinction at 260 mμ was detected in the experiment so that deamination, if it occurred, must be less than 20%. Inspection of the figures for the alteration of extinction at 280 mμ indicates that about 7% deamination of cytidylic acid had occurred after 18 hours hydrolysis, and after 24 hours, 15$\pm$ 5%. Taking the figures for the ratio of extinction at 260 mμ to that at 280 mμ indicates that at 18 hours incubation 5% deamination had occurred, and at 24 hours, 13$\pm$ 2% (see table 51).

Fig. 54. EXTRACTION OF ULTRAVIOLET ABSORBING MATERIAL FROM RAT LIVER BY ALKALI

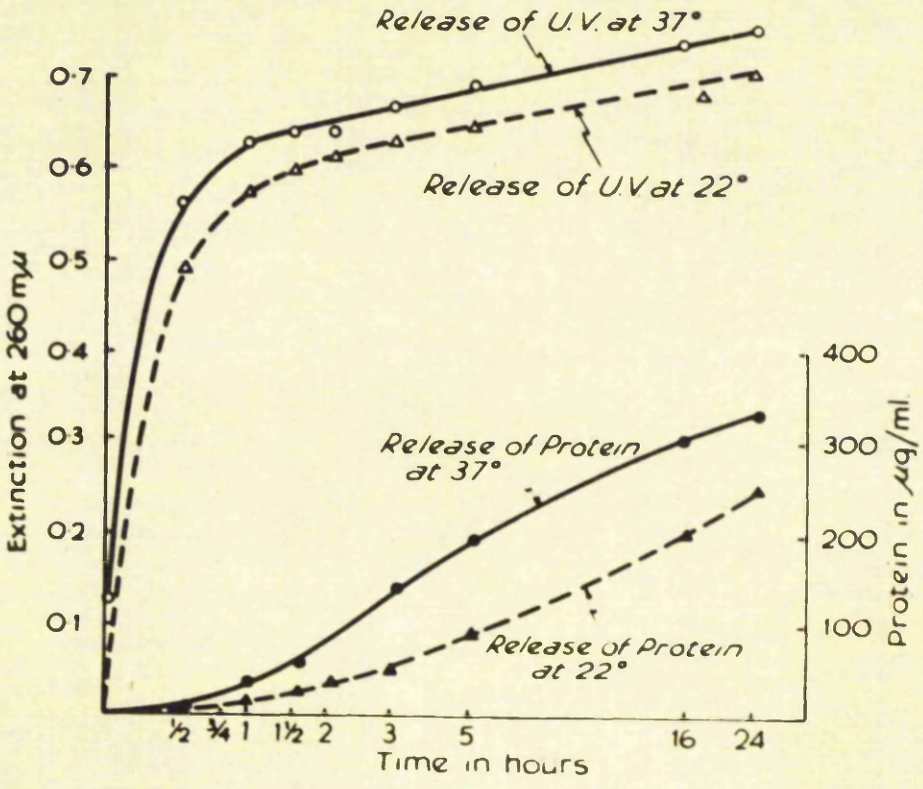
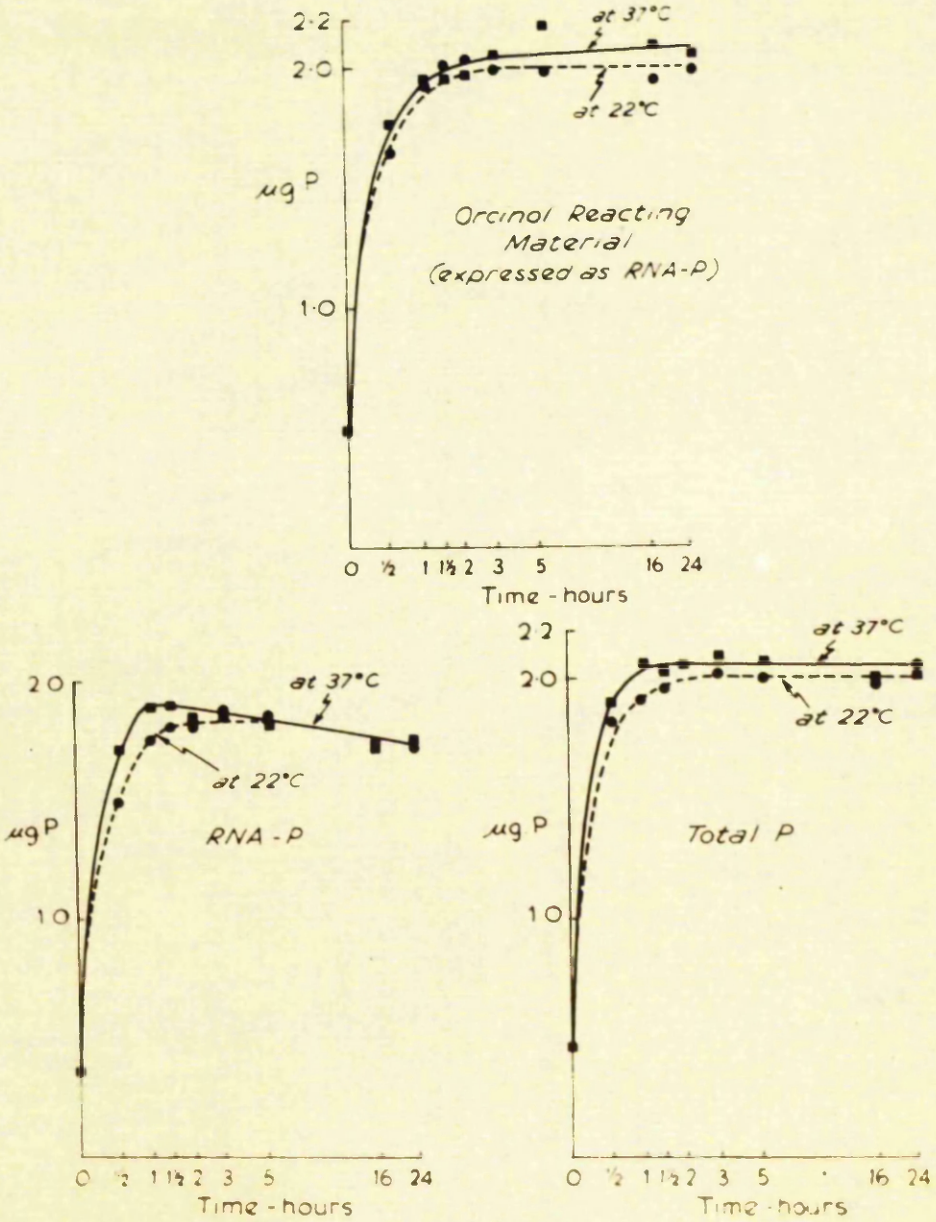


Fig. 55. EXTRACTION OF RNA, (EXPRESSED AS RNA-P), TOTAL PHOSPHORUS, AND ORCINOL REACTING MATERIAL BY 1 N. NaOH



Summary: The Effects of Alkaline Incubation on Rat Liver RNA

1. RNA is rapidly rendered acid-soluble by exposure to 0.3 N KOH at 37°C; less than 15 minutes is required.

2. About 90% hydrolysis of RNA to dialysable fragments occurs within 5 hours at 37°C using 0.3 N KOH and at least 95% hydrolysis to mononucleotides within 18 hours.

3. The hyperchromic effect is complete within ¾ to 1 hour.

4. When RNA is hydrolysed at 37°C by 1 N NaOH for 18 hours or longer, deamination of cytidine will be a source of error in a two-wavelength method for the estimation of RNA in the presence of protein. This is not the case if 0.3 N KOH is used.

The Extraction of RNA from Tissue Residues by Alkali

The study of the "kinetics" of the extraction of RNA from a tissue by alkali is an obvious first stage in the investigation of the determination of RNA by a Schmidt-Thannhauser method: the basis of this study - the effect of alkali on pure RNA - having been completed in the previous section.

After preliminary treatment with acid (with and without lipid solvent extraction), the tissue residues were incubated in 1 N NaOH or 0.3 N KOH for various times and the RNA fraction obtained as described previously.

The first experiment was a study of the effect of temperature on RNA extraction. Tissue residues from which lipids had been extracted were incubated in 1 N NaOH at 22°C and 37°C in air. The results are shown in figs.54 and 55 and should be compared with those of fig.53 which applies to pure RNA. The extinction at 260 mμ

Table 52

Effect of Omission of Lipid Solvents on Estimation of RNA

1. Extraction with 0.3 N KOH) No Lipid Extraction
2. Extraction with N. NaOH)
3. Extraction with lipid solvents then N. NaOH

	Time of incubation (hours)	RNA-P (a) U.V.	RNA-P (b) orcinol	Protein (method of Lowry et al. 1951)	Total P
1.	¾	2.15	1.65	4.1	2.18
	1	2.16	1.81	5.6	2.33
	1½	2.17	1.91	5.9	2.70
	20	2.01	1.83	22.9	4.85
2.	¾	2.16	2.08	5.9	4.46
	1	2.36	1.74	11.2	4.45
	1½	2.46	1.98	17.9	4.97
	20	2.05	2.08	79.9	4.66
3.	¾	2.36	1.92	22.3	2.23
	1	2.35	1.92	25.8	2.27
	1½	2.17	1.89	35.1	2.34
	20	2.04	2.12	91.0	2.34

Fig. 56. EXTRACTION OF (a) ULTRAVIOLET ABSORBING MATERIAL AT 260 mμ (b) PROTEIN, (c) ORCINOL REACTING MATERIAL BY TREATMENT OF "NON LIPID EXTRACTED" TISSUE RESIDUE WITH 0.3 N KOH AT 37°C

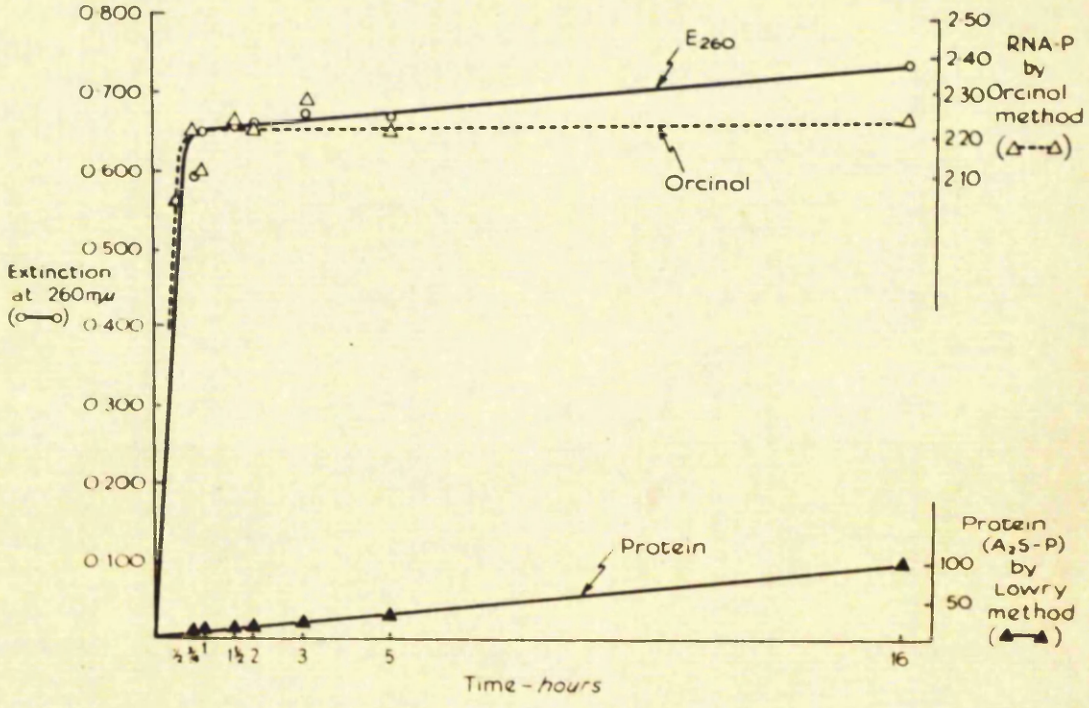


Table 53

Effect of Omission of Lipid Solvents on Estimation of RNA and DNA at 37°C

	(a) (i.e. 0.3 N KOH no lipid extraction)		(b) (0.3 N KOH lipid extraction)		(c) (N NaOH lipid extraction)	
Time of incubation	1 hour	16 hours	1 hour	16 hours	1 hour	16 hours
E_{260}	650	734	664	703	688	793
Protein (µg/ml)	6	100	24	152	76	269
RNA-P by orcinol (µg/ml)	2.43	2.45	2.48	2.42	2.42	2.51
DNA	22.5	-	23.3	23.4	23.0	-

rises rapidly up to 1 hour then increases slowly, whereas the extraction of protein is more gradual, being almost linear. The difference in extinction at 260 mμ with temperature is closely paralleled by the difference in the amount of protein extracted, which is much lower at 22°C. The extraction of phosphorus is almost identical with that of orcinol-reacting material. At 37°C orcinol-reacting material increases slowly with time (almost within experimental error), otherwise the curves suggest that extraction of RNA is very rapid and is complete in one hour. The curve of RNA-extraction versus time is closely similar to the phosphorus and orcinol-reacting material curves (RNA estimation was by ultraviolet absorption methods which will be discussed later).

In the second experiment, the effects of concentration of alkali and omission of previous lipid extraction were studied. The main effect of the presence of lipids is to increase the amount of phosphorus extracted and so clearly to vitiate the use of phosphorus as an index of the RNA present. An additional effect of the omission of lipid extraction is that the amount of protein which becomes acid-soluble on alkaline incubation is reduced. The figures for orcinol-reacting material and RNA (see table 52 and fig.56) again indicate that RNA extraction is complete within one hour of exposure to alkali.

A third experiment was carried out to determine whether 0.3 N KOH was a satisfactory extractant. The results (table 53) in general were the same as those described above. The omission of lipid solvent extraction makes no difference to the amount of

orcinol-reacting material or RNA obtained, but the amount of protein extracted at one hour is very small (tables 52 and 53).

DNA estimation was unaffected, no matter which of the above procedures was adopted (table 53).

Summary: The Extraction of RNA from Tissue Residues by Alkali

Although the above results strongly suggest that RNA is extracted from a tissue residue by 0.3 N KOH (and 1 N NaOH) within one hour at 37°C and that this time of incubation would be suitable for the application of a two-wavelength ultraviolet method of estimating RNA, such a conclusion would be premature without an investigation of the orcinol reaction.

