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STUDIES ON PROTEIN BIOSYNTHESIS.

by

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Thesis submitted for the Degree of
Doctor of Philosophy
of the University of Glasgow,
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GENERAL INTRODUCTION.

The present status of our knowledge of protein synthesis.

For centuries it was recognised that tissues had component fractions which we now recognize as proteins. Many of these were named in quite remote times - thus albumin of blood was known several hundred years ago, and so also casein of milk. Gluten, the protein of wheat flour, was separated in 1734 by Beccari (1746) at the University of Bologna. However, the further recognition of the group as a united class was only possible when chemical analysis became possible at the start of the nineteenth century. In 1838, Mulder (1838) suggested that there was a basic chemical nucleus common to these various nitrogenous compounds, and named the nucleus "protein". This hypothesis was rapidly disproved by further chemical analysis, but the name"protein" was accepted by English-speaking scientists as a convenient name for the group of nitrogenous organic compounds.

Once the proteins had been recognised as important components of the tissues, the question of their renewal from dietary protein was a legitimate field of speculation and inquiry. Thus Liebig (1842) believed in 1842, in common with many of his predecessors, that the tissue proteins were derived from food protein directly by transformation of the latter first into blood albumin and thence into tissue protein.

Little advance on this view took place until it was demonstrated between the years 1860-1900 that the dietary proteins first have to undergo degradation to simpler products during digestion, before passing into the body. This gave rise to speculation as to whether resynthesis of dietary proteins into tissue proteins took place by a reversal of the process of digestion, that is, whether enzymes similar to the proteolytic ferments of the digestive juices were present in the tissue cells, but here carried out a synthetic reaction from the protein degradation products. Experiments were described in which digested proteins were found on incubation with these enzymes to be capable of synthesising larger molecules thought at first to resemble proteins again. These were named "plasteins" the subject has been reviewed by Borsook and Wasteneys (1930).

This theory was eventually seen to be untenable, since the products formed by the reversal of proteolysis were clearly not tissue proteins. Nevertheless, around 1950, it received a certain vogue again when it was observed that some of the proteolytic enzymes were capable of transferring amino acids to and from peptides: transpeptidation (Fruton, 1950; Johnston et al, 1950). In this way, it was possible to envisage how peptides could be indefinitely extended and so attain the size of protein molecules. However, this theory was not capable of supporting the very necessary speculation about why different cells make different

types of protein, and it has in consequence languished since that date.

In the meantime, a development in knowledge of the biological structure of the cell had disclosed a property which was to become a central tenet in a new theory of protein biosynthesis, which had proved to be much more fruitful. Independently, during war-time, Caspersson (1941) in Stockholm with the use of ultraviolet microscopy, and Brachet (1942) in Brussels with histochemical techniques, found in 1941 that ribonucleic acid varied in abundance in different cells, and that the intracellular concentration was a property of the intensity of protein formation by the cell. Ribonucleic acid (RNA) was observed as a component of the nucleus as well as of the cell cytoplasm, and in his book entitled "Cell Growth and Cell Function", Caspersson (1950) describes from ultraviolet measurements of individual cells how the nuclear RNA is manufactured under the influence of chromatin containing deoxyribonucleic acid(DNA), and is then secreted into the cytoplasm where he credits it with the responsibility for protein synthesis control.

Much of the subsequent work on protein biosynthesis has been based on this fundamental observation of a link between RNA and protein formation. In order to achieve further knowledge of the details of the mechanism, it has been necessary to use

cell-free systems which will imitate the mechanism of protein synthesis by the intact cell. All such systems and theories start from the assumption that the cell proteins are made eventually from the free amino acid pool of each cell. In the ensuing sections of this introduction we shall consider first some of the cell structures which are reputed to participate in protein synthesis; secondly, the scheme proposed by Zamecnik and Hoagland; and finally, the background to the present investigation of protein formation in the liver cell and the method of approach will be outlined.

Cell Structure and Protein Biosynthesis.

Most intracellular structures participate to a greater or lesser extent in the task of protein biosynthesis, but the most prominent fraction in this respect is that named originally by Claude (1943a; 1943b) the "microsomes". The intracellular origin and nature of these structures was a matter of dispute until Palade and Siekevitz (1956) provided electron micrographs which showed the microsomes to consist of profiles of vesicles encrusted with electron dense particles. These were identified as pinched off fragments of the endoplasmic reticulum, present in the intact cell.

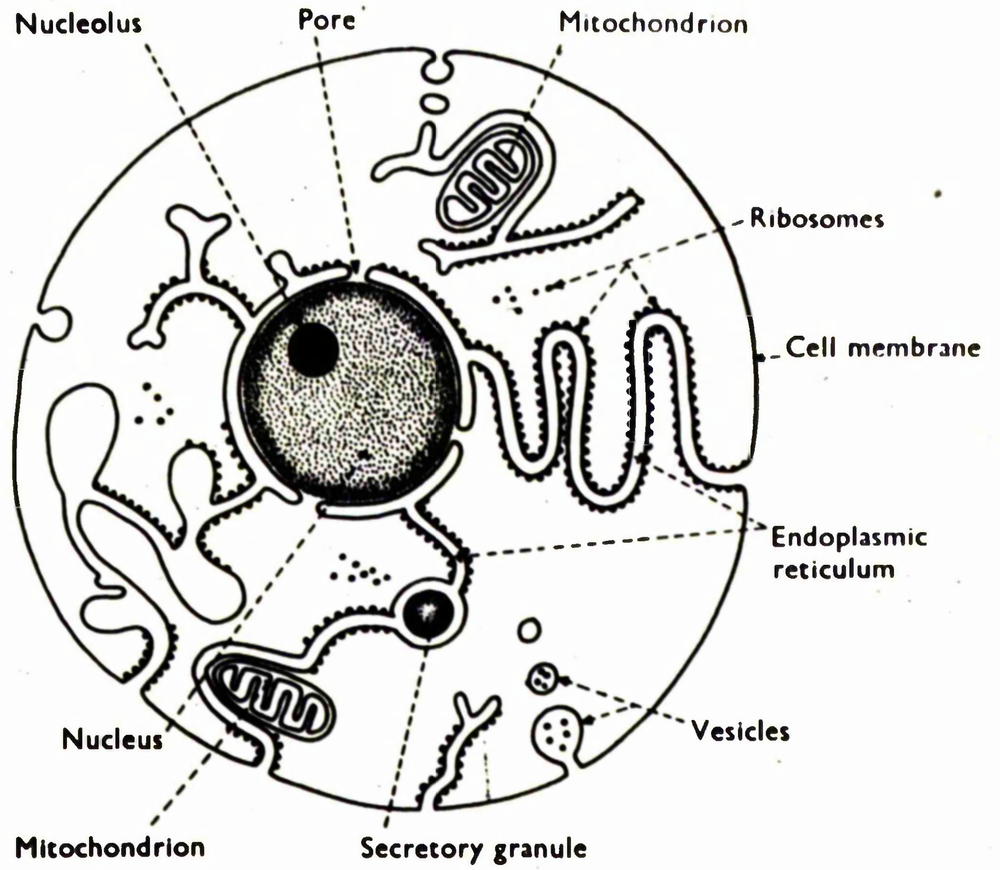
The ergastoplasm, or endoplasmic reticulum, (Porter & Kallman, 1952; Porter, 1953) or alpha-cytomembranes

(Sjostrand,1956), is a complex arrangement of tubules which penetrate throughout the cytoplasm of cells. The degree of organisation of the reticulum varies from simple parallel membranes to three-dimensional lattices in highly differentiated cells. In the electron microscope, the commonly observed picture (e.g. in pancreas) is that of parallel lamellae, on one side of which are numerous electron-dense particles some 15mu in diameter. This is the rough form of endoplasmic reticulum. In some instances, the same membrane is evident without adherent particles, giving a smooth form (Porter, 1961). In the liver which is of peculiar interest to biochemists as an organ of study both forms have been observed. The smooth membranes show no affinity for free particles in the cell sap (Porter & Yamada, 1960) and Moule et al. (1960) have obtained by differential centrifugation a liver fraction which is rich in smooth reticulum. The function of these particlesfree membranes is unknown although they have been noted (Porter, 1961) to occur in liver cells in close relationship to stored glycogen granules. They are also particularly abundant in cells secreting lipids or steroids but it is not known whether the reticulum is actually involved in either of these processes. The rough form consists of three portions; the membrane, the enclosed spaces between the two walls which contain no visible structures, and thirdly the granules aligned along the surface of the membrane adjacent to the cell sap.

Fig.1.

Schematic representation of a 'typical' animal cell.

(Davidson, 1960)



Chemical study of the microsomes (the derivatives in homogenates of the endoplasmic reticulum of the intact cell) shows that the reticulum has three major components, protein, RNA, and phospholipid (Palade & Siekevitz, 1956a). About half of the total RNA of the liver cell is found in this fraction which by itself makes this cell structure a likely candidate for the role of protein biosynthesis through the RNA template mechanism. Exploration of the components of the rough form has been possible by exploiting the differential effect of detergents (Palade & Siekevitz, 1956b) on the microsome sub-structures. Treatment with deoxycholate solubilises the membrane portion leaving the granules which contain 80% of the RNA and 20% of the protein of the original microsomes, still sediment-able. These particles are capable of amino acid incorporation in absence of the membrane (Littlefield & Keller, 1957). Alternatively, the membrane can be prepared free from adherent particles by stripping them of with versene (Palade & Siekevitz, 1956b) or pyrophosphate (Sachs, 1958). The membrane contains most of the original microsomal protein and all the phospholipid. Some 30-40% of the original RNA remains in the membrane fraction prepared with pyrophosphate (Goswami *et al.*, unpublished 1962) and this RNA has been found to have a base composition close to that of nuclear RNA and very different from that of the adherent granules (ribosomal RNA).

The nucleoprotein particles (ribosomes) are also found free in the cytoplasm of some cells (Palade, 1958) , and can function alone in the process of amino acid incorporation into cell protein, which suggests that the membrane may only be present to meet a specific aspect of protein synthesis. The separate existence of ribosomal particles has been questioned, on the grounds that they are artifacts produced during homogenisation from microsomal disintegration. However, Palade and Siekevitz (1956) have separated two post-microsomal pellets which are also ribonucleoprotein particles with a negligible content of phospholipid, and differ in metabolic behaviour from the rib-osome particles prepared from the same cells by treatment of the microsomes with deoxycholate.

There has been no lack of hypotheses on the function of the endoplasmic reticulum. It has been suggested (Porter, 1961) that the membrane is a special modification providing a method of transportation of proteins produced by the particles in glands which secrete protein. This view is supported by the observation that secretory cells are rich in endoplasmic reticulum, e.g. the pancreas. The protein newly synthesised by the ribosomes, may diffuse through the membrane into the cavity of the endoplasmic reticulum which has been shown to be continuous with the external environment of the cell, and also with the nucleus (Hartmann, 1953; Watson, 1955). Protein formed on free ribosomes, i.e. those unattached to reticulum, may represent protein produced for

intracellular purposes and thus require no transport mechanism.

Finally, the origin of the endoplasmic reticulum is a matte of debate. It has already been pointed out that the reticulum is continuous with the nuclear membrane. Porter (1961) suggests that the reticulum originates from the nuclear membrane, and thi has received support from several studies with the electron microscope (Gay, 1960). Evidence obtained in this laboratory (Goswami _et al._, unpublished) shows that RNA associated specifically with the endoplasmic reticulum membrane, after removal of the ribosomes, is close to nuclear RNA in base composition and thus supports this concept of a nuclear origin.

Incorporation of amino acids by the Hoagland-Zamecnik system.

The best authenticated scheme of protein biosynthesis has been pioneered mainly by the laboratories of Hoagland and Zamecnik, and a complicated sequence of steps leading from the free amino acid pool to the site of assembly in protein has been partially elucidated by defined reactions. Ultracentrifugatic has provided an extremely useful tool in solving the problems concerned with the possible site and mechanism of protein synthesis. Disruption of the cell and separation of the particles according to size has permitted the study of subcellular fractior relatively free from one another.

The first evidence of the intracellular localisation of the synthetic process came from the studies of Hultin (1950) who injected labelled amino acids into rats and after an interval,

killed them and separated the liver subcellular fractions. Similar studies on uptake of labelled amino acids into cell fractions were carried out by other workers (Borsook *et al.* 1950; Keller *et al.*, 1954). All three studies demonstrated that the uptake of the amino acids into protein was most intense in the case of the microsomes, even at comparatively short time-intervals. This is consonant with the presence of a high concentration of RNA in the microsomes; earlier work (Caspersson, 1941; Brachet, 1942), showed that cells engaged in intense protein synthesis contain a high concentration of RNA, and it would be reasonable to anticipate that the intracellular site of protein formation would exhibit this high amount of RNA.

The next step, undertaken by Littlefield *et al* (1955), was to sub-divide the microsomes in order to determine which part of their complicated structure was responsible for the amino acid incorporation. This was successfully achieved by using detergents (notably deoxycholate) to separate the ribonucleoprotein particles from the structural protein (reticulum) which was solubilised by the detergent. At short time intervals after injection of labelled amino acids, the deoxycholate insoluble fraction (ribosomes) showed very high labelling, and this levelled off after 5 to 10 minutes following the injection. The soluble fraction became labelled much more slowly, but eventually attained higher levels. It was concluded in consequence that the ribonucleoprotein particles are the initial site of amino acid incorporation.

There remains the problem of the intermediate steps between the free amino acid pool and the labelling of the protein in the ribonucleoprotein particles. The formation of peptide bonds is an endergonic reaction, and in consequence the first step involved the discovery of the mechanism by which the amino acids obtain the necessary energy to undertake this process. Early work by Siekevitz (1952) and by Peterson and Greenberg (1954) showed that incorporation of labelled amino acids by the proteins of the crude homogenate preparation was dependent on an energy generating system which could be provided by actively phosphorylating mitochondria or by glycolysis. It was shown (Zamecnik & Keller, 1954) that the energy produced was used to regenerate ATP, a finding supported by the original suggestion of Lipmann (1941) that the phosphate bond energy of ATP might be used for carboxyl activation of amino acids. Thus amino acid incorporation was dependent on ATP, an energy generating system and the cell sap which provided an essential factor in the process. This soluble fraction was later shown by Hoagland (1955; Hoagland et al., 1956) to catalyse an exchange of ATP with AMP which was much enhanced by the presence of amino acids and was not inhibited by AMP. The high energy of ATP is used to activate the amino acid mediated by the enzymes contained in the cell sap which have been named "amino acid activating enzymes". The activated complex so formed is an amino acid adenylate firmly attached to the enzyme. Attempts to isolate the acyl amino acid adenylate have proved difficult because when free they react

readily with one another or with any available amino groups to form random peptides (Hoagland *et al*., 1957). Nevertheless, their presence as intermediates has been shown by several authors (Karasec *et al*., 1958; Zamecnik *et al*., 1958) and Kingdon *et al*. (1958) have actually isolated a tryptophan adenylate from large amounts of tryptophan activating enzyme. The nature of the bond between the enzyme and activated amino acid is unknown.

The necessity for other factors in the incorporation of labelled amino acids into microsomal particles was indicated from other evidence. It has been found that amino acids can be incorporated into ribonucleoprotein in presence of ATP, an energy-generating system, a fraction prepared from the cell sap (pH 5 enzyme) (Hoagland *et al*., 1957) and GTP (Keller & Zamecnik, 1956). The necessity for GTP was unexplained at this stage and is still only partially understood, since it does not act as an energy source for amino acid activation in place of ATP(Hoagland *et al*.,1956). The occurrence of an intermediate step between amino acid activation and incorporation of the label into protein was indicated by the experiments of Hultin (1956) in which he demonstrated that addition of ^{12}C amino acids to a system which had already started to incorporate ^{14}C-labelled amino acids into protein did not immediately terminate the labelling process, and thus suggested that an intermediate had accumulated. The possibility that this intermediate was poly-

ribonucleotide in nature was demonstrated by Holley (1957) and it was then shown by Hoagland (Hoagland *et al.*, 1958) that the intermediate between the activated amino acid and the ribonucleoprotein site of protein synthesis was an amino acid-RNA complex. The RNA involved, soluble RNA (sRNA) or transfer RNA has quite different base ratios from microsomal RNA and contains unusual bases notably pseudouridylic acid (Dunn, 1959). During these investigations, it was possible to separate some of the components of the system, and to show that the requirement of the system for GTP was at the stage of transfer of the labelled amino acid from sRNA to the ribonucleoprotein particles.

The characteristics of the activating enzyme and sRNA system have been explored extensively in many laboratories. It has been demonstrated that the complex of activating enzyme and amino acid adenylate is formed without competition among amino acids for the activating enzymes (Hoagland *et al.*, 1956). This intermediate readily transfers its amino acid to sRNA without intervention of other enzymes (Berg, 1958; Schweet *et al.*, 1958a; Wong *et al.*, 1959) and also without competition for the sRNA. (Hoagland *et al.*, 1958). Since then, specific activating enzymes for several amino acids have been purified and it is highly probable that there is an individual enzyme for each amino acid (Schweet & Allen, 1958b; Davie *et al.*, 1956; DeMoss *et al.*, 1956).

It was shown by Hoagland (Hoagland *et al.*, 1958) in previous experiments with sRNA that there was no competition among amino acids for a site on the sRNA molecule and it has now been found

that there are specific RNA species for each amino acid although complete separation of each type of RNA is only now being undertaken and has partially resolved some species (Smith _et al._, 1959; Holley & Merrill, 1959; Berg & Ofengand, 1958; Zamecnik _et al._, 1960). Despite these differences in RNA types for each amino acid, all sRNA molecules appear to have a common end-group to which the amino acid is attached. In consequence, the differences in RNA structure for individual amino acids must reside in other parts of the RNA molecule. The end-group common to all sRNA molecules has been identified as a terminal adenylic acid with two cytidylic acid groups in the two adjacent positions of the RNA molecule (Hecht _et al._, 1958; 1959). Some evidence of the existence of this terminal sequence has been previously hinted at when it was observed that AMP could, under certain conditions, be specifically incorporated into sRNA and not into either nuclear or microsomal RNA (Heidelberger _et al._, 1956; Canellakis, 1957; Hecht _et al._, 1959). If the terminal three nucleotides (A-C-C) are removed, no incorporation into sRNA of activated amino acids can take place. The attachment of the amino acid to the terminal AMP residue has been shown by Zachau, Acs and Lipmann (1958) to be through the amino acid carboxyl group to the 2' or 3' hydroxyl group of the ribose portion of the AMP.

The sequence of events up to this point are well documente and provide a clear picture. Amino acids are converted by an energy-dependent enzyme-specific reaction to an amino acid

adenylate which remains complexed with the enzyme. Without intervention of further enzymes, the amino acid is transferred to the terminal adenylic acid of sRNA, to which it is united by an ester linkage. Individual transfer RNA molecules, which have a common functional end group, exist for each amino acid.

The reaction which transfers the amino acids from sRNA to the ribonucleoprotein particles is less well defined. This transfer may be brought about by enzymes in the pH 5 fraction, ATP, an energy-generating system and GTP (Grossi & Moldave, 1959; Hoagland et al., 1958). A more potent source of transferring enzyme was found by Takanami and Okamoto (1960) in the supernatant remaining after the pH 5 enzyme fraction has been precipitated from the cell sap. By fractionation of this material on DEAE cellulose, a partially purefied enzyme was separated which catalysed the transfer of sRNA-attached leucine to ribonucleoprotein, but which had now lost the ability to unite free leucine to sRNA under the conditions commonly used for activating amino acids. This transferring enzyme no longer required ATP or an energy-generating system, although it was still dependent on GTP. A similar system has been studied extensively by Rendi and Hultin (1960). This enzyme was isolated from the S-protein fractic of Sachs (1957). It is capable of catalysing the transfer of leucine from sRNA to microsomes, but is still dependent on ATP and a source of energy. More recently, a heat-labile non-dialysable factor (S-V) has been isolated from the soluble fraction

which promotes incorporation of amino acids from sRNA to microsomes (Fessenden & Moldave, 1961). The ability to transfer different amino acids increases proportionately during purification, thus suggesting that a single enzyme species is involved and not individual enzymes for each amino acid. Enzyme S-V fails to transfer amino acids from sRNA to purified ribonucleoprotein particles prepared by the method of Kirsch et al. (1960), a deoxycholate procedure, unless supplemented by a factor which appears to be protein in nature. This factor was derived from the deoxycholate-soluble fraction of microsomes; by itself, it has no transferring ability. There is some evidence that it is also present in the soluble fraction of the cell, but is rather unstable. The authors think that some evidence of glutathione stimulation of S-V was obtained, which is of some interest, since other transferring systems, namely the S-protein of Sachs and the more highly purified S_{50} protein of Hultin, are also sensitive to the glutathione content of the system.