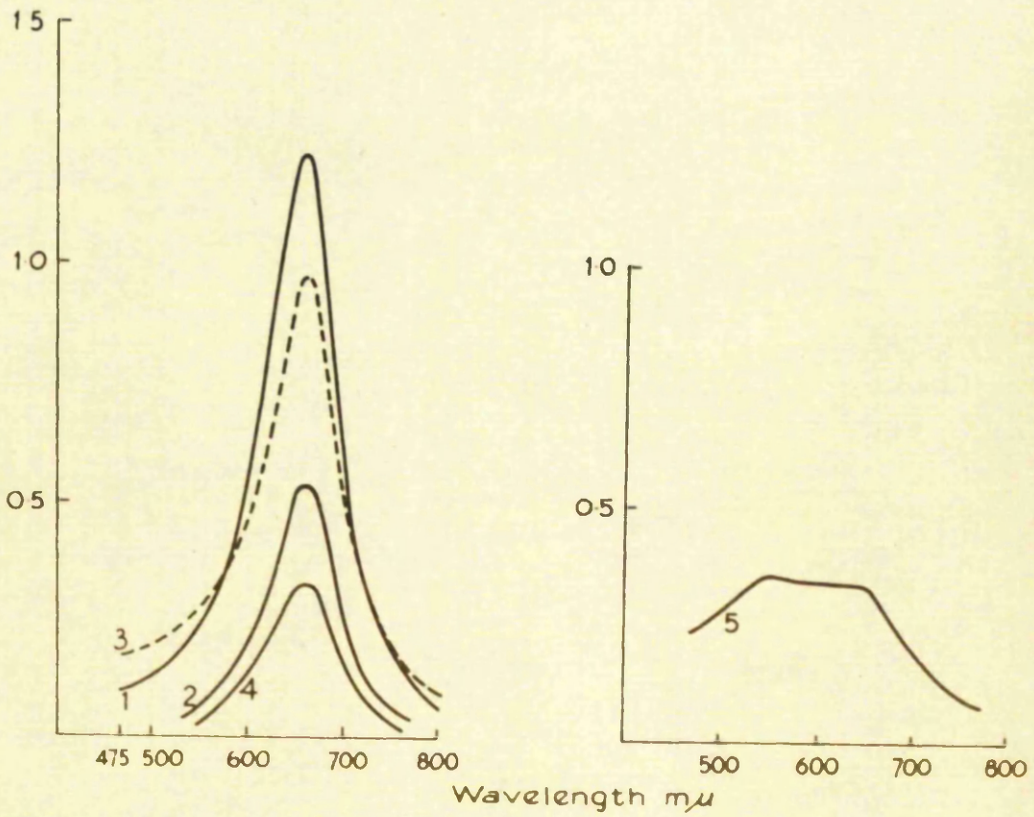
The Estimation of RNA by Reaction with Orcinol

The orcinol reaction, far from being specific for ribose, might be said to be a non-specific sugar reaction (see Hutchison and Munro, 1961). The wavelength of maximum absorption of the colour complex of the orcinol reaction apparently varies with the reacting substance so that "two-wavelength" methods have been suggested for the elimination of errors due to interfering substances (Brown, 1946). The validity of the estimate of RNA obtained by the orcinol method therefore depends on the possibility of the presence of interfering substances in the RNA fraction and the possibility of correcting for them.

The suspicion that glycogen was present in the RNA fraction has been strengthened by the work of Koritz and Munck (1960). These authors confirmed the finding of Stetten, Katzen and Stetten (1958) that TCA extraction does not remove all the tissue glycogen and that

Fig. 57. SPECTRA OBTAINED WITH THE ORCINOL REACTION

1. Pure Ribose.
2. Pure RNA (from Rat Liver).
3. Acid-soluble fraction from Rat Liver (following incubation in N. NaOH at 37°C for 18 hours).
4. Acid-soluble fraction from Rat Liver (1 hr. incubation in 0.3 N KOH at 37°C).
5. Acid-soluble Polypeptide material (1.25 mg./ml.)



KOH can extract additional glycogen. The finding of 3% glucose following hydrolysis of the acid-soluble polypeptide material with 6 N HCl at 100°C for 4 hours strongly suggests that some glycogen, or polysaccharride, will always be found in RNA fractions.

Examination of various absorption spectra obtained with the orcinol reaction is illuminating. The acid-soluble polypeptide material gives a spectrum which although not that of pure glucose, would interfere in the determination of RNA (fig.57), and comparison of the spectrum of pure ribose with that of an RNA fraction obtained after 18 hours hydrolysis in 1 N NaOH illustrates the presence of a shoulder in the latter spectrum obviously due to the presence of acid-soluble peptide material. The shape of the absorption spectrum of the acid-soluble peptide material suggests that there may be two reactive components, in which case a "two-wavelength" method of correction for this would be inapplicable. Slight differences in the absorption spectra of ribose, pure rat liver RNA, and those of RNA fractions obtained following various incubation times are also illustrated in fig.57.

Calculation shows, assuming that chromogenic material is extracted in parallel to acid-soluble peptide, that the interference could give rise, in RNA fractions obtained after 18 hours incubation in 1 N NaOH at 37°C, to errors ranging from +6 to +17%, with an approximate average of + 10%. Comparison with an experimental error of about 5% indicates that in the previous "extraction kinetic" studies, only at the later times could serious divergence arise. Statistical analysis of the figures used to plot the orcinol-reacting material of the 18 hour digest (fig.55) showed that the gradient of

the line differed in a positive direction from zero to a significant extent, demonstrating progressive extraction of interfering material. It is of course possible that the interfering material present in these samples could prolong the colour development time (see Albaum and Umbreit, 1947), thus reducing the apparent interference effect and giving fortuitous good agreement among different samples obtained by different conditions of incubation.

A minor difficulty in the orcinol reaction may arise if ribose is used as a standard. Although it is mainly purine nucleotide ribose which reacts, pyrimidine ribose does so also (Ibsen, Coe and McKee, 1958); this will to some extent give rise to difficulties in converting extinction readings to accurate RNA values.

It may be concluded then that although the orcinol reaction is not an ideal colorimetric method for the estimation of RNA, it may be used as a rough check for other methods since the estimate of RNA obtained should not differ from the true RNA content by more then -8 to +20%. Taking note of the absorption spectra, it is unlikely that the results obtained using the RNA fraction following one hour of incubation with 0.3 N KOH, will be more divergent from the true RNA figure than $\pm$ 8%.

The Estimation of RNA Utilising its Ultraviolet Absorbing Properties

The estimation of RNA by making use of its ultraviolet absorbing properties has been attempted many times (see for example, Warburg and Christian, 1942; Logan, Mannell and Rossiter, 1952; Burdon and Smellie, 1960; Tsanev and Markov, 1961; to cite only a few), although rarely has there been any regard to the possibility

of interference and its correction. Tsanev and Markov (1961) state: "It should be considered obligatory in nucleic acid determination to utilise only procedures that eliminate the interfering substances." The investigation of the presence of "concomitants" in the RNA fraction by Hutchison et al. (1956), particularly the polypeptide concomitants; the demonstration by Tsanev and Markov (1961) of polypeptide material in the acid-soluble fraction; and the finding of Davidson and Smellie (1952) - among others - that rat liver RNA-P is approximately 75% of the total acid-soluble phosphorus, all indicate that the estimation of RNA by utilising its ultraviolet absorbing properties requires careful preliminary investigation.

If there are only two substances present which contribute significantly to the ultraviolet absorption of the acid-soluble fraction (i.e. RNA and protein), then extinction readings at two wavelengths will be required in order to be able to calculate the concentration of each present (see Dawes, 1956).

The "Two-wavelength" Method of Estimating Each of the Components of a Two-component Mixture

Using the following symbols:-

λ_1 , the 1st. wavelength at which extinction is measured.

λ_2 , the 2nd. wavelength at which extinction is measured.

E_1 , the extinction of the protein-RNA mixture at λ_1.

E_2 , the extinction of the protein-RNA mixture at λ_2.

P_1 , the specific extinction coefficient of protein at λ_1.

P_2 , the specific extinction coefficient of protein at λ_2.

r_1 , the specific extinction coefficient of RNA at λ_1.

r_2 , the specific extinction coefficient of RNA at λ_2.

C_p , the concentration of protein in the mixture.

C_r , the concentration of RNA in the mixture.

it is obvious that:

$$E_1 = r_1 C_r + p_1 C_p, \text{ and } E_2 = r_2 C_r + p_2 C_p.$$

solving for C_r gives:

$$C_r = \frac{p_2 E_1 - p_1 E_2}{p_1 r_2 - p_2 r_1} = \frac{1}{\frac{p_1}{p_2} r_2 - r_1} E_1 - \frac{1}{r_2 - \frac{p_2}{p_1} r_1} E_2$$

so that,

(equation 1) $C_r = A. E_1 - B. E_2$, where A and B are constants. Note that when $p_1 = p_2$, (the conditions selected by Tsanev and Markov (1961)), $C_r = \frac{1}{r_1 - r_2}(E_1 - E_2)$, the equation used by Tsanev and Markov to calculate the RNA concentration in solutions.

Since it is possible to estimate the concentration of protein present ($= C_p$) directly by the method of Lowry et al. (1951) the result obtained with the "two-wavelength" method may be checked using the following relationship:-

(equation 2) $$C_r = \frac{E_{260} - C_p . p_{260}}{r_{260}}$$ in which the subscript 260 indicates the wavelength (mμ) at which the property is measured.

The data for the rat liver RNA and acid-soluble peptide material can be used to construct a nomograph (see later). This was done and the figures obtained using it compared with those from the first two-wavelength method with satisfactory results.