There is reason to believe that the sRNA undergoes some metabolic changes at the time of donating amino acids to the microsomal template. Experiments with labelled sRNA have shown that there is an ATP-GTP-dependent transfer of nucleotides from the sRNA to microsomal RNA during the process (von der Decken & Hultin, 1958; 1959), a finding confirmed by Bosch et al. (1959) who were however unable to show a requirement for soluble enzyme or GTP. The combined use of labelled amino acids and labelled

RNA has enabled Hoagland and Comly (1960) to show that transfer RNA is alternately taken into the microsomes and released into the cell sap, a cycle which apparently takes place whether the sRNA is free or has an amino acid terminally attached. There is no evidence to suggest that a mononucleotide triphosphate forms an intermediate in the process and it seems more probable that polynucleotide material is transferred.

One factor which has cast doubt on the importance of this mechanism of protein biosynthesis has been an inability in many of the experimental systems to demonstrate net protein formation coincident with amino acid incorporation into the microsomal particles. It has been suggested by Webster (1959) that this is due to the lack in the system of an enzyme which will release the formed protein from the template. Using pea-seedlings, he has demonstrated the occurrence of an enzyme catalysing release of protein into the soluble part of the cytoplasm. Schweet _et al._ (1958a) have shown that ribonucleoprotein particles derived from reticulocytes can incorporate radioactive amino acids into a soluble protein in the presence of ATP, an energy-generating system and a fraction containing activating enzymes. The protein so formed was characterised as haemoglobin or a molecule very like it.

The scheme of Hoagland and Zamecnik is the best authenticated system for protein biosynthesis at present available. However, a considerable amount of evidence has accumulated to suggest that there are other methods of amino acid incor

poration into protein which do not appear to involve sRNA or even the conventional activating enzymes. These systems are more appropriately reviewed later, when we come to present our own data, and accordingly details of their mechanisms will be deferred until the general discussion at the end of this thesis.

The Influence of Diet on Liver Cell Structure in Relation to Protein Synthesis.

From the foregoing summary of the mechanism of protein synthesis, as presently understood, it is apparent that ribonucleic acid is an integral component of the biosynthetic pathway at several points. There is now a considerable body of evidence to demonstrate that the RNA content of the liver cell is determined by diet, and particularly by the protein content of the diet. When a rat receives a protein-free diet or is fasted, the amount of RNA in the liver declines sharply during the first one to two days by about 20%. This change in RNA content is accompanied by a loss of protein and phospholipid of about equal magnitude, which led Kosterlitz (1947) to postulate the presence of a type of "labile liver cytoplasm" consisting of these three components. However, Munro (1954) has criticised this concept on the grounds that the loss of RNA, protein and phospholipid differ in different fractions of the liver cell and not, as would be anticipated from a single entity, by parallel losses of all three. Wikramanayake, Heagy and Munro (1953) showed that the loss of protein occurred from all parts of the cell during protein deficiency, the

loss of phospholipid was from the microsomal and lighter fractions and the loss of RNA was confined to heavy microsomes. These data were confirmed by calculation of the data of Seifter, Muntwyler and Harkness (1950) for protein depleted liver cells.

This loss of RNA suggests that the protein level in the diet exerts a controlling influence over some aspects of RNA metabolism. It might at first sight be supposed that the loss of RNA from the liver during protein deficiency is due to a decline in the rate of RNA synthesis. However, there is evidence to show that this is not so (Campbell & Kosterlitz, 1948, 1952; Munro *et al*, 1953). When rats are fed on a diet deficient in protein for a few days and then receive ^{32}P by injection, the uptake of ^{32}P by the liver is considerably higher than the level observed in control animals fed on a diet containing adequate amounts of protein. It would thus appear that the diminished amount of RNA in the livers of the protein-depleted animals undergoes a more rapid turnover of a compensatory character, so that the *total* number of new RNA molecules made in a given time remains constant irrespective of protein depletion.

Why, then, does the amount of RNA in the liver become reduced, if synthesis continues undiminished in intensity during protein deficiency? The data obtained by Munro *et al*., (1953) and supplemented by the more extensive experiments of Clark, Naismith and Munro (1957) show that the detailed course of

protein depletion on RNA metabolism affords a clue to the reason. When rats are transferred from a normal diet to a diet deficient in protein, the amount of RNA in the liver falls very quickly, and from the second or third day onwards tends to level off. However, the compensatory increase in ^{32}P uptake by RNA, mentioned above, does not immediately take place for during the period that the RNA content of the liver is diminishing rapidly, uptake of ^{32}P and other precursors is greatly diminished. This low level of uptake of precursors can readily be explained if we assume that nucleotides, released by RNA breakdown, are flooding the precursor pools and diluting the incoming isotope in newly synthesised molecules of RNA. Thus, adenine nucleotides released by RNA breakdown would dilute ^{14}C-labelled adenine in the acid-soluble adenine in the pool, and so on. Clark et al., (1957) provided other evidence substantiating this view of RNA breakdown during the earliest stages of protein depletion by showing that alantoin excretion is raised during this period. (See Fig. 2)

Of considerable interest was the finding that these changes are rapidly reversed by feeding protein. The uptake of isotopes is increased several-fold (20-fold in the case of ^{14}C-glycine into guanine) by giving a meal of casein 3 hrs. beforehand, and the excretion of allantoin diminishes.

From these findings, we can conclude that part of the liver cell RNA becomes unstable when the supply of amino acids from the diet is interrupted and this RNA begins to break down.

Fig. 2.

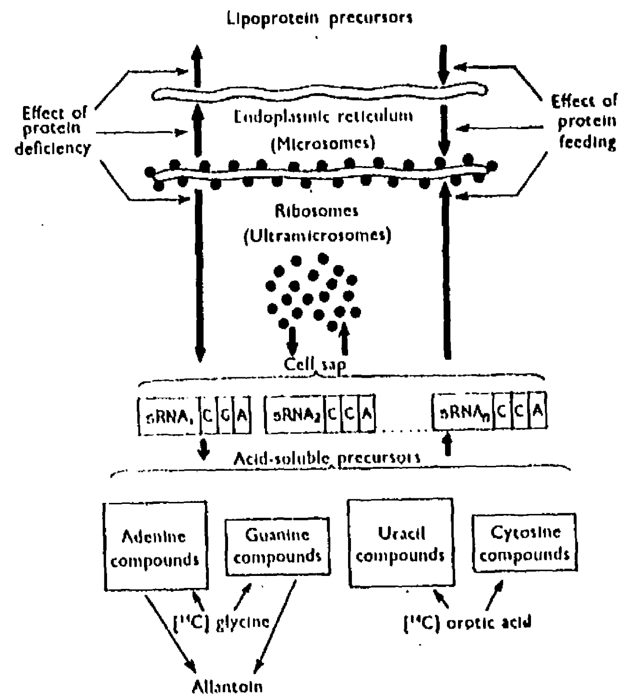


Fig. 2. Schematic representation of the influence of protein intake on ribonucleic-acid metabolism in liver-cell cytoplasm. sRNA, cell sap ribonucleic acid; C, cytidylic acid; A, adenylic acid; the subscript numbers (*1*, *2*, . . . *n*) denote separate species of sRNA corresponding to different amino acids.

The process of breakdown is sharply halted by feeding protein, presumably by stabilising the labile material. Where is this labile RNA to be found? The evidence cited above shows that the loss of RNA from the liver cell is confined to the microsome fraction, and presumably its restoration on re-feeding protein represents the restitution of this material to the microsomes. This is in good agreement with the electron microscopical evidence on the effects of diet on liver cell endoplasmic reticulum. Thus Fawcett (1955) and Bernhard and Rouiller (1956) have independently demonstrated that protein depletion causes loss of reticulum from the liver cell, and Fawcett has additionally shown that the feeding of a protein-rich meal (but not of a low protein meal) results in the formation of new reticulum visible within six hours of food being administered. This new reticulum was observed to be of the smooth type. Porter (1961) has concluded otherwise, considering that the smooth reticulum appears as a response to deposition of glycogen granules in the cytoplasm. This divergence of opinion requires further investigation. If Fawcett's conclusion is valid, the agranular membrane appears in response to the feeding of protein, and thus the ribosomal granules are not the focus for the reticulum formation but rather the granules adhere to the reticulum once it has been formed.

What form do the breakdown products take? Munro and Clark (1959a) observed that omission of a single essential amino acid from the diet caused quantitatively different effects on the

labelling of the purine and pyrimidine bases of liver RNA from common precursor pools. They concluded that dilution of isotope as the result of RNA breakdown probably affected cytidylic acid and adenylic acid more extensively than the other two bases, and they identified this with loss of terminal groups of sRNA, which are cytidylic and adenylic acids. This pointed to the possibility that breakdown of microsomal RNA releases sRNA into the cell sap, and that the feeding of protein reverses this process, more sRNA being transferred to microsomes.

Protein depletion affects the RNA of the nucleus. It has been demonstrated by Stenram (1953) that protein depletion causes enlargement of the nucleolus, including deposition of more RNA in that organelle (Stenram 1958b). When protein is provided in the diet the nucleolus becomes smaller. In view of what has been said above about the formation of the reticulum from the nuclear membrane, it would appear rational to conclude that the nucleolus is enlarged during protein deficiency because the nucleolar contents are not being used in membrane formation. When protein becomes available, the nucleolus diminishes in size because its contents are devoted to formation of the new membrane. This puts the nucleolar apparatus in the interesting position of possibly being the cellular element responsive to the supply of amino acids On the other hand, it will be remembered that by the electron microscope Fawcett noted outgrowth of new reticulum from pre-existing masses in the cytoplasm when protein had been fed six hours beforehand.

Thus the picture of the response to protein administration is by no means uncomplicated

Finally Clark & Munro (1959b) demonstrated that the malignant liver cell (hepatoma) which is deficient in endoplasmic reticulum found in normal cells, showed no response to dietary changes. The protein and RNA content did not change with dietary alterations and incorporation of precursors into RNA was not subject to dietary control. In addition, the nucleolus which is found to be large in these cells (Stenram, 1958a), does not become smaller when protein is fed. In normal tissue the endoplasmic reticulum is the sub-cellular component most affected by diet and thus, the measure of resistance to diet found in the tumour cells may be attributed to the absence of reticulum.

Plan of the Present Experiments.

From the discussion of results obtained in this laboratory, it is apparent that protein intake affects some fractions of the liver cell RNA, and this has been identified as the microsome Furthermore, it was concluded from the available evidence that, when protein is withdrawn from the diet, the microsomal RNA breaks down and releases sRNA into the cell sap. In consequence, the amount of sRNA in the cell sap might be expected to increase during the period of initial protein depletion, and when protein is fed again, to diminish. Thus, the starting point for the present experiments was an investigation of the effect of protein intake on the amount and amino acid carrying capacity of the liver cell sap RNA. Following this, we subdivided liver cell sap

by more prolonged centrifugation into a post-microsomal pellet and a final cell sap preparation. The effect of protein intake on each of these fractions was then examined and found to differ. The post-microsomal pellet had some interesting properties, and it was studied in more detail, these results being reported in the second section of this thesis.

SECTION I.

As explained in the introductory section to this thesis, the initial experiments were carried out to explore whether the cell sap of the liver cell changes in its RNA content in relation to the protein intake of the animal before death. The evidence already cited shows that the endoplasmic reticulum begins to disintegrate as soon as the absorption of amino acids from the gut ceases, and in consequence one looks for evidence of breakdown products in animals which have been fasting overnight. In the case of animals which have been depleted of protein by feeding a protein free diet for some days, this process has already occurred, so that fasting no longer induces this breakdown process. Consequently, the study of cell-sap RNA was undertaken in three dietary groups of rats : (a) Animals which had been protein-depleted by a short period on a protein-free diet; (b) Animals which had been receiving adequate amounts of protein, but were fasting overnight; and (c) Animals similar to those in the preceding group but fed protein a few hours before killing and thus were actively absorbing amino acids.

From each group of animals, livers were obtained and the cell-sap was prepared and studied for its content of RNA and for its ability to accept labelled amino acids. It soon became apparent that a sub-component of the cell-sap (post-microsomal pellet) was varying with dietary conditions and the experimental exploration of this fraction was continued.

EXPERIMENTAL METHODS.

Animals and diets. Male albino rats of about 3-4 months of age and weighing approximately 180 g. were selected from stock bred in the departmental animal house. They were housed individually in cages located in a room which was thermostatically controlled at 25°C. For five days prior to killing, they were fed on either a diet containing an adequate amount of protein, or on a protein free diet. The amounts of individual nutrients in those diets and the composition of the meals are shown in Tables 1 & 2. The diets were fed as two meals daily. At 9 a.m., all animals received a vitamin-mineral-roughage mixture together with some of the dietary carbohydrate and fat; at 5 p.m. they ate the remainder of the carbohydrate and fat together with any protein provided by the diet. After a few days on these regimens, the rats had learned to consume their meals promptly. Consequently, when they were ready for use on the morning of the sixth day of the experiment, they were in the fasting state, having consumed their final meal at 5 p.m. on the preceding evening. All of the animals on the protein-free diet were killed while still in the fasting state. Some of the animals on the protein containing diet however, were fed an additional meal of 2.5 g. casein (solubilised with 0.1 g. $NaHCO_3$) 2 hrs. before killing.

In this way, three nutritional groups of rats were studied :-

(a) Animals made protein-deficient by a short period on a protein free diet.

Table 1.

Composition of evening feeds given to rats.

Constituent	Adequate Protein Diet	Protein Free Diet
Margarine	0.42	0.42
Starch (Potato)	0.69	1.89
Glucose	0.69	1.89
Casein	2.40	-

4.2g were given daily to each rat.

Table 2.

Feeding Schedule.

Constituent	Group of rats			Time & Duration of Feeding
	(a) Protein Free	(b) Adequate Protein (Fasted)	(c) Adequate Protein (Fed Protein)	
	g.	g.	g.	
V.M.R.*	1.0	1.0	1.0	9 a.m.
Glucose	3.8	3.8	3.8	5 days
Protein Free Diet	4.2	-	-	5 p.m.
Protein containing Diet	-	4.2	4.2	5 days
Casein	-	-	2.5	8 a.m.
$NaHCO_3$	-	-	0.1	6th day only

* V.M.R. = Vitamin, mineral roughage mixture. (Munro & Naismith, 1953)

(b) Animals fasting 18 hrs. after an adequate intake of protein.

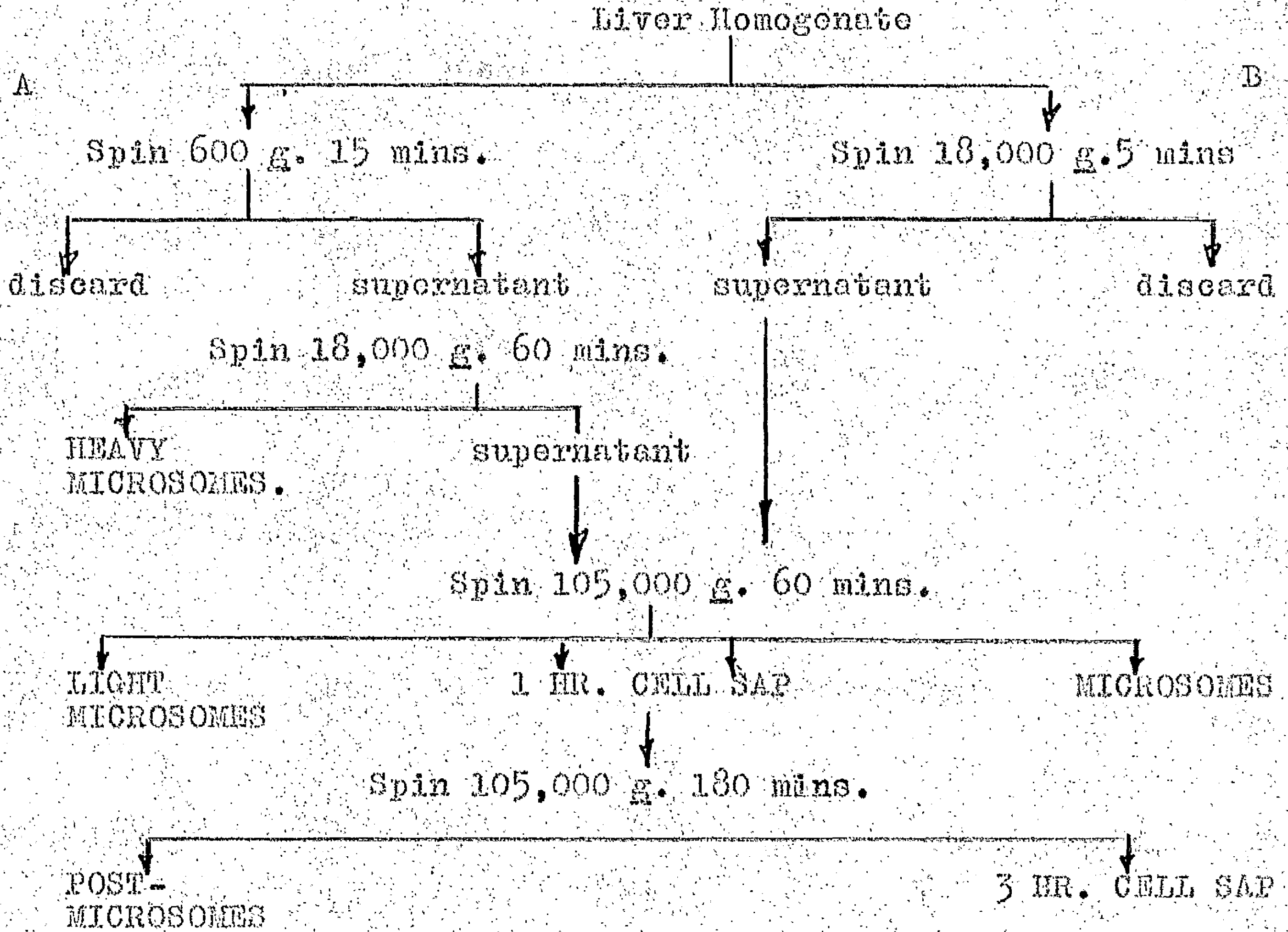
(c) Animals similar to those in the preceding group but actively absorbing amino acids from a meal of protein.

Removal of liver and preparation of cell fractions. The animals were killed by a blow on the head and the liver removed expeditiously and washed with ice-cold suspending medium. It was then blotted dry, weighed briefly and roughly minced with scissors in a chilled beaker. The pooled livers from three to four rats were then homogenised in 2.5 volumes of suspending medium in a cold Potter-Elvehjem (1936) homogenizer. The medium selected was that described by Rendi and Campbell (1959) and contained 0.01 M $MgCl_2.6H_2O$, 0.03 M $KHCO_3$, 0.025 M KCl, 0.02 M K phosphate buffer pH 7.8 (KH_2PO_4 : K_2HPO_4, 1 : 9), and 0.35 M sucrose.