Fig. 58. ABSORPTION SPECTRA OF LIVER ACID SOLUBLE FRACTION

1. After 18 hours hydrolysis in 1 N NaOH at 37°

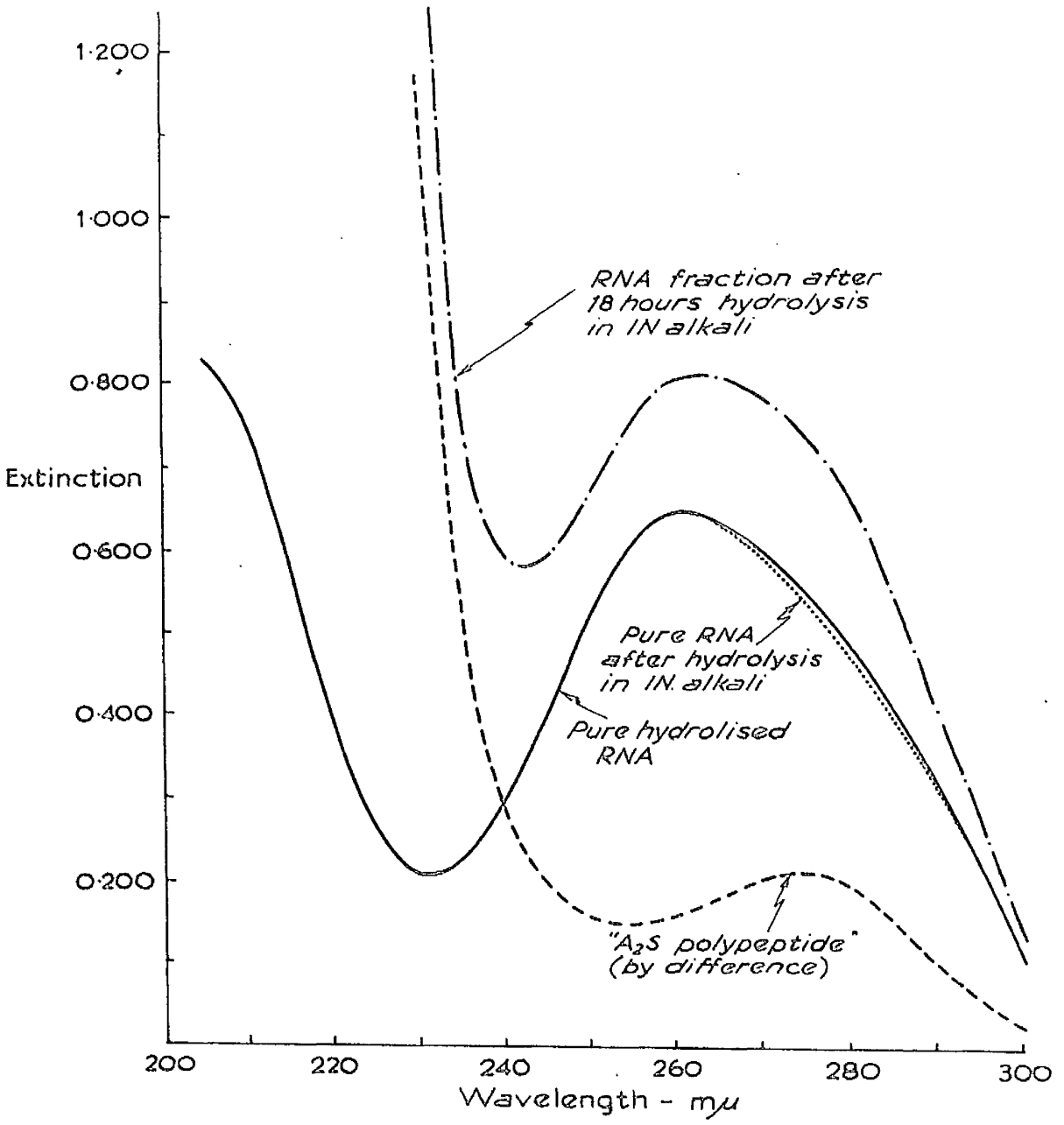


Fig. 59. ABSORPTION SPECTRA OF LIVER ACID SOLUBLE FRACTION

2. After 1 hour hydrolysis in 0. 3 N KOH at 37°

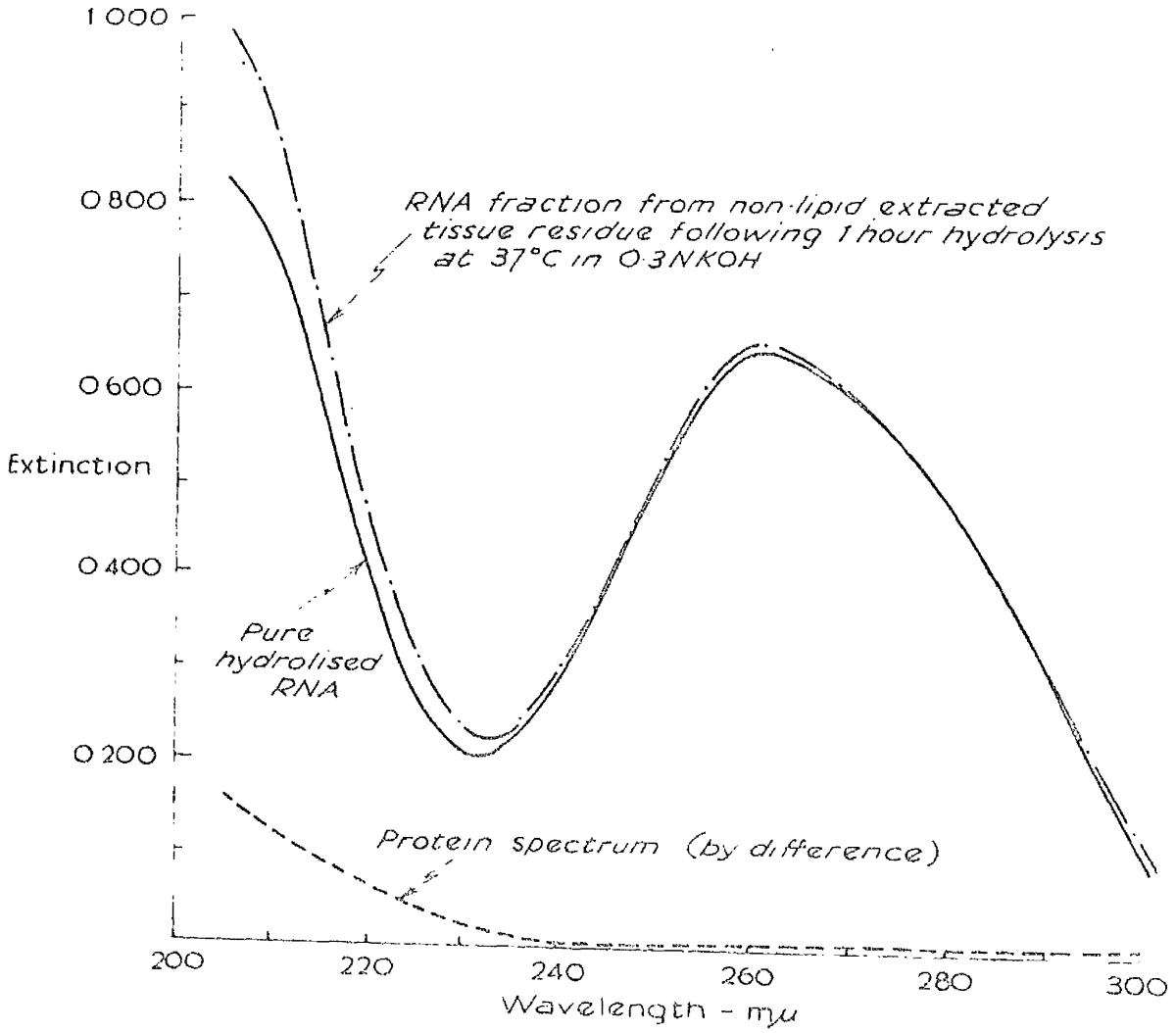
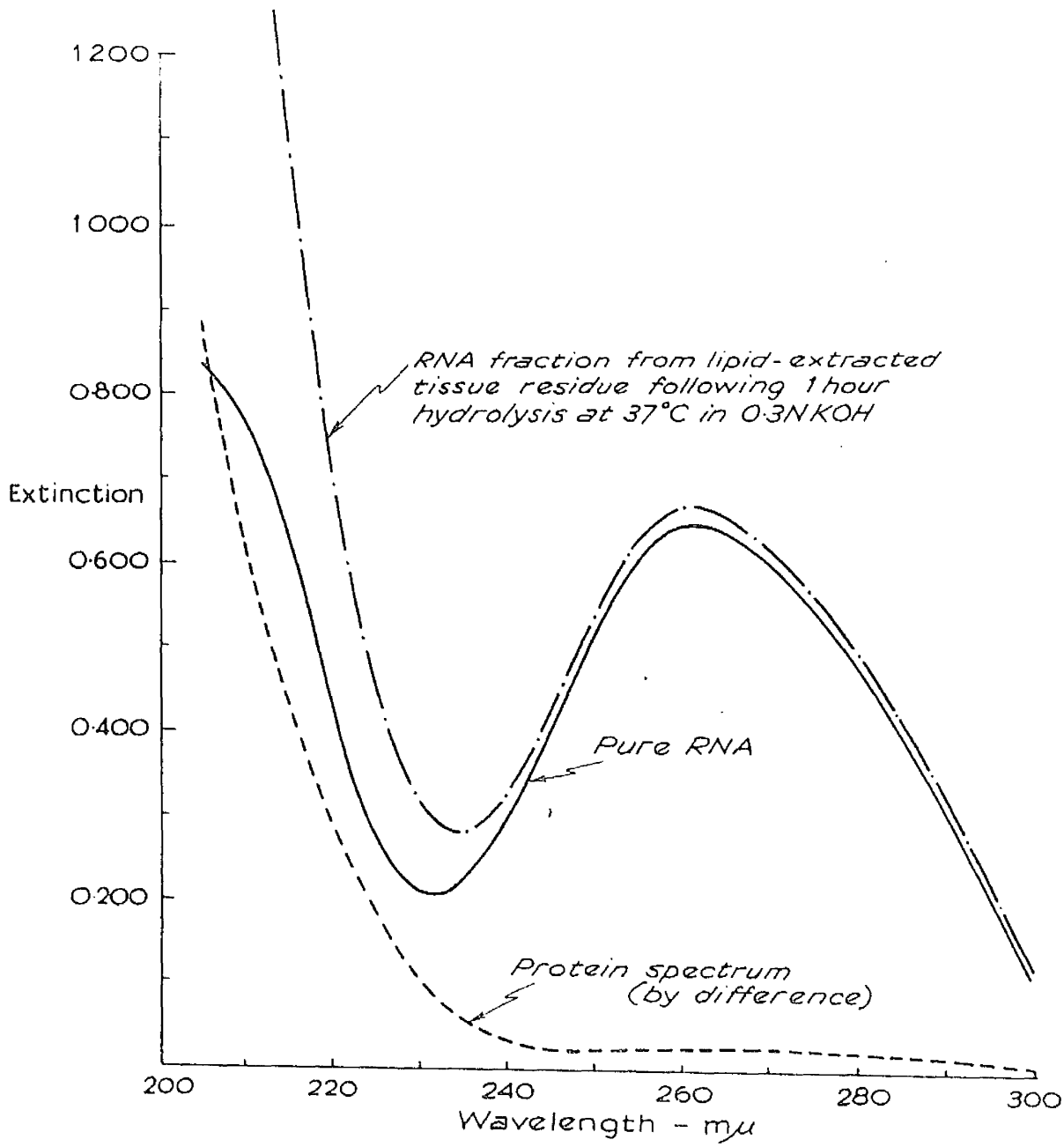


Fig. 60. ABSORPTION SPECTRA OF LIVER ACID SOLUBLE FRACTION

3. After 1 hour hydrolysis of lipid-extracted tissue residue in 0.3 N KOH at 37°



In a "two-wavelength" method for the estimation of RNA the choice of wavelengths at which extinctions are read is important. Since RNA is the component of main interest, $\lambda_{max.}$ of RNA (= 260 mu) is usually selected as one of the wavelengths. The other may be $\lambda_{max.}$ protein, $\lambda_{min.}$ protein, or $\lambda_{min.}$ RNA. There is the alternative of selecting two wavelengths of equal specific extinction of protein (i.e. the situation where $p_1 = p_2$, that used by Tsanev and Markov, 1961). Beaven and Holiday (1952) have presented data for amino acids confirming what was to be expected on theoretical grounds, that the method utilising the wavelength of maximum extinction of each component is that with the minimal experimental error. The only advantage of the method adopted by Tsanev and Markov (1961) is that it requires only the absorption spectrum of the protein, and knowledge of the specific extinction coefficient of the protein is unnecessary.

Inspection of the absorption spectra of rat liver RNA and acid-soluble polypeptide material (fig.49) and various RNA fractions (figs.58, 59, 60) indicate that the "2λmax." method can be applied with acceptable accuracy only when the amount of protein present is considerable (that is from about 75 to 300 μg protein per ml), and that in these circumstances the "λ max.-λmin." method is inapplicable because the extinction of the mixture at "λ min." of RNA (232 mμ) is greater than 1.000 (see fig.58). When the amount of protein present is small (that is from 0 - 75 μg per ml), the "λ max.-λmin." method is most useful as the extinction at "λ min." is in the measurable range, and the specific extinction of protein

Table 54

Test of the Application of the Laws of Lambert and Beer to Mixtures of Protein and RNA

Actual content of mixture		Calculated extinction of mixture	Extinction readings of mixture		RNA content calculated by 2-wavelength method	Nomograph (Fig.61)	RNA content calculated from Nomograph derived from data of Warburg and Christian (1942)
RNA (µg/ml)	Protein (µg/ml)	at 260 mµ	at 260 mµ	at 280 mµ			
5.02	42	0.186	0.194	0.154	5.11	5.7	6.7
5.84	20	0.185	0.192	0.145	5.70	6.0	6.9
11.0	84	0.363	0.376	0.300	9.9	10.5	13.1
11.6	40	0.372	0.374	0.283	11.3	11.2	13.3
13.0	147	0.549	0.540	0.440	13.0	13.7	18.3
14.8	34	0.464	0.478	0.356	15.1	14.9	17.2
15.0	127	0.566	0.566	0.449	14.8	15.0	19.3
17.4	59	0.543	0.561	0.423	16.9	17.1	20.2
		average difference = 3%			average difference = 2%		

Fig. 61.

NOMOGRAPH

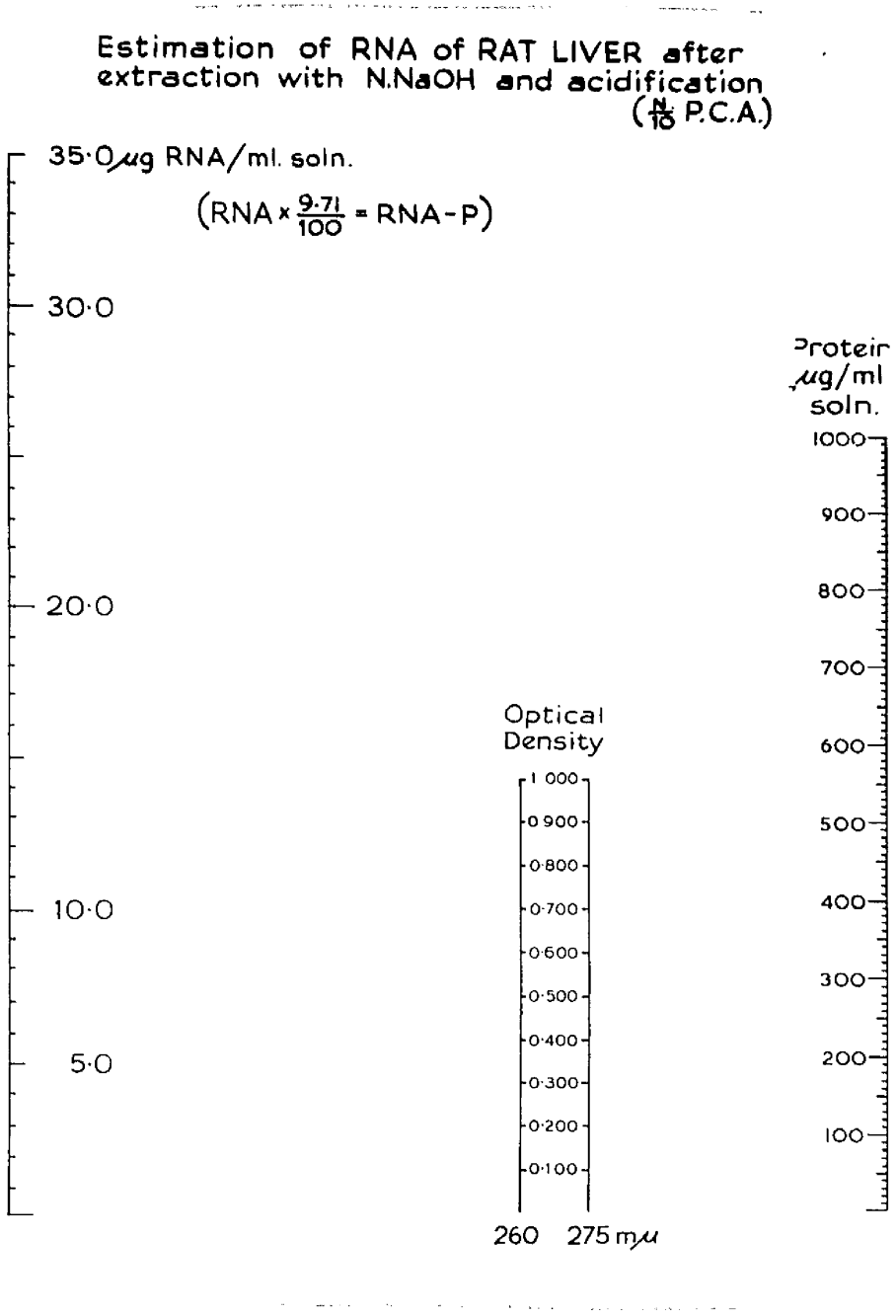


Table 55

Data on Ribonucleic Acids

(Yeast and Rat Liver)

1. Base Ratios

	Adenine	Guanine	Cytidine	Uracil
Yeast	10	11	8	10
Rat Liver	10	18	14	10

2. Analysis

	% N	% P	N/P ratio
Yeast	16.14	9.64	1.67
Rat Liver	16.49	9.71	1.70

3. Orcinol Reaction

	Purine Ribose as % Total ribose in Mol.	1 G purine ribose = G RNA	1 G purine ribose = G RNA-P
Yeast	56.4	3.65	0.352
Rat Liver	53.8	3.84	0.373

4. Optical Properties (Based on extinction of constituent nucleotides at pH 2)

	$\frac{260}{280}$ at pH 2	ϵ_p at 260 mμ
Yeast	1.632	10.93×10^3
Rat Liver	1.374	10.57×10^3

All data were obtained by calculation from the quoted base ratios which together with some additional information were obtained from Magasanik (1955).

at " λ min." of RNA is about 2 to 4 times that at " λ max." of RNA. The error of reading at the steeply-sloping part of the spectrum is acceptable because fairly large errors in the protein correction result, in this case, in only a small error in the final RNA figure.

The first essential requirement for the ultraviolet spectrophotometric estimation of RNA in the presence of protein in acid-soluble fractions from rat liver tissue is that the Beer-Lambert laws hold in the conditions used. Solutions containing varying amounts of pure rat liver RNA which had been hydrolysed for 18 hours in 1 N NaOH at 37°C and acid-soluble polypeptide material were prepared and the extinctions of the mixtures at various wavelengths noted (table 54). The amount of RNA present was computed from equation 3 - RNA-P = 6.8 (1.648 E_{260} - 1.458 E_{275}) - and the nomograph (fig.61). Obviously, the Beer-Lambert laws hold.

The second essential requirement for the estimation of RNA by ultraviolet spectrophotometry is appropriate standards. Some basic data calculated from the published (Magasanik, 1955) base ratios of rat liver and yeast RNA is shown in table 55, and the absorption spectra of hydrolysed rat liver and yeast RNA are compared in fig.49. Rat liver RNA has a "shoulder" at 275-280 mμ due to its greater cytidine and guanine content and a smaller E_{260}:E_{280} ratio than yeast RNA. This difference will obviously vitiate results obtained for rat liver using a two-wavelength method based on yeast RNA as standard, which Tsanev and Markov (1961) appear to have done. This error is further illustrated by using the nomograph published by the California Foundation to estimate RNA of rat liver.

Since it is based on the original data of Warburg and Christian (1942) obtained for yeast RNA and a single enzyme protein an error of +25% which varies with the amount of protein present (see table 54) is not surprising.

During the studies of the extraction of phosphorus, orcinol-reacting and ultraviolet-absorbing material, and RNA into the acid-soluble (RNA) fraction of rat liver tissue, the value for RNA which was obtained using 'equation 3' (i.e. a "two-wavelength" method) was observed to attain a maximum after about one hour extraction then to decline gradually to about 80% of the one-hour value at 18 hours (fig.55). This single observation indicates that the "two-wavelength" method must be more rigorously investigated.

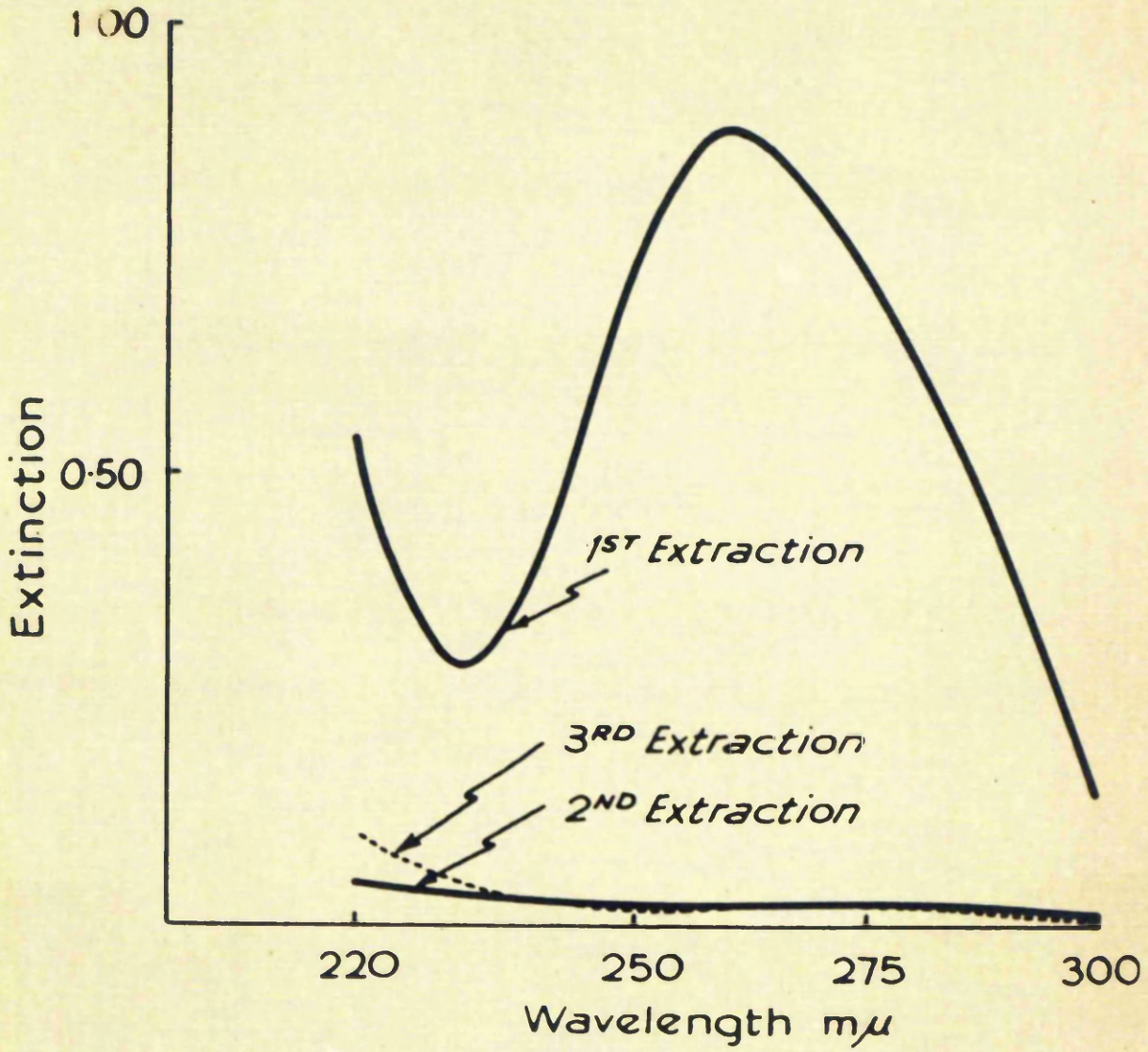
Summary of Section on Methods of RNA Estimation

The following essential conclusions can be reached from the foregoing data and results:-

Since the orcinol-reacting material "plateaus" at ¾-1 hour extraction with 0.3 N KOH or 1 N NaOH at 37°C, and the phosphorus content of the lipid-extracted residues behaves similarly, it can be concluded:

1. that the extraction of RNA under the above conditions is completed at one hour (in agreement with Scott et al., 1956). Furthermore, (a) the extinction at 260 mμ of the acid-soluble fraction increases very rapidly up to ¾-1 hour of incubation then increases only very slowly (figs.54 and 56); (b) the hyperchromic effect of RNA due to alkaline incubation is complete in ¾-1 hour (fig.53); (c) the extinction at 260 mμ of RNA does not alter with up to 24 hours of alkaline incubation (fig.53); (d) the "laws" of

Fig. 62. EFFECTS OF SUCCESSIVE EXTRACTION OF RAT LIVER MICROSOMES WITH 0.3 N KOH AT 37°



Lambert and Deer hold for RNA and protein mixtures in solution under the conditions used in this investigation (table 54);

(e) there is a gradual extraction of protein during alkaline incubation (figs.54 and 56).

2. From these observations, it can be deduced that the extinction at 260 mμ of the extracted RNA attains a maximum at one hour of extraction with alkali and thereafter remains constant.

3. The increase in extinction at 260 mμ of the acid-soluble (RNA) fraction with increasing time of extraction with alkali is due to the progressive extraction of protein.

In order to confirm that extraction of RNA by 0.3 N KOH at 37°C is complete in one hour, rat liver microsomes were prepared as described in Part 2 and subjected to preliminary precipitation and washing with PCA, then incubated for one hour in 0.3 N KOH. The acid-soluble (RNA) fraction was then prepared in the usual way and the precipitate re-incubated in alkali. A second acid-soluble fraction was prepared and the precipitate again subjected to alkaline incubation, following which a third acid-soluble fraction was obtained. The ultraviolet absorption spectra of these acid-soluble fractions were plotted using the 'Cary' recording spectrophotometer (fig.62). In addition, protein was estimated in each fraction by the method of Lowry et al. (1951). It is obvious from the spectra alone that extraction of at least 99% of the RNA was achieved in one hour and that further incubation extracts protein. The protein content of the first acid-soluble fraction was less than 10 μg per ml.

Examination of the absorption spectra (fig.59) of RNA fractions obtained after one hour incubation in 0.3 N KOH at 37°C of non-lipid extracted tissue residues indicates that there is only a small amount of protein present, (the ratios of extinction at 260:280 and 260:232 mμ are almost the same as those for pure rat liver RNA). The direct determination of protein by the method of Lowry et al. (1951) confirmed this; more than 10 μg protein per ml was never obtained (tables 52 and 53). If $E^{1\%}_{1cm}$ at 260 mμ for protein is taken as 10, the amount of protein present in these acid-soluble fractions would give an extinction at 260 mμ of about 0.006. Thus in RNA fractions prepared in this way, taking the extinction at 260 mμ as equivalent to the extinction of the RNA present will result in an error of +1%, which is of the order of the experimental error. However, when using this procedure routinely, it is advisable to check on the amount of protein present. This may be done by direct estimation (by the method of Lowry et al., 1951) or, more simply by using the following equation to calculate the RNA content of the solution;

$$\text{RNA-P} = \frac{6.8}{100}(54.42\ E_{260} - 23.13\ E_{232}). \text{ - (equation 4).}$$

The extinction values for protein (in terms of μg protein per ml) at 232 mμ equals 2.43×10^{-3}, and at 260 mμ is 1.03×10^{-3}; these values were calculated from the data of fig.56 by equating gradients (of extraction of protein).

Similarly, using the data for acid-soluble fractions obtained from lipid-extracted tissue residues incubated in 0.3 N KOH for one hour, the error in taking the extinction at 260 mμ of the acid-soluble fraction as equivalent to RNA is +3%. Although this is small, it is

Table 56

The Effect of Alkaline Incubation on the Ultraviolet Absorbing Properties of Acid-Soluble Fractions and the Reaction in the Lowry Procedure

A. Lowry Procedure

Sample	Acid-soluble protein μg per ml solution before re-incubation	0.3 N KOH 18 hours	N NaOH 18 hours
*1	7	6	5
1.5	9	6	-
1 LK	25	20	19
1 LN	76	62	58

B. U.V. extinction at 232 mμ ($\times 10^{+3}$)

1	164	170	174
1.5	164	186	201
1 LK	216	207	223
1 LN	298	302	363

C. U.V. extinction at 260 mμ ($\times 10^{+3}$)

1	523	536	553
1.5	519	550	564
1 LK	537	533	549
1 LN	544	552	586

* The samples are acid-soluble fractions. Figures refer to hours initial incubation; 1 and 1.5 are acid-soluble samples from non-lipid extracted residues incubated in 0.3 N KOH at 37°C for 1 and 1.5 hours respectively.

1 LK and 1 LN are acid-soluble samples from lipid extracted tissue residues incubated in 0.3 N KOH and N NaOH for 1 hour at 37°C.

outwith the experimental error. By successive approximations, using equation 4 to obtain a preliminary estimate of the RNA present, by estimating protein directly, and comparing absorption spectra (fig.60), the specific extinction coefficients of the appropriate acid-soluble peptide material at 232 and 260 mμ were estimated to be 3.0×10^{-3} and 0.9×10^{-3}.

Sufficient evidence has now been assembled to indicate that the decrease in the RNA value obtained in the "extraction kinetics" studies (fig.55) is due to variation in the ultraviolet absorption properties of the acid-soluble peptide material. This was confirmed experimentally in two ways. Duplicate samples from the same homogenate were treated to give acid-soluble fractions; the first was obtained following treatment of the non-lipid extracted residue with 0.3 N KOH for one hour at 37°C, the second, after incubation of the lipid-extracted residue with 1 N NaOH at 37°C for 18 hours. The extinction of the former at 260 mμ gave the RNA content of the tissue (to ± 1%). The absorption spectrum of the RNA could then be drawn and 'subtracted' from the spectrum of the acid-soluble fraction obtained from 1 N NaOH treatment, to give the absorption spectrum of the interfering material (fig.59). As expected, the spectrum so obtained was similar to that of the acid-soluble polypeptide material prepared previously. However, the spectrum was equivalent to only 230 mg. of acid-soluble protein compared with the figure of 270 mg. obtained by direct estimation of protein. In the second experiment acid-soluble fractions obtained by treatment with 0.3 N KOH and 1 N NaOH were re-incubated in alkali for 18 hours. The results (table 56) demonstrate clearly that alkaline digestion reduces the

Table 57

Comparison of Methods of Estimating RNA and DNA

	Non lipid extracted 0.3 N KOH 1 hr.	Lipid Extracted 0.3 N KOH 1 hr.	N NaOH 18 hrs.	Approximate Experimental Error
E_{260}	(a) 100%	102%	130%	3%
$\frac{E_{260} \text{ protein}}{\text{Total } E_{260}}$	2% (maximum)	3.5% (mean)	30% (mean)	-
Orcinol as % RNA-P (measured by u.v.-a)	100%	100%	107%	5%
DNA	97%	100%	101%	5%
$\frac{\text{Total P}}{\text{RNA P}}$		137%		

Figures are the means of 16 duplicate samples.

Table 58

Error due to Extraneous Ultraviolet Absorbing Material in Acid-Soluble Fractions

Time of incubation	N NaOH at 22°C	N NaOH at 37°C	0.3 N KOH	
			(a) lipids extracted	(b) lipids not extracted
1 hour	3%	7-10%	2 - 3%	0.5 - 1%
2 hours	10%	15%	-	2%
16-24 hours	22-27%	22-36%	9 -18%	4 - 15%

chromogenicity of polypeptide or protein in the method of Lowry et al. (1951) and simultaneously alters the extinction at 232, 260 and 275 mu.

Summary of Experimental Results

1. Owing to the difficulty in obtaining a standard acid-soluble peptide material, there can be no satisfactory two-wavelength method of estimating RNA in acid-soluble fractions, obtained following prolonged (5-18 hours) alkaline digestion.

2. If the period of treatment with alkali is longer than one hour, the errors introduced into the estimation of RNA from the extinction at 260 mμ will be considerable, up to 35% with 18 hours incubation in 1 N NaOH at 37°C (see tables 57 and 58).

3. The only satisfactory method of RNA estimation based on ultraviolet spectrophotometry requires the omission of lipid-solvent extraction and incubation with 0.3 N KOH at 37°C for one hour in an air oven. In this method the extinction at 260 mμ gives an estimate of RNA with about 1% error. That protein is not interfering may be checked by (occasional) direct estimation of protein, or taking an additional reading at 232 mμ and using equation 4 to calculate the RNA content.

4. The alternative methods of RNA determination (i.e. orcinol or phosphorus determination) also require the use of the one hour incubation period in order to be reliable. The experimental error of these methods was greater than with ultraviolet spectrophotometry due to there being more steps involved.

Table 59

Comparison of Methods of RNA Estimation

(Figures are expressed as mg-P)

	RNA (E_{260} method)	Orcinol	Total P	RNA % / Total P
	1.89	1.81	2.22	85
	1.81	1.74	2.17	83
High Protein Diet	1.63	1.74	2.11	77
	1.76	1.89	2.03	87
	1.69	1.62	2.23	76
	1.78	1.88	2.40	74
High Protein Diet injured	1.76	1.86	2.28	77
	1.85	2.00	2.53	73
	1.64	1.69	1.94	84
	1.75	1.74	2.23	79
Low Protein Diet	1.92	2.07	2.34	82
	1.63	1.90	1.97	83
	2.06	1.98	2.26	91
Low Protein Diet injured	1.87	1.92	2.38	79
	1.96	2.01	2.34	84
	1.87	1.73	2.12	88
	2.11	1.89	2.26	93
	2.00	1.91	2.20	91
	1.68	1.60	2.06	82
			Mean =	83%

Comparison of the Ultraviolet Spectrophotometric and Other Methods

A comparison of the methods of estimating RNA in liver was carried out using animals receiving high and low protein diets and some from each group which had been subjected to injury. RNA was estimated, using one hour incubation in 0.3 N KOH, by the extinction at 260 mμ and the orcinol method, and the results compared with the total phosphorus of the acid-soluble fraction following lipid extraction. There was good agreement between the orcinol and ultraviolet absorption methods and the RNA-P (by the ultraviolet method) was approximately 80% of the total acid-soluble phosphorus, (table 59).