The scheme for fractionation of the homogenate was based on that used by Douglas and Munro (1959) for separation of pancreatic homogenates in a slightly different medium, and was an extension by them of the scheme originally proposed by Schneider (1949) for fractionation of liver cell homogenates from 0.25 M sucrose. The cell fractions were prepared from the livers of our animals by differential centrifugation at $0^{o}C$ as shown in the flowsheet (Fig. 3). In the first experiments, the separation of cell debris, mitochondria and microsomes was carried out using lustroid centrifuge tubes in the super-speed attachment of an MSE refrigerated centrifuge. In the later experiments, this separation was carried out in the Spinco

Fig. 3.

Scheme of liver cell fractionation.



preparative ultracentrifuge (Model L) using the rotor 30. The Spinco rotor 40 was used for all separations at higher speeds. In order to reduce contamination of the fractions, at each stage in the fractionation process only the middle portion of the supernatant fluid was retained. The upper turbid layer (fat) and the portion of the supernatant fluid directly above the pellet were removed by Pasteur pipette and discarded. The pellet was then surface-washed with a small volume of the suspending medium, the sides of the tube dried free of medium with filter-paper and the pellet was finally suspended in a small volume of medium and stored in the cold for future use. The identity and homogeneity of the individual particulate fractions was checked by electron microscopy. This and other evidence regarding the nature of the particles will be considered later in the second section of results.

In the studies on pH 5 enzymes, the 1 hour cell-sap was not further separated into post-microsomal pellet and final cell-sap.

The centrifugation schedule was shortened in many of the later experiments, as only the three hour cell-sap and post-microsomal pellet were of interest (Fig. 3 B). In such cases the separation of light and heavy microsomes was combined into a single centrifugation in the Spinco ultracentrifuge.

<u>Chemical studies on the cell fractions.</u> (a) RNA estimations. The measurement of RNA was done by a modification of the method of Littlefield <u>et al.</u> (1955). Portions of the fractions to be

analysed were precipitated by addition of 5 ml. of 0.4 N perchloric acid (PCA) at 0°C. After separation at 0°, the precipitated residue was extracted twice for 20 mins. at 70°C with 5 ml. 0.4 N PCA. During extraction the tubes were agitated at approximately 5 min. intervals. The amount of RNA was determined by the difference in absorption at 260 and 290 mu of the combined extracts. Yeast RNA prepared by the method of Schucher and Hokin (1954) was used as a standard. Ideally, the RNA standard should have been the RNA from each cell fraction of liver, but this imposed considerable extra work, and it was felt that, since the results are all comparable within an experimental series, the yeast standard was adequate. Table 3 shows that irrespective of the source of the RNA extracted from different fractions of the liver cell, it does not vary markedly in its UV absorption characteristics. The variation in base ratios does not produce variations in the 260/290 absorption ratio. Thus the relationship of the ultraviolet absorption to the RNA content using a common standard was deemed permissible.

(b) Estimation of protein. This was carried out by the method of Lowry et al (1951). The reagents were as follows :

A. 2% Na_2CO_3 in 0.1 N NaOH.

B. 0.5% $CuSO_4$ in 1% Na or K tartrate.

C. Alkaline copper solution. (Mix 50 ml. solution A with 1ml. solution B.)

D. Diluted Folin-Ciocalteu reagent

Table 3.

U.V. absorption characteristics of liver RNA extracted with Phenol from Different Cell Fractions and degraded in either 0.3N alkali at 37°C or 0.4N perchloric acid at 70°C.

Fraction	Phenol Extracted RNA			
	Hydrolysed for 18 hrs. with 0.3N KOH		Extracted twice with 0.4N PCA at 70°	
	$\frac{260}{280}$	$\frac{260}{290}$	$\frac{260}{280}$	$\frac{260}{290}$
3 hr. Cell Sap	1.34	2.27	1.39	2.27
Post-Microsomes	1.38	2.30	1.39	2.30
Light Microsomes	1.34	2.18	1.35	2.15
Heavy Microsomes	1.38	2.25	1.35	2.24

(The concentrated Folin-Ciocalteu reagent was titrated with N NaOH using phenolphthalein as indicator; on the basis of this titration, the Folin-Ciocalteu reagent was diluted to make it 1 N).

Up to 1 ml. of the protein containing solution was added to 5 ml. of reagent C and after 10 mins. had elapsed, reagent D was added rapidly and with instantaneous mixing; the speed of mixing was critical for full colour development, a few seconds delay giving a reduced colour formation. The blue colour was found to be completely developed after standing for 20 mins. and to remain constant for at least a further 30 mins. The optical density was read 30 mins. after the addition of reagent D, at 750 mu in a Unicam SP 600. The protein content was obtained from a calibration curve prepared in this laboratory using bovine serum albumin as standard. The method accurately estimates a range of from 40-400 ug. protein.

Uptake of labelled amino acids by cell fractions.

The pH of each fraction was adjusted to 5 in accordance with the procedure commonly used for precipitating soluble RNA and activating enzymes from the cell sap, to examine amino acid activation. The pH 5 fraction was then incubated with an energy source and a labelled amino acid and the amount of radioactive uptake assayed.

(a) Preparation of pH 5 fraction. The pH of the 1 hr. and 3 hr. cell sap was adjusted to pH 5.1-5.2 by the dropwise addition of N acetic acid at 0° with constant stirring; the

procedure was controlled with a glass electrode. The precipitate was spun down at 2,000 r.p.m. for 10 mins. in a refrigerated centrifuge. The supernatant fluid was decanted and the sides of the tube dried out with filter paper. The precipitate was suspended in medium and stored frozen at -10°C; in this form the pH 5 fraction from 1 hr. and 3 hr. cell sap retained its activity for several weeks. Before acid precipitation of the post-microsomal pellet, it was suspended in approximately 50 ml. of phosphate buffer (Rendi and Campbell, 1959) and treated as above. After one to two days storage the post-microsomal pellet lost most of its activity.

(b) The incubation conditions of Rendi and Campbell (1959) were used. The pH 5 fractions were incubated in air for 10 mins. at 37°C with constant shaking in 1 ml. volume with $MgCl_2.6H_2O$ 10 umoles, $KHCO_3$ 30 umoles, KCl 25 umoles, K phosphate 20 umoles pH 7.8, sucrose 350 umoles, DL-leucine-1-^{14}C 1 uC (Radiochemical Centre, Amersham) and ATP 10 umoles (Sigma, disodium salt). Initially, samples of pH 5 fractions from different animals were adjusted to give either equal RNA content or equal protein content. In later experiments in this series, only RNA content was made similar.

(c) Preparation of samples for counting. The reaction was terminated at the end of incubation by addition of 5 ml. ice cold 0.4 N PCA. After 30 mins., the precipitate was washed three times with 0.2 N PCA at 0°C, twice with 5 ml. ethanol:ether:chloroform 2:2:1 at room temperature, and once with ether, also at room temperature (Rendi and Campbell, 1959). The lipid-free dry

precipitate was plated directly (See below) as a measure of the total leucine incorporated by the pellet. To obtain the amount of leucine incorporated into protein, parallel samples were incubated and washed as above. The lipid-free dry powder was extracted twice with hot PCA (0.4 N) at 70°C for 20 mins.. The protein residue remaining after hot acid extraction was dissolved in N NaOH and plated. The UV absorption of the PCA extract was measured at 260 and 290 mu to estimate the amount of RNA present at the end of incubation.

(d) Determination of radioactivity. This was carried out by the lens paper method of Garrow and Piper (1955). The dried protein powder was dissolved in 0.3 ml. N NaOH by heating in a boiling water bath for 10 mins.. This protein containing solution was put onto lens paper discs on planchettes using Pasteur pipettes. The procedure was repeated with a further 0.3 ml. N NaOH, the washing being added to the lens paper disc. In the initial experiments the lens paper discs were supported on polythene planchettes, 3.1 cm^2. in area, and allowed to dry in air for a minimum of 18 hrs. before counting with an end window Geiger Muller tube. In the later experiments, the samples were plated on lens paper discs on stainless steel planchettes, 5.15 cm^2. in area, and dried by heating intensely under infra red lamps. These samples were counted with a gas-flow windowless Nuclear-Chicago automatic counter.

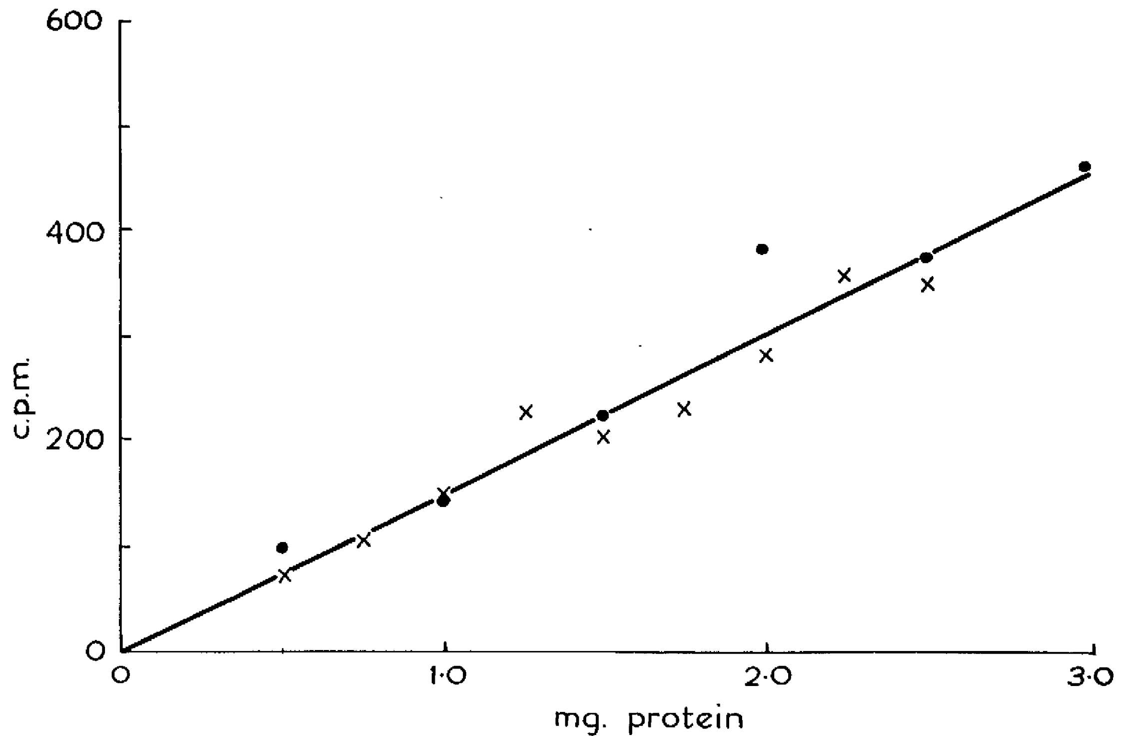
The results were calculated as counts/min./incubation tube or counts/min./mg. protein or RNA.