Discussion

The reasons for the rejection of the Schneider (1945) procedure for estimating nucleic acids of rat liver have been summarised in the introductory section.

A detailed discussion of possible methods of determining RNA in the acid-soluble fraction of the Schmidt-Thannhauser procedure has been published (Hutchison and Munro, 1961). A very convenient and simple approach would be to measure the ultraviolet absorption of the acid-soluble fraction. However, after the conventional period of 15-18 hours digestion in 1 N alkali at 37°C, the proportion of ultraviolet absorption at 260 mμ due to protein degradation products can be quite considerable. Thus De Decken-Grensen and De Decken (1959) removed the contaminants by passing the acid-soluble fraction through an ion-exchange resin and observed that ultraviolet absorption was reduced by as much as 70% in some cases (plant tissues etc).

With bacteria, Jones, Rizvi and Stacey (1958) observed that about 30% of the absorption of the RNA fraction was due to non-nucleotide contaminants, mainly peptides. Scott, Fraccastoro and Taft (1956) give data for the ultraviolet absorption of RNA fractions obtained at different times during digestion of liver, kidney, and spleen samples in 1 N alkali at room temperature. Inspection of their graphs shows that, although all the RNA became acid-soluble during the first hour of incubation, continuation of digestion to 24 hours contributed an additional 15-25% to the absorption at 260 mμ. The data presented here (fig.54) shows a similar phenomenon. In the case of digestion for 24 hours in 1 N alkali at 37°C, the additional ultraviolet absorption added 35% to the apparent RNA content of the fraction (table 58).

Tsanev and Markov (1961) have attempted to correct for absorption by these contaminants by taking readings at 260 and 286 mμ and computing the RNA from these. The equation used to make this calculation requires data for the absorption at these two wavelengths of the RNA of the tissue being analysed, and of the peptide contaminants present in the RNA fraction. The absorption of RNA at 286 mμ relative to its absorption at 260 mμ is not constant for different tissues, as Tsanev and Markov assume, but may vary considerably as shown in fig.49. Yeast RNA differs considerably from liver RNA due to the "shoulder" around 280 mμ in the liver RNA produced by the large amounts of guanylic and cytidylic acids. This no doubt accounts in part for the low estimates of RNA content of liver obtained by Tsanev and Markov with their procedure.

The second source of error in their method, is failure to allow for deamination of cytidylic acid by 1 N alkali at 37°C, which will reduce absorption at higher wavelengths. The present data show (tables 49, 50,51; fig.53) that RNA itself, like pure solutions of cytidylic acid (Davidson and Smellie, 1952; Marrian, Spicer, Balis and Brown, 1951), undergoes this deamination when incubated in 1 N alkali at 37°C. This effect can be avoided by using 0.3 N alkali at 37°C (table 51), or by reducing the temperature of incubation in 1 N alkali to 22°C (see Marrian et al., 1951). The third problem which the procedure of Tsanev and Markov raises, is the provision of a representative sample of the protein degradation products contaminating the RNA fraction after prolonged alkaline digestion. They attempted to resolve this by removing the nucleic acids from their tissue samples with hot PCA and then subjecting the residue to alkaline digestion. The acid-soluble material obtained at the end of 17 hours digestion was taken to represent the peptide material which usually contaminates the RNA fraction of the digest. The peptide material obtained in this way was found to have a similar ultraviolet absorption spectrum when prepared from a variety of tissues. The present investigation produced data (table 56) for reincubation of the RNA fraction which suggest that the ultraviolet absorption of a given amount of this material changes during continued alkaline digestion. Attempts to isolate a sample of protein degradation products from the RNA fraction resulted in production of material which was clearly not representative, since correction factors based on its spectrum gave erroneous values for RNA (fig.55). It would thus appear that the two-wavelength

procedure is subject to too many sources of error to be a reliable method of estimating RNA in the acid-soluble fraction obtained after prolonged alkaline digestion of tissue residues.

The alternative approach, introduced by Scott et al. (1956), is to carry out alkaline digestion under conditions which result in only negligible release of protein into the acid-soluble fraction of the digest. Scott et al. used 1 N alkali for one hour at room temperature to achieve this. If a short period of digestion is used, three conditions must be satisfied. First, it must be shown that all the RNA has been extracted from the tissue in a form no longer precipitable by acid. Scott et al. (1956) provide evidence for several mammalian tissues that their conditions of digestion fulfil this criterion, and the present data on liver demonstrate that digestion in 0.3 N alkali for 1 hour at 37°C is adequate for liver samples. Under these circumstances, essentially all the orcinol-reacting material is solubilised (fig.55) and re-digestion of the precipitate obtained on acidification of the alkaline digest failed to extract further RNA (fig.62). Secondly, the period of digestion must be adequate to degrade the RNA to a stage at which its ultraviolet absorption is maximal (i.e., hyperchromic effect complete); unless this is so, the ultraviolet absorption of the extracted RNA may be variable. The preliminary data obtained with pure RNA demonstrates that incubation with 0.3 N KOH at 37°C for one hour is fully adequate to achieve completion of the hyperchromic effect. It does not, however, give complete hydrolysis to mononucleotides.

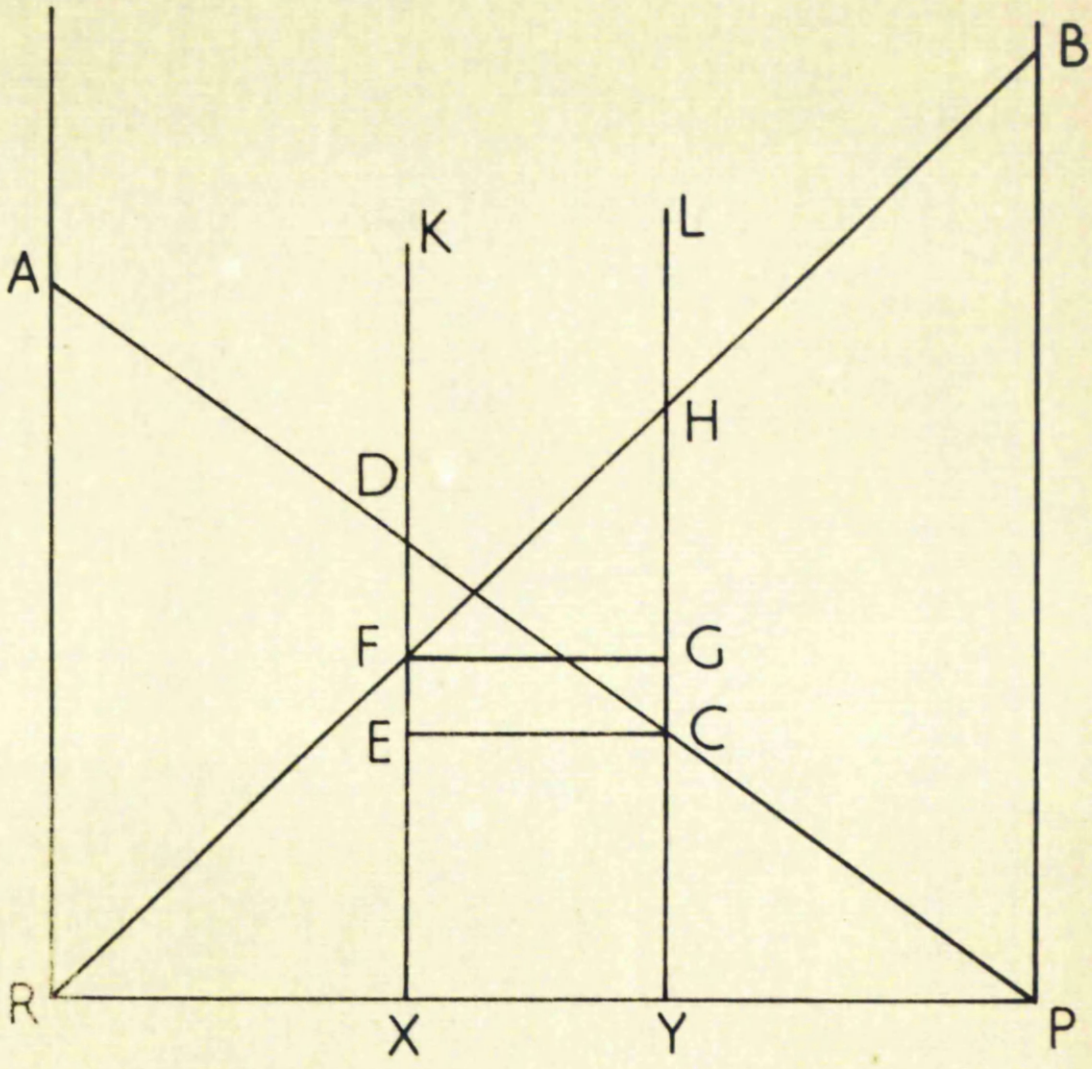
Finally, the conditions of digestion must be such that release of protein in an acid-soluble form is an insignificant

source of error. From the data provided by Scott et al. (1956), it is not possible to determine the extent of the contamination of the acid-soluble fraction by protein-breakdown products after one hour of digestion in 1 N alkali at 22°C. Some evidence is presented here which shows that the conditions of incubation can have a considerable effect on the amount of protein released during one hour of incubation at 37°C (table 56). Digestion of liver in 0.3 N KOH without previous removal of lipids resulted in release of only 6 µg protein per ml of the acid-soluble fraction. An approximate idea of the magnitude of the error in ultraviolet absorption at 260 mµ caused by the presence of this amount of protein can be obtained from the specific extinction ($E^{1\%}_{1cm}$) of the peptide material isolated from the acid-soluble fraction of liver. At 260 mµ this is 7.5, so that 6 µg of protein per ml would account for only 0.8% approximately of the observed ultraviolet absorption after one hour digestion in 0.3 N KOH. On the other hand, digestion of a lipid-extracted sample of liver for one hour in 1 N NaOH released 76 µg protein per ml, which would correspond to about 8% of the total ultraviolet absorption of the acid-soluble fraction. To obtain an accurate estimate of the RNA content of the tissue, it is thus necessary to use the lower concentration of alkali. It is also advantageous to dispense with the use of lipid solvents, provided that adequate care is taken to avoid carrying over significant amounts of acid from the initial cold acid treatment of the tissue, which must, of course, be with PCA.

Finally, it appears that no matter which of the incubation procedures described here is used, the estimate of the DNA content of the tissue is the same (table 57).

The reliability of the procedure recommended above for the estimation of tissue nucleic acids - that is, separation of RNA and DNA by incubation in 0.3 N KOH at 37°C for one hour - has been confirmed by D. Ribbons and J. Paul of this department. The former used E.coli and the latter used an isotopic method of checking that extraction of RNA from tissue culture cells was complete.

Fig. 63. CONSTRUCTION OF A NOMOGRAPH



Note on the Construction of a Nomograph

In order to construct a nomograph which is to be used to estimate the concentrations of two substances by reading extinctions at two wavelengths λ_1 and λ_2, it is essential to have the following data, (retaining the symbols as on p.200) r_1, r_2, p_1, and p_2. Four vertical lines must be erected on a base line; the positions of two are arbitrary, the position of the other two are determined by the relations of r_1 : r_2, etc. Similarly, two of the scales are arbitrary, fixing the other two.

In the present construction (see fig.63), graph paper was used, the extinction scales and the base line were fixed; that is KK and LY were precalibrated identically in terms of extinction, and the positions of the RNA and protein concentration scales (AR and BP) fixed on the baseline RP. Thus the positions of the extinction scales on the base line (i.e. the positions of X and Y on RP) are to be found, and the concentration scales AR and BP calibrated. This is done in the following way:

By similar triangles DXP, CYP, $\frac{DX}{CY} = \frac{XP}{YP} = \rho_r$,

and by similar triangles, HYR, FXR, $\frac{FX}{HY} = \frac{RX}{RY} = \rho_p$, so that,

$\frac{RP - XP}{RP - YP} = \rho_p$. Substituting $XP = \rho_r YP$ gives $\frac{RP - r^{YP}}{RP - YP} = \rho_p$, and

rearranging, $RP - \rho_r YP = \rho_p RP - YP\,\rho_p$.

Thus, $RP(1 - \rho_p) = YP(\rho_r - \rho_p)$; which gives

$$YP = RP \frac{1 - \rho_x}{\rho_x - \rho_p} \quad \text{and} \quad XP = \rho_x YP.$$

Similarly it can be shown that $RY = \frac{1 - \rho_x}{\rho_p - \rho_x}$.

Since $\rho_x = \frac{x_1}{x_2}$ and $\rho_p = \frac{p_1}{p_2}$, this determines the positions of the extinction axes on the base line RP.

The scales of AR and BP are determined by producing PC through D to A (C and D are extinctions of RNA at λ_2 and λ_1), giving C_x equal to the length of AR. In the same way, extending RF through H to B gives C_p equal to the length of BP.

Using the data for rat liver RNA and acid-soluble peptide material obtained after digestion in 1 N NaOH at 37°C for 18 hours the nomograph of fig.61 was constructed.

Errors in using this nomograph will be fairly large as the extinction axes are rather close, there was some deamination of cytidylic acid, and the preparation of the interfering protein is not truly representative. However, it does illustrate how inappropriate for rat liver is the nomograph published by the California Foundation which was based on the data of Warburg and Christian, (1942).

The Estimation of Tissue Proteins and Nucleic Acids, and Lipids

1. Preliminary stages

The first stage in the preparation of a tissue sample for analysis is usually acid precipitation, with the aim of removing free amino acids, peptides and nucleotides; all of which could otherwise at a later stage be estimated as protein or nucleic acid. This is of particular importance in the case of nucleic acid estimation, since it can be calculated (from the data of Goodlad and Munro, 1959) that the ratio of adenine nucleotides to RNA in rat liver is 1:10.

Acids have been used to precipitate tissue and other proteins for many years. Miller and Van Slyke (1922) investigated the efficacy for protein precipitation of several reagents which had been recommended before that date. After using tungstic acid, picric acid and trichloracetic acid (TCA) at various concentrations, they concluded that 2.5% TCA was the most suitable precipitant for the separation of plasma proteins from the peptides of plasma, and that the time of contact (from 15 minutes to 24 hours) with TCA before separation did not influence the result. The efficacy of TCA as a plasma protein precipitant has been confirmed in a number of investigations (Jackson, Sherwood, and Moore, 1927; Martens, 1928; Christensen and Lynch, 1946a, 1946b). However, there seems to have been little published on the use of TCA as a tissue protein precipitant. Black (1958) found that 10% TCA was effective in the separation of free amino acids and peptides from the protein of liver and muscle. From his data it can be computed that the free

amino acid-N plus peptide-N was only 1% of the total protein-N of the organ. Thus it would appear that the precipitation of protein as a preliminary to estimation of the protein content of an organ, may be unnecessary. Munro and Downie (unpublished results, 1962) found that concentrations of TCA from 2.5-20% were equally effective in precipitating protein from rat liver homogenates.

Perchloric acid (PCA) as a protein precipitant was introduced by Neuberg, Strauss and Lipkin (1944). It was found that the minimum concentration necessary for the precipitation of various proteins varied; for example, 0.1 N for ovalbumin and 0.7 N for casein. Again, there is lack of evidence on the efficiency of PCA as a precipitant of tissue proteins.

As a precipitant, PCA has some advantages over TCA:-

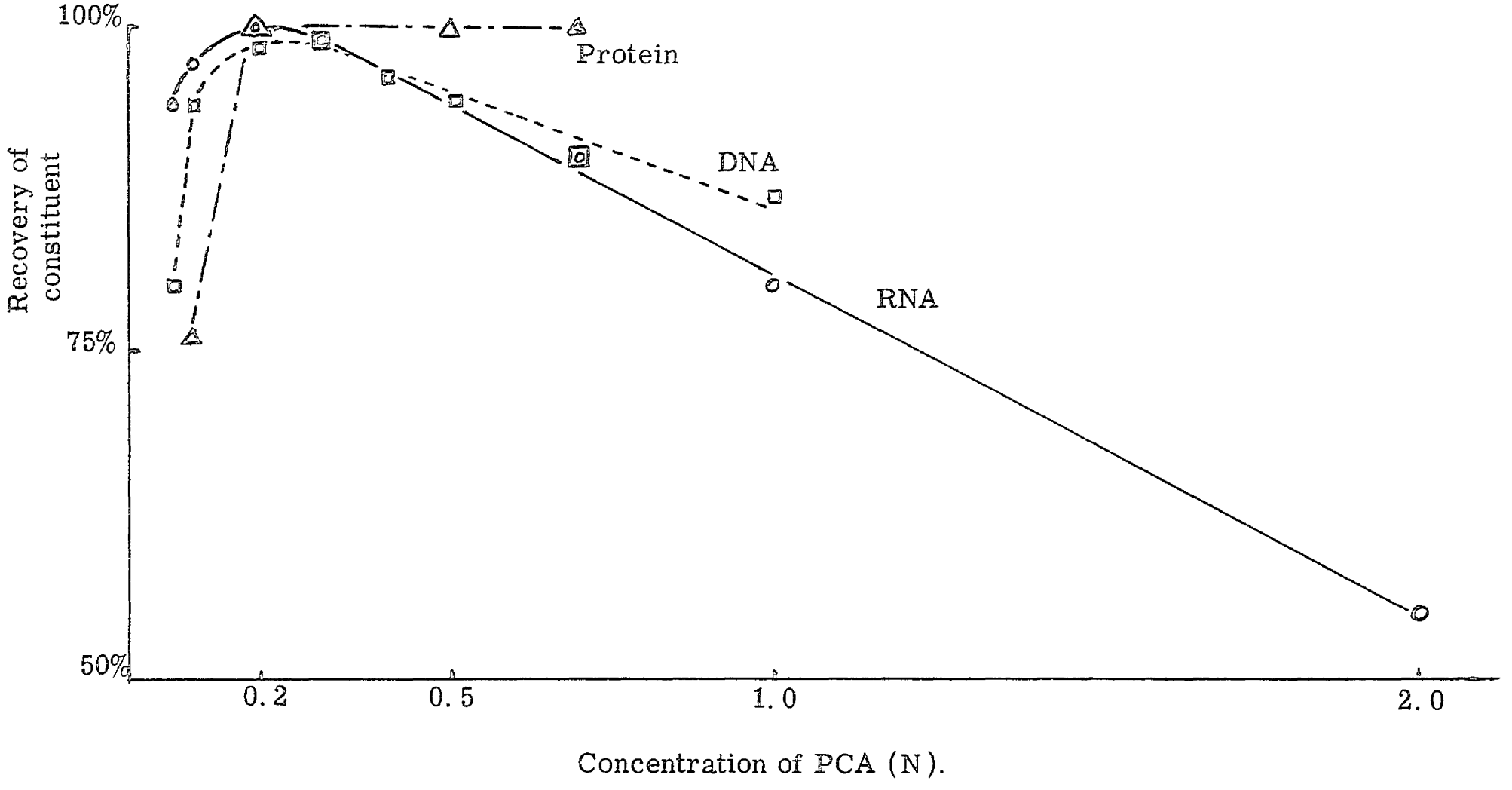
1. it has negligible ultraviolet absorption;
2. the potassium salt is insoluble;
3. there is no report of its interfering in the orcinol reaction.

The main danger in the use of PCA, destruction of nucleic acid (see Hutchison, Downie and Munro, 1962), can readily be eliminated by the use of the correct concentration of PCA, precipitation at $0^{\circ}C$, and by avoiding storage of the tissue in contact with PCA.

<u>The determination of the optimal concentration of acid for the precipitation of RNA, DNA and protein of rat liver tissue</u>

<u>Procedure</u>: The tissue was homogenised in 20 volumes ice-cold distilled water, and samples transferred to 15 ml. centrifuge tubes. Precipitation and 2 washings were carried out using the appropriate concentration of acid. For the estimation of protein, a portion of the precipitate was dissolved in 0.3 N KOH and made up to a suitable

Fig. 64. RECOVERY OF TISSUE CONSTITUENTS FROM RAT LIVER AFTER ACID PRECIPITATION



volume with distilled water. Protein was estimated by the methods of Gornall et al. (1949) and Lowry et al. (1951). RNA was estimated by using the ultraviolet absorption at 260 mμ of the acid-soluble material remaining after digestion of the acid-washed tissue residue in 0.3 N KOH at 37°C in an air oven for 1 hour. DNA estimation was by the method of Cerriotti (1955). Protein and DNA were also estimated directly on the tissue homogenate samples.

Results

In several experiments the effects of using concentrations of PCA ranging from 0.07 N-2.0 N, and concentrations of TCA varying from 1%-30% were studied. Fig.64 shows that there is an optimal concentration (0.2 N) of PCA for the precipitation of RNA and DNA, and that 0.2 N PCA is also satisfactory for the precipitation of protein. In the case of RNA it is obviously impossible to estimate it directly on the tissue homogenate, so that the figure of 100% represents the maximum obtained with the above method. Precipitation of the homogenate with 80% boiling ethanol gave a figure about 5% lower than this. The figure of 100% for protein and DNA was that obtained on the whole, non-acid precipitated homogenate sample.

When TCA was used in the preliminary stage, somewhat lower figures for protein and DNA were obtained: in the case of RNA the method outlined above cannot be used when TCA is the precipitant.

Ogur and Rosen (1950) claimed that RNA could be exposed to 0.2 N PCA for several hours without loss of RNA due to hydrolysis etc. This observation was confirmed; after several washings with 0.2 N PCA there was no loss of RNA, but after 10 washings with 2.0 N PCA, up to 60% of the RNA was solubilised. Thus unless there is a small

amount of extremely labile RNA in the cell, it is likely that the precipitation of tissue RNA by 0.2 N PCA is quantitative. Whether this in fact represents 100% of the RNA of the cell is impossible to say, as there is no method for the direct estimation of RNA in a tissue homogenate and RNA cannot be isolated quantitatively from an organ such as the liver.

It can thus be concluded that:

1. PCA as a precipitant of tissue protein and nucleic acid gives better results than TCA.
2. The optimal concentration of PCA is 0.2 N.
3. At this concentration of PCA, 100% of the protein and DNA (within the limits of error of the estimation procedures) can be precipitated, and the maximal recovery of RNA obtained.

2. Factors influencing the selection of a method of protein estimation

From the survey of the literature on methods of estimating protein, two methods which would be suitable for application to the determination of tissue proteins emerge; those of Gornall, Bardawill and David (1945) - a biuret method, and Lowry, Rosebrough, Farr and Randall (1951).

These two methods were applied to the estimation of the protein content of liver, using bovine plasma albumin as standard, in order to select the most satisfactory procedure.

Procedure: Duplicate 5 ml samples from a 1 in 20 cold aqueous homogenate of liver were treated in parallel in two ways for the estimation of protein.

Table 60

Comparison of Methods of Estimating the Protein Content of Liver

Method	Direct (on diluted whole homogenate)	Indirect (after acid precipitation)	Protein content of supernatant after acid precipitation
Biuret	6.14	4.63 (75%)	0.78
Lowry et al. (1951)	5.39	5.32 (99%)	-

Each figure is the mean of several determinations.

The figures in brackets are the percentage recovery of protein, i.e.

$$\frac{\text{Indirect method}}{\text{direct}} \times 100$$

(a) Direct method: 5 ml 0.2 N KOH was added and the solution made up to 10 ml. 1 ml samples were taken for the biuret procedure and 1 ml samples diluted to 20 ml with water, after which a 1 ml sample was subjected to the procedure of Lowry et al. (1951) for the estimation of protein.

(b) Indirect method: To the 5 ml sample, 2.5 ml 0.6 N PCA was added and after standing and centrifuging, the precipitate was washed twice with 0.2 N PCA. The supernatant and washings were combined and made up to 10 ml in 0.1 N KOH from which a 1 ml sample was then subjected to the biuret procedure. The precipitate was dissolved in 10 ml 0.1 N KOH and a 1 ml sample taken for the estimation of protein by the biuret method. A further 1 ml sample was diluted as before to 20 ml with water and 1 ml taken for protein estimation by the method of Lowry et al. (1951).

Results

The results are summarised in table 60 and clearly indicate that the biuret method is unsatisfactory in comparison with the method of Lowry, not only because the recovery of protein is poor with the biuret procedure (75%) but because the quantity of biuretogenic material in the supernatant obtained after acid precipitation, when summed with the protein estimated after acid precipitation does not yield a result equal to the protein content of the whole homogenate.

The procedure of Lowry et al. (1951) in contrast, yields a satisfactory recovery of protein.

Conclusion: The method of Lowry, Rosebrough, Farr and Randall (1951) is suitable for the estimation of liver protein and may be satisfactorily applied following acid precipitation, or directly, on the suitably

diluted homogenate.

3. The Estimation of Lipids

In the Schmidt and Thannhauser (1945) and the Schneider (1945) procedures for the estimation of nucleic acids, the tissue is first extracted with cold acid then with lipid solvents. For example, Davidson, Frazer and Hutchison (1951) followed the initial TCA precipitation step with successive extractions with: acetone, ethanol, chloroform, ethanol-ether (3:1) twice, and finally ether. Although Schneider (1945), Greenbaum and Slater (1957), Cooper and Loring (1957) and Hutchison, Downie and Munro (1962) have not observed any loss of RNA attributable to the lipid solvent extraction procedure, the reports of Venkataraman and Lowe (1959) and Venkataraman (1960) of losses of RNA following lipid-solvent extraction prompted the following investigations: (a) whether RNA and protein are extractable by lipid solvents and (b) suitable methods of tissue lipid estimation.

The effects of lipid solvents on the determination of RNA and protein

(a) Experimental methods

Samples of a single rat liver homogenate in ice-cold water (prepared as described in part 1) were precipitated with increasing concentrations of either PCA or TCA. Duplicate samples were then digested in 0.3 N KOH for 1 hour at 37°, others were extracted with lipid solvents (acetone, ethanol etc. as described by Davidson et al., 1951), before being subjected to alkaline digestion for one hour. DNA and protein were precipitated at a concentration of 0.2 N PCA as previously described and the RNA content of the acid soluble fraction determined from the extinction at 260 mμ. In the case of the TCA-treated samples, it was necessary to estimate RNA by the orcinol

Fig. 65. EFFECTS OF LIPID EXTRACTION FOLLOWING PRECIPITATION WITH PERCHLORIC ACID ON RECOVERY OF RNA.