The following experiment was carried out to check that the self absorption of the sodium hydroxide was constant when varying amounts of protein were plated. Samples of the same radioactive protein of varying known weights were dissolved as described above in N NaOH. 0.6 ml. samples were plated using the lens paper technique also described above and counted using the Nuclear-Chicago gas-flow counter. The radioactivity measured is shown as a function of protein content in Fig. 4. An approximately linear relationship is shown up to 3 mgs. Above this value the counting efficiency falls off and completely plateaus above 20 mg. protein. Only the lower portion of the graph is shown since in the vast majority of the work samples containing 1 mg. protein were used, and in all cases, the protein content fell on the portion of the graph shown.

Phenol preparation of RNA. The method of Kirby (1956), as modified by Hoagland *et al.* (1958), was used to prepare pure samples of RNA from the fractions shown in Fig.3. An equal volume of 90% (w/v) phenol was slowly added, with continuous agitation, to the suspension of RNA-containing material in phosphate buffer, pH 7.8 and shaken in a mechanical shaker for 1 hr. at $0^{o}C$, a temperature at which Bosch *et al.* (1960) found the recovery of RNA to be superior; in addition, this temperature reduces the risk of degradation by ribonuclease. The aqueous layer was re-isolated by centrifugation at 15,000 *g* at 0° for 10 mins. and removed by Pasteur pipette. The phenol layer was washed twice with distilled water. Residual phenol was removed

Fig. 4.

<u>To test the technique used for counting ^{14}C-protein for uniform self absorption.</u>



Different known weights of the same radioactive protein were plated as described in the text in the same volume of N NaOH (0.6 ml.) and counted on the Nuclear-Chicago windowless counter.

from the combined aqueous layer by extracting three times with ether. The appropriate volume of 20% potassium acetate at pH 5 was added to the RNA-containing solution to adjust the solution to 2% with respect to acetate. Absolute ethanol, 2.5 volumes at $-10^{\circ}C$ was then added to bring about precipitation. After standing for 18 hrs., the RNA was sedimented at 2,000 r.p.m. for 10 mins., redissolved in water and dialysed against running water for four hours at $0^{\circ}C$. (Visking dialysis tubing, ½" diameter was soaked in distilled water overnight before use). In some experiments, the RNA was precipitated twice with ethanol before dialysis.

Fractionation of RNA on Ecteola columns. Ectoola (Serva, Heidelberg, capacity 0.41 m.eq./g.) was used. This ion-exchange resin was prepared for use by washing alternately with N NaOH and N HCl, the procedure being carried out several times. Between each wash with acid or alkali, the resin was wased with distilled water until the pH was neutral (Goldthwaite, 1959a). The glass column was 1 cm. internal diameter, and the height of Ectoola in it was adjusted to give a column length of 3.5 cms.. This was adequate to separate some 300-800ug. of RNA (Goldthwaite,1959a). The gradient system consisted of a constant-volume mixing chamber containing 250 ml. of 0.01 M potassium phosphate pH 6.85, into which flowed 250 ml. of M NaCl buffered with 0.01 M potassium phosphate 6.85. Approximately 5 ml. fractions were collected from the column, but owing to the uncertainties of the fraction-collector, the

volume in each tube had to be measured. The optical density of each tube at 260 mu was determined. After the 250 ml. of NaCl had been collected from the column, 50 ml. of N NaCl was passed through the column. The third eluant, a gradient of NH_4OH prepared by running 200ml. of N NH_4OH into 250 ml. of N NaCl was then run through the column. Finally, 50 ml. of N NaOH was run through. The rate of flow throughout was adjusted to approximately 1 ml. per min., using positive pressure applied to the mixing vessel at the top of the column.

Quantitative measurement of nucleotide composition of RNA.

The nucleotide composition of RNA from the cell sap and post-microsomal pellet was examined in relation to diet. Specimens of RNA prepared by the phenol procedure were hydrolised in 0.3 N KOH for 18 hrs. at 37^{0}C and the nucleotides were separated by the paper chromatographic procedure of Lipshitz and Chargaff (1960), the amount of each nucleotide being measured by the ultraviolet absorption of the material eluted from the paper. This procedure allows separation quantitatively of not only the four main nucleotides but also of minor components of RNA. In the case of cell sap RNA, up to 10 separate nucleotides were identified and measured. We are indebted to Mr.G.C.Barr for carrying out this part of the study, using his modification of the procedure of Lipshitz and Chargaff (1960) to obtain quantitative measurement of the nucleotides in the presence of ultraviolet absorption by the paper, by means of a two-wave length procedure.

RESULTS.

Studies on cell-sap prepared by centrifuging at 105,000 g for 1 hr.

In the first series of experiments, the amount of RNA recovered from the cell sap after 1 hr. centrifugation was related to the diet previously fed to the animal (Table 4.) The animals fed the protein containing diet but fasted for 18 hrs. before killing showed the largest amount of cell-sap RNA this being about 20% more than that observed in the cell-sap of the protein-depleted group. A more remarkable observation is however, that feeding protein to animals of this series only two hours before killing reduced the quantity of RNA almost to the level obtained in the protein depleted series. This observation coincides with the hypothesis which initiated the work carried out namely that fasting of animals previously receiving an adequate protein intake results in breakdown of liver cell RNA, with consequent appearance of the fragments in the cell sap. Feeding of protein, by terminating breakdown or initiating reformation of RNA in the cell particle fractions, should thus result in a fall in cell sap RNA, and this in fact was observed. The original hypothesis (Munro and Clark, 1960) implied that the RNA translocated between cell sap and particles as a result of these dietary changes was in fact sRNA (Fig. 2.) Consequently, the capacity of the RNA in the cell sap to take up labelled amino acids should not alter qualitatively with diet, but only in its amount.

This point was investigated by following uptake of labelled

Table 4.

Influence of diet on the RNA content of 1 hr. Cell Sap.

(Each value is a mean of at least 2 animals.)

Dietary State			
Protein Free	Adequate Protein (Fasted)	Adequate Protein (Protein Fed)	
mg. RNA / 100g Initial Body Weight.			
1.62	2.47	1.77	
3.20	3.50	2.90	
2.05	3.26	2.75	
3.08	3.71	4.05	
2.09	2.13	1.55	
2.41	3.01	2.62	Mean

Table 5.

Incorporation of ^{14}C-DL-leucine by pH 5 enzyme prepared from Livers of Three individual Rats on Stock Diets.

Three adult male rats were taken at random from stock, and pH 5 enzyme was prepared from the livers individually. The incubation system contained 10 uM ATP, 1 uC ^{14}C-DL-leucine the pH 5 fraction (with RNA equalised in all tubes) and phosphate-buffered medium, pH 7.8 (Rendi & Campbell, 1959) to give a total volume of 1 ml. per tube. Control tubes were carried out with medium in place of ATP. Incubation was continued for 10 mins., aerobically at 37° with constant shaking. All samples were counted to 1000 cts. using an end-window counter. For estimation of RNA at the end of incubation, duplicate tubes were incubated and, after washing with cold perchloric acid and lipid solvents, were extracted with hot perchloric acid and the RNA content determined by UV measurements; the protein residue left after extraction was then counted to give the amount of incorporation of leucine into protein.

The data are expressed as cts./min./mg. RNA or protein.

Table 5.

Rat No.	pH 5 enzyme added		Incubated in presence of ATP				Incubated in the absence of ATP	
	RNA per tube	Protein per tube	Cts./min./flask	Cts./min./mg. protein added	Cts./min./mg. RNA added	Cts./min. mg./RNA isolated	Cts./min./flask	Cts./min./mg. RNA
	mg.	mg.	cts.	cts.	cts.	cts.	cts.	cts.
1.	0.265	4.5	123 (20)	27	470	460	5 (6)	20
2.	0.265	3.0	117 (10)	39	440	430	6 (6)	30
3.	0.267	4.6	123 (10)	27	460	490	7 (7)	30

The figures in brackets represent radioactivity left after extraction with perchloric acid. The RNA isolated at the end of incubation was 104%, 104% and 95% respectively of the amount added.

leucine under the conditions prescribed by Rendi and Campbell, (1959) for study of attachment of labelled amino acids to sRNA. In order to verify that the conditions under which our experiments were carried out were satisfactory a pilot study was made in which pH 5 fractions from 1 hr. cell sap preparations from three rats taken from stock diet sources were prepared and incubated with ^{14}C-leucine and ATP (Table 5). An adequate level of labelling of the cold PCA precipitable material was obtained with all three preparations and essentially all of this was extractable with hot PCA. In the absence of ATP, labelling was minimum. It can be concluded that under the conditions of our experiment the uptake of labelled amino acid is confined to acid-extractable material consistent with RNA, and is ATP-dependent. The uptakes by the pH 5 preparations made from these three animals show a remarkable degree of uniformity, whether calculated as counts/mg. RNA added to each tube, or as counts/mg. RNA recovered at the end of incubation.

Table 6 shows a series of experiments in which samples of cell sap from animals receiving different diets were incubated under the above conditions. The picture obtained is essentially the reverse of that obtained in the quantitative studies - the animals fasted after receiving a diet containing protein show a distinctly lower uptake of ^{14}C-leucine per mg. RNA initially present in the incubation mixture. It would

Table 6.