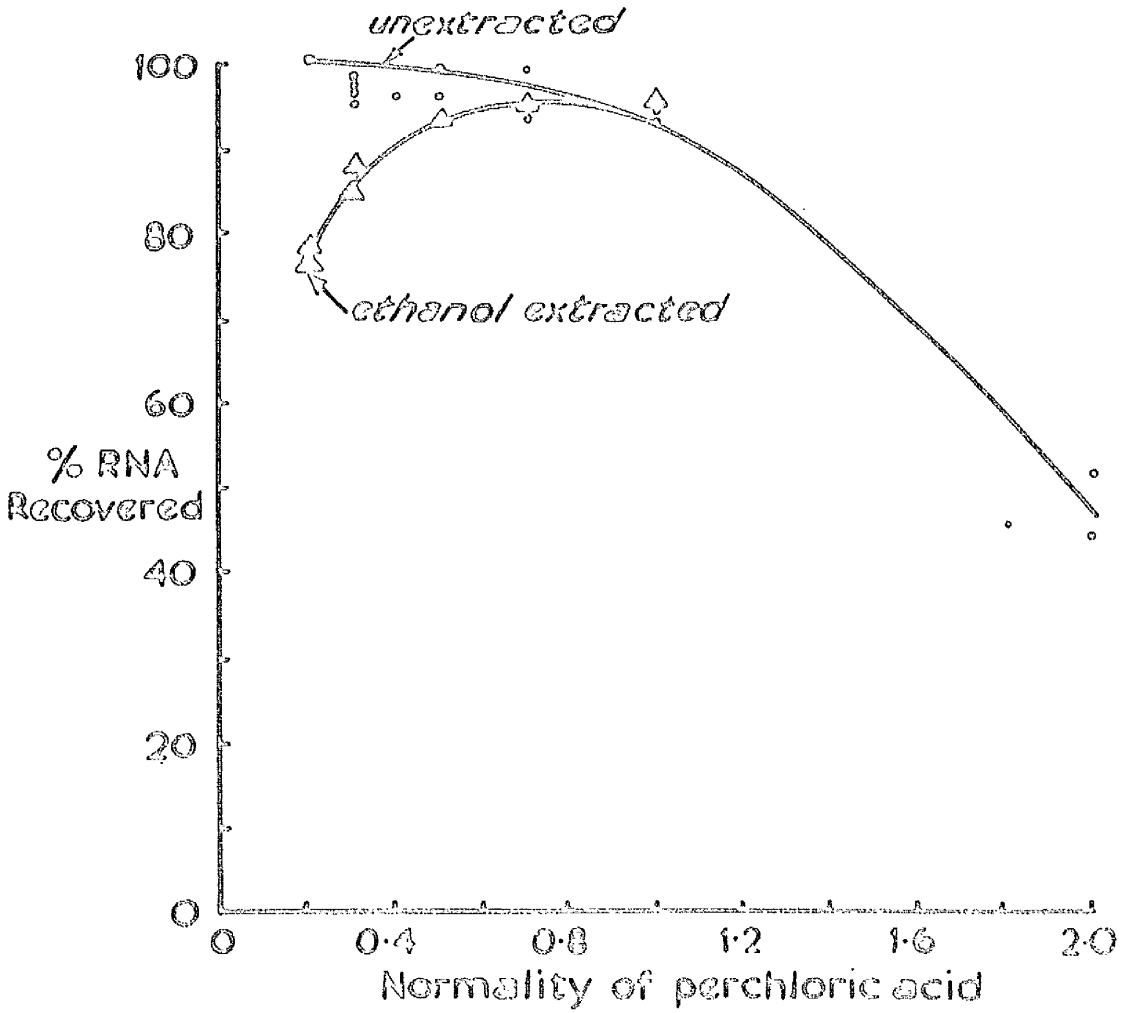
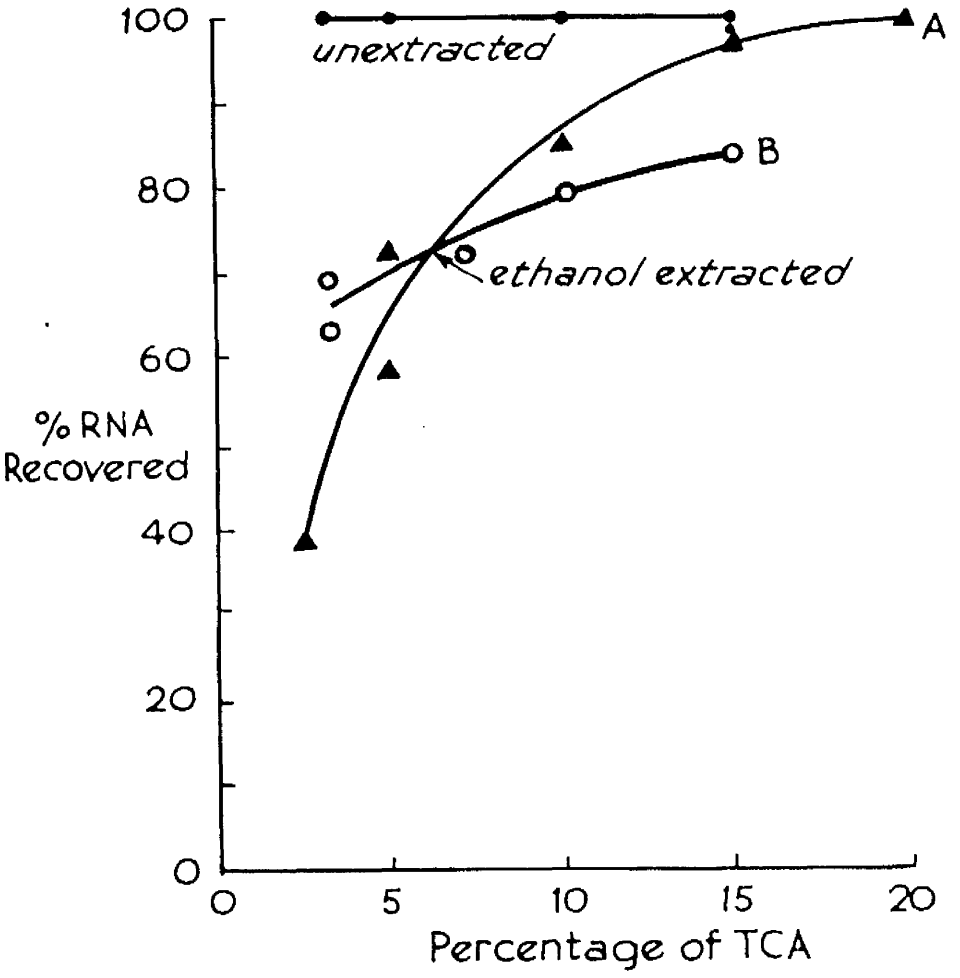


Fig. 66. EFFECTS OF LIPID EXTRACTION FOLLOWING PRECIPITATION WITH TRICHLOROACETIC ACID ON RECOVERY OF RNA



method previously described (p.190).

Results

The results (figs.65 and 66) demonstrate that at the lower concentrations of acid used, considerable quantities of RNA are extracted by lipid solvents. There is no concentration of acid following which lipid solvents may be applied to the precipitate and 100% recovery of RNA obtained.

Using a similar method, it was shown that when lipid-solvents extracted 9% of the RNA of the sample, approximately 35% of the protein was extracted. The addition of 1.5% potassium acetate to the first - ethanol-extraction - stage reduced the loss of RNA and protein to 2% and 5% respectively. Varying the concentration of potassium acetate did not improve those recoveries.

Conclusion: From these results it is apparent that when the optimal concentration of acid (0.2 N PCA) is used for the initial acid-precipitation stage of tissue nucleic acid and protein analysis, it is inadvisable (and unnecessary) to follow this step with the extraction of lipids.

(b) Methods of lipid extraction

From the foregoing experimental evidence, it is obvious that the determination of lipids together with nucleic acids and protein on a single tissue sample is an analytically unsound procedure. Also the multi-stage extraction of lipids is tedious and there is no evidence relating to the efficiency of the extraction method. However, Folch, Lees and Sloane-Stanley (1957) claimed that a single extraction of a tissue sample with 20 volumes of chloroform-methanol (2:1) gave complete extraction of lipids, it was decided to investigate their method

By determining the amount of saponifyable material in the residue after extraction and by following the extraction of lipid-phosphorus it was concluded that two extractions of 2:1 chloroform-methanol (20 volumes) were necessary for quantitative extraction of lipids from liver and liver microsomes. The slightly modified procedure which was found to be satisfactory is described in detail in the appendix.

Discussion and Conclusions

The foregoing literature surveys and experimental work should permit the introduction of methods of tissue analysis which will give reliable results. It is now apparent, for example, that the standard method of determination of nitrogen in biological materials should be based on the micro-Kjeldahl method. In addition, the only satisfactory procedure for the determination of protein is that of Lowry et al. (1951). RNA may be determined accurately by a simple ultraviolet absorption method provided digestion in alkali is not prolonged and that preliminary acid precipitation is carried out with 0.2 N PCA. Similar conditions may be used in the preliminary treatment of the tissue for DNA estimation by the method of Cerriotti (1955).

The estimation of lipids is best carried out directly on the tissue homogenate using the two stage extraction modification of the method of Folch et al. (1957), principally because the multi-stage extraction methods are tedious and because there is no published evidence of the recovery of lipids using these methods. A further advantage of the chloroform-methanol extraction procedure is that the lipid extract can be washed free of non-lipid contaminants (Folch et al., 1957). This gives another indication that it is

impossible to achieve good analytical results when attempting to estimate lipids, protein and nucleic acids simultaneously. Any reliable scheme of tissue analysis requires separate samples for determination of lipids, protein, and nucleic acids.

GENERAL DISCUSSION

The change in the rate of turnover of plasma albumin in response to change in the protein content of the diet may be a form of protective mechanism. As the protein content of the diet is reduced, the rate of turnover of protein decreases. Thus nitrogen will be conserved and the survival time following protein depletion prolonged. Although there is no data on the turnover rate of the microsome proteins, the analytical data obtained from animals on diets of differing protein content is consonant with the changes in turnover of plasma albumin.

It is unfortunate that the technical difficulties involved in the study of the synthesis of a single protein from microsomes made it necessary to study the incorporation of leucine into the whole microsome protein because it is probable that microsome proteins are synthesised at different rates just as are those of the plasma, so that the study of a single microsome protein might have led to more satisfactory results in some experiments. Nevertheless, it is obvious that feeding protein immediately before removal of the liver leads to a stimulation of protein synthesis in microsomes. The failure to demonstrate a greater uptake of leucine in vitro by microsomes prepared from the livers of animals fed on adequate amounts of protein in comparison with those from deprived animals (when both groups had been fasted for 18 hours) indicates that the stimulation due to feeding protein is not prolonged. That breakdown of polysomes occurs in this period of fasting confirms this hypothesis.

Despite a not entirely clear-cut picture, the results of the experiment in which Actinomycin D was administered prior to the feeding of casein are suggestive of messenger RNA being involved in the response of the microsomes to dietary protein.

In current terms it may be supposed that the feeding of protein inhibits a suppressor mechanism so that, in response to dietary protein, messenger RNA is synthesised or leaves the nucleus, and an increase in the activity of polysomes or microsomes results.

The alternative possibility is that the presence of amino acids in some way stabilises the polysomes or microsomes, perhaps by inhibiting ribonuclease. Although this hypothesis also fits much of the experimental data, it would require a complex explanation for the effects of Actinomycin D in inhibiting the response of microsomes to the feeding of casein to the experimental animals. In addition, it is not readily reconcilable with the increase in turnover of plasma albumin in response to a high protein diet, as it is not unlikely that this effect is general and that there is a parallel increase in the turnover of the proteins of the microsome and possibly of messenger RNA.

There are several possible experiments which would contribute evidence to the foregoing discussion. For example, the administration of ^{32}P or ^{14}C-orotic acid to rats receiving different diets or fed protein shortly before removal of the liver and preparation of polysomes would yield information on whether there is an increase in messenger RNA in the polysome fraction in response to feeding. A careful study of the effects of ribonuclease on polysome fractions

prepared from the livers of animals on different diets would contribute some evidence on the lability of polysomes. It would also be interesting to know whether polysomes occur to any extent free in rat liver or whether they are derived from the Rough-Surfaced Vesicles.

Finally, it would appear to be possible that the stimulation of the microsome fraction or protein synthesis in general requires the complete complement of amino acids (Munro and Clark, 1959). If so, this would require a complex 'recognition mechanism' at the level of the suppressor.

Summary

Plasma protein metabolism in relation to diet and injury

1. Protein depletion, which leads to loss of RNA and protein from the liver, leads to a decrease in the turnover rate of plasma albumin in albino rats.

2. When the experimental animals were subjected to injury those which had been maintained on a diet of high protein content responded with an increase in the excretion of nitrogen in the urine. This did not occur to a significant extent in animals deprived of protein. Injury of animals on a high protein or low protein diet led to no detectable differences in the liver composition of rats, nor did it affect the turnover rate of plasma albumin.

3. Although it is possible that in normal animals a significant quantity of albumin may be catabolised in the gastro-intestinal tract and that the presence of serum albumin and globulin in bile can be demonstrated by immuno-electrophoresis, the amounts of these serum proteins present in normal bile are such as to exclude the possibility that the bile is the source of the serum protein which is catabolised in the gastro-intestinal tract.

Studies in protein synthesis

4. The microsome fraction of rat liver (which is the site of synthesis of plasma albumin among other proteins) is affected by the protein content of the diet: animals deprived of protein have less liver microsomal protein, RNA and phospholipid than the livers of animals fed adequate protein.

5. Rat liver microsomes can be solubilised by treatment with a mixture of 1% sodium deoxycholate and 0.05 M sodium pyrophosphate (pH 7.4). This treatment however, does not render the isolation of the proteins of the microsomes simple since, on removal of the deoxycholate and pyrophosphate aggregates are formed of 's' values 36 to 4s.

6. The preparation of pure samples of plasma albumin from plasma or serum was examined. Satisfactory separation may be achieved using DEAE-Sephadex and column electrophoresis or by acid-ethanol extraction. Neither of these procedures is satisfactory for the preparation of albumin from microsomes although the second procedure yields a sample of albumin which is contaminated with RNA.

7. The in vitro uptake of radioactive leucine by microsome fractions from rat liver was studied. When livers are obtained from fasted animals the activity of the microsome fraction of the animal deprived of protein is greater than that of the protein-fed group. In contrast, feeding casein 2 hours before removal of the liver and preparation of the microsome fraction leads to an increase in the incorporation of leucine into the microsomal protein. It is probable that this effect is inhibited by the previous administration of Actinomycin D.

8. Sucrose density gradient studies of the 'polysome' fraction of rat liver indicate that the fasting of animals which had been previously fed a normal amount of protein leads to breakdown of polysomes and accumulation of free ribosomes.

The estimation of tissue constituents

9. Various analytical procedures were investigated as part of the work performed in this project. After reviewing the literature relating to protein and nitrogen estimation, methods of determining the protein and nitrogen content of samples of biological origin were selected which proved on experimental investigation to be reliable.

10. Among available procedures, protein may be estimated by a very sensitive method which depends on the preliminary formation of a biuret complex and subsequent development of a blue colour complex with the Folin phenol reagent. This method is equally applicable to aqueous homogenates of liver or to acid precipitates of liver homogenates.

11. A simple method for the estimation of RNA in liver was devised. This depends on precipitation of the tissue with acid, then hydrolysis of the RNA in 0.3 N KOH at 37° for 1 hour, followed by acidification with perchloric acid; the RNA content of the tissue is then computed from the extinction of the now acidified hydrolysate at 260 mμ.

12. The optimal concentration of perchloric acid for the precipitation of liver RNA, protein and DNA was established as 0.2 N. The extraction of lipids of liver with lipid solvents invariably removes some RNA and protein. There is a satisfactory method of tissue lipid estimation based on a chloroform-methanol extraction procedure.

APPENDIX

Analytical Methods

Nitrogen Estimation by the Micro-Kjeldahl Method

References: (a) review by A. Fleck and H.N. Munro, 1964 - in press Clin.Chim.Acta.

(b) H. Markham (1942), Biochem.J., 36, 790.

Sensitivity: 0.5 to 2.0 mg N.

Accuracy: 1%

Reagents: (all Analar)

1. H_2SO_4 (concentrated) (nitrogen-free)
2. 4 N H_2SO_4
3. HgO (red)
4. Mercury catalyst solution:

4 gm HgO in 100 ml 4 N H_2SO_4

5. K_2SO_4 (anhydrous)
6. Zinc dust
7. NaOH 40%
8. De Wesselow's indicator:-

0.02% Methyl red in 50% ethanol ... 100 ml)
0.1 % Methylene blue ... 15 ml) mixture

9. Standard H_2SO_4 and NaOH 1 N stock and 0.01 N
10. Nitric acid (concentrated)

Apparatus: As described by Markham (1942).

Procedure: Place quantitatively material containing 0.5 to 2.0 mg N in a digestion flask, add 1.5 ml concentrated H_2SO_4, then 1.0 ml catalyst solution and 1.2 gm K_2SO_4. Wash down the sides of the flask

with a little distilled water. Transfer the flask to the digestion rack, heat gently to boil off the water then digest over maximum heat for ½ hour (after 10 minutes digestion clearing should have occurred). Simultaneously, duplicate blanks should be treated in the same way.

Note: (a) It is advisable to do duplicate N estimations and essential to have at least 2 estimates of the blank, as error in the latter vitiates the whole group of N estimations.

(b) ½ hour digestion is adequate even for refractory materials.

(c) If excess carbonisation occurs, due for example to the presence of sucrose, intermittent addition of H_2O_2 after slight cooling of the digestion flask will aid digestion. A blank must be treated in the same way, as H_2O_2 is liable to contain N.

(d) If, when cool, the digest solidifies, digestion must be repeated with a smaller quantity of K_2SO_4 (excess salt leads to solidification on cooling, too high a b.p. of digest with consequent loss of N).

After digestion, wash down the sides of the cool flask with water and dissolve the K_2SO_4, then transfer quantitatively to the Markham apparatus, washing the flask twice. Add 0.2 gm Zinc dust to the apparatus and wash twice with washings from digestion flask. Follow with 10 ml 40% NaOH. Steam distill in the usual way for 3 minutes. Trap the NH_3 in 10 ml 0.01 N H_2SO_4 (20 ml if more than 1.0 mg N), and titrate with 0.01 N NaOH using De Wesselow's indicator (end-point is green colour after purple to colourless change). The acid versus alkali titration should be checked before doing the "blank" titration. Zinc Amalgam may be removed at the end of a day by dissolving in concentrated HNO_3.

Laboratory Methods

Estimation of Protein

References: O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, (1951), J.Biol.Chem., 193, 265.

Specificity: Few substances interfere significantly; uric acid, guanine, xanthine are examples. However, the method is satisfactory for protein in tissue.

Sensitivity: (In this version), 25-500 µg protein in 1 ml.

Principle: Modification of the Folin method for estimation of tyrosine, the reaction takes place in alkaline solution in the presence of Cu; the method is very sensitive.

Reagents: 1. 2% Na_2CO_3 in 0.10 N NaOH.

2. 1.0% $CuSO_4\ 5H_2O$ in 2% Na or K tartrate.

3. 2% $CuSO_4\ 5H_2O$ in 2% Na or K tartrate.

4. Mix equal volumes reagent (2) and reagent (3) to give the copper reagent immediately before making up reagent (5).

5. 1 ml copper reagent (2) in 50 ml reagent (1). (This solution to be made up fresh each day).

6. Folin-Ciocalteau phenol reagent diluted so that it is 1 N with respect to acid (i.e. about 1 in 3 dilution).

Standards: Protein solution of the type to be estimated. (Bovine serum albumin, 100 µg/ml is convenient).

Method: Take 25-500 µg protein solution in not over 1 ml

(make up to 1 ml if necessary); add 5 ml alkaline copper reagent (5) and stand for 10 minutes. Add 0.5 ml Folin reagent, shaking immediately. After standing at room temperature for 30 minutes, read at 750 mμ.

Note: The calibration curve is linear up to only 100 μg protein (extinction approximately 0.320).

Nucleic Acids (of rat liver)

References: (a) A. Fleck and H.N. Munro (1962), Biochim.Biophys.Acta, 55, 571.

(b) T. Hallinan, A. Fleck and H.N. Munro (1963) Biochim.Biophys.Acta, 68, 131.

(c) W.C. Hutchison and H.N. Munro (1961), Analyst, 86, 768.

(d) G. Ceriotti (1952), J.Biol.Chem., 198, 297.

Sensitivity: for method as described 0.7-3.5 mg RNA (70-350 µg RNA-P). May be simply adjusted 1/10 for the range 70-350 µg RNA.

Reagents: 0.3 N KOH, 0.6 N PCA, 0.2 N PCA.

Procedure: 1. Make a 1/20 homogenate of rat liver in ice-cold distilled water (use a Nelco Blendor for 5 minutes at full speed) with ice in the jacket).

2. Pipette 5 ml (= 250 wet weight tissue) into a centrifuge tube, add 2.5 ml ice-cold 0.6 N PCA, mix and stand 10 minutes in cold. Centrifuge, discard supernatant and wash precipitate twice with 0.2 N PCA.

3. Drain off excess acid by inverting tube over filter paper briefly, then add 4 ml 0.3 N KOH and incubate in an air oven at 37°C for one hour.

4. After incubation, cool in ice, add 5 ml 0.6 N PCA, stand 10 minutes in cold, then centrifuge. Wash precipitate twice with 5 ml 0.2 N PCA. Transfer supernatant and washings to 100 ml cylinder, add 10 ml 0.6 N PCA and make up to 100 ml with water (or, add 2 ml 0.6 N PCA and make up to 50 ml for smaller amounts of RNA). This gives the RNA (or acid-soluble) fraction 0.1 N PCA.

5. Dissolve the precipitate in 5 ml 0.3 N KOH, transfer to a 50 ml cylinder, add 12 ml 0.3 N KOH and make up to 50 ml with water. This gives the DNA fraction in 0.1 N KOH.

6. RNA estimation: Read the extinction of the RNA fraction at 260 mμ. Extinction of 1.000 = 35.18 μg RNA = 3.412 μg RNA-P/ml (whole rat liver RNA)

7. DNA estimation: Take 2 ml of the DNA fraction for estimation of DNA by the method of Ceriotti.

Note 1: Keeping solutions (for stages 6 and 7) overnight in the cold will not introduce more than 5% error.

Note 2: The method has been adopted, essentially unmodified, for the preparation of labelled microsomal proteins etc. free of RNA for radioactive assay.

Comments

1. Concentrations of PCA greater than 0.2 N hydrolyse RNA even at 0°C.

2. 0.2 N PCA also gives full precipitation of proteins (but must not be used if N determinations are to be carried out).

3. Extraction of the acid precipitate with lipid solvents must be avoided since there is no satisfactory method of removing lipids which does not also remove protein and RNA.

4. Increase in time of hydrolysis and concentration of alkali (or temperature greater than 37°C) leads to errors in ultraviolet absorption due to hydrolysis of protein. The conditions of incubation, i.e. 1 hour at 37°C in an air oven, should be adhered to strictly.

5. For complete hydrolysis of RNA to nucleotides, 16-18 hours in 0.3 N KOH at 37°C is necessary; this is not a requisite in ultraviolet estimation of total RNA.

6. The method has been applied to various tissues and cell fractions with success.

7. The specific extinction quoted is for whole rat liver RNA and may not be applicable for RNA's of other tissues or for cell fractions.

Laboratory Methods

Estimation of DNA

References: Ceriotti, G. (1952), J.Biol.Chem., 159, 211.

Specificity: almost solely for DNA (deoxyribose); arabinose is the only interfering chromogen which gives a colour not extractable with $CHCl_3$. There is a sharp absorption peak at 490 mμ.

Sensitivity: (2.5) 5-15 μg DNA/ml

0.4 -1.2μg DNA-P/ml

Principle: Many substances produce colours when heated with indole in the presence of concentrated HCl; apart from arabinose, only DNA (deoxyribose) gives a colour which is not extracted from the aqueous phase by $CHCl_3$.

Reagents:

1. Indole. 0.04% w/v in distilled water. (store in refrigerator).
2. HCl concentrated (sp.gr. 1.19) Analar.
3. $CHCl_3$. (anaesthetic grade -see note 3).

(N.B. The reaction should be carried out in 15 ml pyrex glass-stoppered centrifuge tubes).

Standards: DNA - usually departmental stock. Calf thymus DNA prepared by the method of Kay et al. (Ref.1) or commercial DNA. About 20 mg DNA dissolved in 50 ml distilled water with the aid of a little NaOH - this gives Stock Standard solution. 2 ml of this stock solution in 50 ml of water gives standard solution for routine use. (See note 1) (16 μg DNA/ml).

Method: To 2 ml DNA solution, add 1 ml indole reagent, 1 ml concentrated HCl and mix (total volume 4 ml). Place in a boiling water bath for 10 minutes, then cool in running water. Extract with 4 ml $CHCl_3$ 3 times (using a Pasteur pipette and discarding the $CHCl_3$ layer). On the last extraction, or previously if the layers do not separate clearly, centrifuge at 500 rpm for 5 minutes, before removing the $CHCl_3$ phase. Read the aqueous layer at 490 mμ (see note 2).

Note 1: The standard solution on being made up from the stock solution should be subjected to the same treatment as the material being estimated; e.g. treated in N PCA at 70°C for 10 minutes.

Also, if the results are to be expressed in terms of DNA-P it is essential to estimate the P content of the standard solution by Griswold's method (Ref.2).

Note 2: The aqueous layer may be allowed to stand for several hours at room temperature and, provided the pink colour which slowly appears is extracted with $CHCl_3$ before reading, the same optical density will be obtained.

Note 3: The $CHCl_3$ despite the author's remarks to the contrary, should not be specially purified before use. (Anaesthetic grade seems to be satisfactory as it contains no phosgene).

References: 1. Kay, E.R.M., Simmons, N.S. and Dounce, A.L., (1952) J.Am.Chem.Soc., 74, 1724.

2. Griswold, B.L., Humoller, F.L. and McIntyre, A.R. (1951), Anal.Chem., 23, 192.

Preparation of microsome protein for assay of ^{14}C

References: (a) A. Fleck and H.N. Munro (1962), Biochim.Biophys.Acta, 55, 571.

(b) T. Hallinan, A. Fleck and H.N. Munro (1963), Biochim.Biophys.Acta, 68, 131.

(c) W.C. Hutchison and H.N. Munro (1961), Analyst, 86, 768.

Reagents: 0.3 N KOH, 0.6 N PCA, 0.2 N PCA, 0.25 PCA with 1 mg/ml DL or L-leucine.

Procedure: The frozen samples obtained after incubation (0.2-0.5 ml in a 15 ml centrifuge tube) were allowed to thaw at 0°C, then 5 ml 0.25 N PCA (containing leucine) was added and mixed (use a Vortex mixer throughout). After standing for 10 minutes, the precipitate was centrifuged down and the supernatant discarded. The precipitate was then washed twice with 0.2 N PCA and finally the excess acid carefully drained off before the addition of 4 ml 0.3 N KOH and incubating for 1 hour at 37°C (in air). After incubation, the tubes were cooled to 0°C, and 1.0 ml 2.4 N PCA added. The precipitate was centrifuged down (after 10 minutes standing) and washed twice with 0.2 N PCA. If it was desired to estimate RNA, the supernatant and washings were combined and made up to a suitable volume (50 ml) in 0.1 N PCA. The excess acid was drained off the protein precipitate which was dissolved in 0.3 ml 0.3 N KOH, warming to room temperature if necessary. When the protein had dissolved, the solution was

cooled to $0^{o}C$ and centrifuged to remove potassium perchlorate before applying 0.2 ml to a lens paper on a planchette, drying and assaying ^{14}C in a Nuclear Chicago gas flow counter. When the quantity of protein plated out in this way was less then 2 mg, conditions of infinite thinness could be assumed.

Phospholipid Phosphorus Estimation

References: J. Folch, M. Lees and G.H. Sloane-Stanley (1957), J.Biol.Chem., 226, 497.

Reagents: 1. $CHCl_3$:CH_3.OH - 2:1

2. NaCl 0.73% in water

3. NaCl 0.56% in water

4. "Pure solvents - upper phase." This is the upper phase from $CHCl_3$: CH_3OH, NaCl (0.56%)- 8:4:3. (Avoid the use of separating funnels in case of contamination with stopcock grease).

Procedure: Transfer 0.5 ml (see Note 1) of 1 in 3 aqueous homogenate of liver to a 10 ml ground glass stoppered centrifuge tube. Add 10 ml $CHCl_3$: CH_3OH and shake thoroughly at intervals for 10 minutes. Transfer the solvents quantitatively to a 30 ml ground glass stoppered centrifuge tube (see Note 2). Re-extract as before with another 10 ml solvents and combine the extracts. Add 4.5 ml 0.73% NaCl to the extract (see Note 3), shake and centrifuge for 10 minutes at 1500rpm before removing and discarding the upper layer. Carefully, without disturbing the interface, wash down the walls and interface with 4.5 ml "pure solvents upper phase" - repeat this twice and discard the washings each time.

Transfer the washed extract quantitatively to a 25 ml measuring cylinder, wash out the centrifuge tube twice with approximately 3 ml methanol and after adding the washings to the extract, make up to 25 ml with methanol.

Transfer a sample of this $CHCl_3$:CH_3OH (3 ml from liver is satisfactory - see Note 4), extract to a pyrex test tube, evaporate to dryness at 100°C then add 0.4 ml 72% PCA and digest in the sand bath at 200°C for 1 hour etc. - proceed as for the Allan phosphorus estimation.

Notes 1: Solvents should be added in the ratio 20 volumes to one of aqueous phase.

2. It is usually unnecessary to centrifuge prior to this stage.

3. The ratios 8:4:3 of $CHCl_3$:CH_3OH:H_2O must be preserved throughout the procedure.

4. For microsomes from 4 gm liver in 10 ml, take 0.5 ml for extraction and 5-10 ml of the lipid extract for P estimation.

Estimation of Phosphorus

References: R.J.L. Allen (1940), Biochem.J., 34, 858.
(The method is specific for inorganic phosphorus).

Specificity: there are few sources of interference.

Sensitivity: 5-30 µg P in 4.0 ml (1/5 procedure).

Reagents: Use borosilicate glass test tubes throughout.

1. PCA 72%.

2. Reducing agent. 1% amidol in 20% sodium metabisulphite (filter before use - keep in dark bottle and store for not more than 2-3 days).

3. Ammonium molybdate 0.3%.

Standards: Stock P solution - 1 mg P/ml (BDH).
25 µg P/ml and 5 µg P/ml are useful concentrations.

Procedure: The aliquot of P-containing material (containing 5-30 µg P) should be concentrated to approximately 0.5 ml or evaporated to dryness at 100°C before adding 0.4 ml 72% PCA and digesting in a sand bath at 200°C for 1 hour. Cool and wash down walls of tube with (accurately pipetted) 4 ml H_2O. Add 0.4 ml amidol, then 0.2 ml molybdate solution, mixing at each step. 5-20 minutes later read the extinction at 725 mµ (in the SP 600).

For inorganic phosphorus estimation, omit the digestion stage and make up the phosphorus solution accurately to 4 ml with water before adding 0.4 ml PCA, then amidol and molybdate as before.

[O.D. of 1.00 = 39.66 µg P - calibration curve is linear to O.D. of 1.5]

Assay of ^{131}I

^{131}I is a β and γ emitting isotope of half-life 8.1 days. Equipment was obtained from Nuclear Enterprises (Edinburgh), and consisted of NE5301 (EHT supply), NE5202 (amplifier), NE5102 (pulse height selector) and 'Ekco' scaler N530D. A sodium iodide-thallium activated crystal scintillator with a specially made re-entrant well-type beaker was used. The beaker was cast in this department from "Araldite," and gave an efficiency of 34%.

Procedure: Place an active sample over the crystal. Plot counts against EHT with different pulse heights. At EHT > 900v counts above background were obtained. At pulse heights > 3v increase of EHT produces no significant change in total counts. Repeat for various amplifier gains.

Let s = sample counts, b = background counts and plot $\frac{(s - b)^2}{b}$/EHT for various pulse heights. The optimum conditions were obtained with:-

amplifier gain = 150, EHT = 11,000v, pulse height = 4v

Note: It is possible to use this equipment as a γ -ray spectrometer.

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