Influence of Diet on the Uptake of ^{14}C-DL-leucine by the pH 5 Enzyme Fraction of the Liver Cell Sap.

(First series of experiments - data expressed on the basis of the initial RNA content of the tube.)

The livers of 4 rats were pooled in each experiment. The pH 5 enzyme prepared from the dieted animals (sufficient to provide an equal amount of RNA in each tube) was incubated as shown in Table 5.

Dietary group of rats			
Protein Free	Adequate Protein (Fasted)	Adequate Protein (Protein Fed)	
Total Counts/minute/mg. RNA added			
490	300	320	
530	350	490	
560	390	560	
290	350	520	
290	260	330	
720	640	730	
480	380	490	Mean

have been desirable to record these data also in relation to the amount of RNA recovered at the end of incubation, since this is essentially what is counted, and not the amount initially present. Thesecexperiments were carried out at a time when we were having difficulty in freeing the RNA at the end of incubation from the large excess of ATP used as an energy source. However, simply by washing the precipitated material three times with cold acid instead of twice complete removal of contaminating ATP was achieved. The success of this washing procedure is demonstrated in Table 7, where tubes incubated with ATP or without ATP show identical average recoveries of RNA.

Using this improved technique for RNA recovery, the experiments with ^{14}C-leucine uptake by the pH 5 enzyme were repeated (Table 8) and demonstrated once more that diet influenced the incorporation of the label in relation to the amount of RNA in the sample. In this instance, the data are calculated as counts/mg. RNA recovered at the end of incubation, thus validating the results given in Table 6, where the basis of computation is the activity per mg. RNA initially present.

In view of the similarity of the pictures obtained in Tables 6 & 8 we have thought it justifiable to pool the data for radioactive uptake,,and the overall findings are recorded in Table 9, where it will be seen that the higher recoveries of RNA in the cell sap of animals fasting after the adequate diet is counterbalanced by an equivalent reduction in the ability of this RNA

Table 7.

Recovery of RNA from pH 5 enzyme preparations following incubation with and without ATP.

pH 5 enzyme fractions from the livers of dieted animals were incubated with or without ATP, under the conditions given in Table 5.

Dietary group from which pH 5 enzyme was prepared.	Initial RNA added to incubation	Incubated with ATP		Incubated without ATP	
		Final RNA content	Recovery	Final RNA content	Recovery
	ug.	ug.	%	ug.	%
Protein Free Diet	360	348	97	290	81
	360	329	91	284	80
Adequate Protein (fasting)	356	308	87	378	106
	364	332	91	330	91
Adequate Protein (protein fed)	356	278	78	296	83
	316	290	92	290	92
Mean Recovery			89		89

Table 8.

Incorporation of ^{14}C-DL-leucine by pH 5 Fraction of Rat Liver Cell-sap after various Diets.

(Second series of experiments - data expressed on basis of final RNA content of tubes.)

Conditions of incubation are as in Table 6.

Dietary Group of Rats			
Protein Free	Adequate Protein (Fasting)	Adequate Protein (Protein Fed)	
Counts/Minute/mg. RNA recovered after incubation			
277	199	172	
318	188	203	
413	241	377	
281	240	336	
325	217	272	Mean

Control tubes incubated in absence of ATP showed negligible incorporation.

to incorporate labelled amino acids. If the amount of RNA is multiplied by the radioactivity (last column of Table 9), we see that the several dietary groups now become equal, which implies that the total amount of biologically active RNA in the 1 hr. cell sap is constant, but that diet varies the total quantity by adding or subtracting inert RNA.

Thus the theory that RNA enters the cell sap when there is breakdown of RNA in other parts of the cell, usually the endoplasmic reticulum, and gives rise to sRNA, is invalid. The extra RNA obtained under such conditions contributes nothing to the ability of the cell sap to accept labelled amino acids, but merely dilutes it, so that the specific activity falls. It can thus be deduced that the additional RNA observed under these circumstances represents breakdown fragments other than sRNA.

Goldthwaite (1959a) separated the cell sap into a further fraction (post-microsomal pellet) and a final cell sap by spinning the 1 hr. supernatant fraction for an additional 3 hrs. at 105,000 g. This centrifugation procedure provided a simple method subdividing the 1 hr. cell sap and a more than reasonable chance of separating the RNA types since Hoagland et al. (1958) had already observed that further centrifugation of the cell supernatant obtained after spinning for 1 hr. at 105,000 g caused sedimentation of 50% of the RNA without any loss of the total soluble RNA activity in the pH 5 fractions. Therefore we divided the 1 hr. cell sap into post-microsomal pellet and

Table 9.

Effect of Protein Intake on the RNA of Rat Liver Cell Sap Prepared by Centrifuging for One Hour at 105,000g.

(Mean data from 7 experiments.)

Dietary Group	Amount of RNA/ 100g. Body Weight	Uptake of ^{14}C-leucine by pH 5 enzyme	Total Uptake per 100g. body weight
	mg.	c.p.m./mg.RNA	
Protein Depleted	2.41	403	975
Adequate Protein (Fasting)	3.01	299	901
Adequate protein Fed Protein 2 hrs.	2.62	381	999

The total activity was obtained by multiplying the specific activity by the amount of RNA in the fraction per 100mg. body weight.

The changes in the specific activity with diet are statistically significant at the 5% level by analysis of variance.

supernatant (3 hr. cell sap) to determine in which fraction the RNA was being influenced by diet.

Studies on cell sap and post-microsomal pellets prepared by centrifuging for 3 hrs. at 105,000 g.

3 hr. cell sap and post-microsomal pellet were prepared from animals receiving the diets as before. Table 10 shows the total amounts of RNA recovered in each fraction per unit of standard body weight. The 3 hr. cell sap is not significantly affected by the diet fed. The amount of RNA is essentially uniform throughout. On the other hand, the RNA content of the post-microsomal pellet is greatest in animals fasting after an adequate protein intake, being almost twice the amount observed with the protein depleted rats, and this quantity tends to fall in the animals fed protein 2 hrs. before sacrifice. These changes are more conclusively demonstrated by expressing the amounts of RNA in the pellet as a ratio of the quantities recovered in the 3 hr. cell sap. The pellet RNA represents only 0.28 in the protein depleted group, rises to 0.41 in the group fasting after a diet adequate in protein and falls again to 0.33 in the series fed protein just before death. These differences are significant statistically.

The effect of dietary condition on the uptake of radio-active amino acids by the cell sap after 3 hrs. centrifugation and by the post-microsomal pellet was examined independently. Before incubation, all fractions were submitted to acid precipitation under the conditions dictated for preparations

Table 10.

Effect of protein Intake on the amount of RNA in Different Fractions of Liver Cell Sap after Centrifuging for a further 3 hours at 105,000g.

(Mean data from three experiments.)

Dietary Group	Amount of liver RNA/100g. body wt.		
	In cell sap	In postmicrosomal pellet	$\frac{\text{Postmicrosomal pellet RNA}}{\text{Cell Sap RNA}}$
	mg.	mg.	
Protein Depleted	3.19	0.88	0.28
Adequate Protein (Fasted)	3.17	1.31	0.41
Adequate Protein (Fed)	3.65	1.23	0.33

The ratio of postmicrosomal pellet RNA to cell sap RNA is significantly affected by diet. (P 0.01 by analysis of variance.)

Table 11.

The Amount of RNA found in the pH 5 precipitable Fraction of 1 hour and 3 hour Cell Sap and Post-microsomal Pellet.

The RNA was measured in each fraction before and after precipitation at pH 5 and the recovery in the pH 5 precipitate was calculated as a percentage of the initial content.

The results are the mean of two experiments.

Dietary Group	% of original RNA from each cell fraction found in pH 5 precipitate		
	1 hr. Cell Sap	3 hr. Cell Sap	Post-microsomes
Protein Free	67	65	107
Adequate Protein (Fasted)	76	76	98
Adequate Protein (Fed Protein)	74	87	108

of the pH 5 enzyme from the 1 hour cell sap and so a preliminary study on the recovery of RNA in the pH 5 precipitable material was carried out. Table 11 shows that precipitation at pH 5 resulted in total transfer of the RNA of the pellet fraction to the pH 5 preparation without loss. However, as noted by Hoagland _et al._ (1958) for 1 hr. cell sap, the precipitation of RNA is incomplete, so that only about 70% of the total RNA of the three hour cell sap is retained in the pH 5 fraction. This means that, when, as is customary, pH 5 enzyme is isolated from cell sap prepared at 105,000 g. for 1 hour, the product will inevitably concentrate the post-microsomal material which is completely precipitated under these conditions of pH and will thus result in a preparation which does not really represent the distribution of RNA between post-microsomes and sRNA in the 1 hr. cell sap.

Using the pH 5 fractions made from the post-microsomes and from the 3 hr. cell sap, we have studied the uptake of ^{14}C-leucine under the conditions appropriate to the labelling of sRNA. Table 12 shows the results. The uptake of label into the RNA of the 3 hr. cell sap preparation was high and essentially unchanged by dietary conditions. The amino acid incorporated would appear to be attached to RNA, sRNA, since only 4% of the radioactivity is in a hot acid-stable form. The post-microsomal pellet incorporation was about 20% of that in the 3 hr. cell sap and was signif-

icantly affected by dietary conditions. The post-microsomal pellet from the adequate protein dieted animals, whether fasting or in the post absorptive state, had an inferior amino acid incorporating ability compared with that of the protein deficient preparation. This observation is valid whether total or hot acid stable radioactivity is considered.

A further study was made on post-microsomal pellets twice resuspended in phosphate buffer and reseparated at 105,000 g. for 180 mins., in order to test the effect of washing the pellet completely free from any possible contaminants from the cell sap. Table 13 shows that the amounts of RNA recovered and the uptake of ^{14}C-leucine by the pellet prepared in this way still show the influence of the preceding diet. The reduction in activity may be due to the more prolonged preparation schedule since we have found that even in the cold the ability of the pellet to incorporate amino acids declines rapidly.

When the overall picture of the amount of RNA and radioactivity is considered (Table 12) it is seen that the reduction in radioactive uptake in the group receiving the adequate diet and fasted is offset by the increase in amount, suggesting as in the earlier series of experiments that the extra RNA is contributing nothing to the total activity in this fraction, but is causing dilution of the RNA already present by adding biologically inert material. Thus, we reach the point at which it would appear that the products of RNA breakdown in the particulate fractions of the liver

Table 12.

The Effect of Protein Intake on the Uptake of ^{14}C-DL-leucine by the pH 5 Fraction from the 3 Hour Cell Sap and from the Post-microsomal Pellet of Rat Liver.

3 hr. cell sap and post-microsomal pellet containing equal amounts of RNA were incubated separately for 10 mins. as described in Table 5. The specific activity was computed from the amount of RNA remaining at the end of incubation. Figures in parenthesis are the amounts of activity remaining after extraction with hot perchloric acid, expressed as cts./min./mg RNA extracted. The total activity of the fraction was obtained by multiplying the specific activity by the amount of RNA in the fraction per 100g initial body weight. (See Table 10). Each entry represents the mean result obtained in three experiments, in each of which the livers of 3 or 4 rats on each diet were pooled. Both fractions had negligible activity in absence of ATP.

Table 12.

Dietary group	RNA/100g body weight		Specific Activity		Total Activity	
	mg.		Cts./min./mg. RNA		Cts./min./total fraction	
	Cell Sap	Post-microsomes	Cell Sap	Post-microsomes	Cell Sap	Post-microsomes
Protein Depleted	3.19	0.88	4580 (143)	970 (110)	14600 (455)	850 (97)
Adequate Protein (Fasting)	3.17	1.31	4500 (165)	640 (88)	14300 (520)	840 (73)
Adequate Protein (Fed Protein)	3.65	1.23	4040 (124)	670 (118)	14800 (450)	830 (86)

Diet has a significant effect on the specific activity of the post-microsomal fraction (P 0.01 by analysis of variance). There are no significant effects of diet on the total activity.

Table 13.

The Effect of Washing on the Incorporation of Amino Acids by the Post-microsomal Pellet.

Conditions were as described in Table 12.

Dietary Group	mg. RNA/100 g. Body Wt.		Cts./min./mg. RNA		Pellet RNA / 3 hr. Cell Sap RNA	
No. of Washes	1	3	1	3	1	3
Protein Free (Fasted)	0.88	0.39	970	725	21	14
Adequate Protein (Fasted)	1.31	1.35	640	345	15	8
Adequate Protein (Fed Protein)	1.23	1.14	670	306	17	7

cell, resulting from withdrawal of protein intake, are appearing in the post-microsomal fraction of the cell.

In order to demonstrate that these differences in radioactivity were in fact due to changes in the nature of the RNA in the post-microsomal pellet we attempted to extract the RNA from the cell sap and from the pellet and to study its various properties relating these to diet.

Properties of RNA from different cell fractions.

Hoagland *et al.* (1958) have shown that pH 5 enzymes will catalyse amino acid incorporation into soluble RNA which fortuitously is also contained in this fraction . Addition to this system of extra sRNA purified by phenol extraction increases the level of amino acid incorporation in proportinn to the RNA added. This mechanism provides a means of testing the amino acid incorporating ability of different RNA preparations.

It is possible that variation in response to diet of the amino acid incorporating activity of the post-microsomal pellet may be due to changes in the essential qualities of the RNA. If this is so, the amino acid incorporating activity of a standard pH 5 enzyme preparation will be augmented to different degrees by the addition of RNA purified from different post-microsomal preparations. Therefore, RNA extracted with phenol from the 3 hour cell sap and post-microsomal preparation from the livers of dieted animals was incubated with a standard pH 5 enzyme preparation to test the combined

ability of these fractions to incorporate amino acids (Table 14). The uptake of ^{14}C-leucine is increased to the same extent (about 40%) when equivalent amounts of RNA, phenol extracted from the 3 hr. cell sap of rats receiving different diets, were added to the enzyme preparation. Addition of RNA prepared from the post-microsomal pellet of the animals from the same dietary groups failed in all instances to stimulate, and rather depressed the uptake of labelled leucine by the pH 5 enzyme. Thus, no further studies on the heterogeneity of post-microsomal RNA could be made in this way since the pellet RNA appeared to have no amino acid accepting ability such as sRNA possesses.

Another attempt to identify some soluble RNA in the post-microsomal RNA or to detect some biological activity of this RNA in the system was made. The pH 5 fractions from 1 hr. cell sap, 3 hr. cell sap and post-microsomal pellet obtained from stock animals were incubated with ATP and ^{14}C-leucine. Examination of the radioactivity levels in relation to RNA content showed the 3 hr. cell sap (543 c.p.m./100 ug. initial RNA) to be more active than the 1 hr. cell sap (422 c.p.m./ 100 ug. initial RNA) and the mixture of 3 hr. cell sap and post-microsomes (295 c.p.m./100 ug. initial RNA). RNA, phenol extracted from the 3 hr. cell sap, was universally utilised by these enzyme systems (Fig. 5) to accept labelled leucine the activity being approximately doubled in each case. Among the enzyme systems tested only the 3 hr. cell sap showed any

Table 14.

The Biological Activity of the Purefied RNA from Post-microsomes and 3 Hour Cell Sap.

A standard pH 5 Enzyme Preparation (100 ug. RNA) from rats on a mixed diet was incubated under conditions as in Table 12. Additions were made as noted of phenol extracted RNA (100 ug. RNA) from the fractions and diets shown. (Data in brackets represents the activity remaining after hot perchloric acid extraction.)

Table 14.

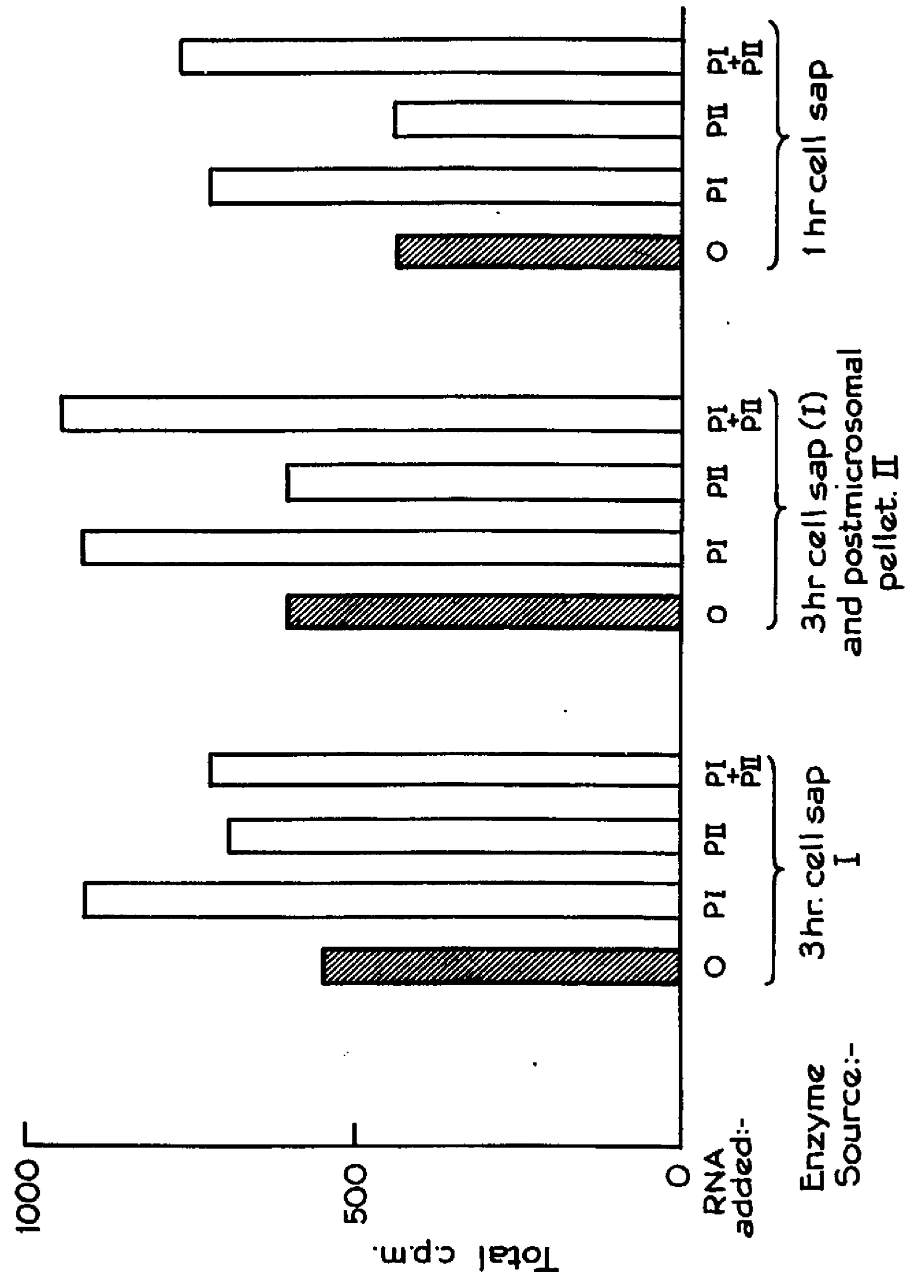
Source of RNA	Dietary Group	Counts per tube	RNA recovered	Cts./min. less 261	RNA less 85ug.	Cts./min./ mg. excess RNA
			ug.			
-	-	261 (18)	85	-	-	-
3 hour Cell Sap	Protein Depleted	342 (21)	154	+81	+69	1175
	Adequate Protein (Fasted)	349 (13)	157	+88	+72	1220
	Adequate Protein (fed Protein)	348 (22)	171	+87	+86	1010
Post-microsomal	Protein Depleted	228 (15)	144	0	+59	0
	Adequate Protein (Fasted)	215 (12)	156	0	+71	0
	Adequate Protein	210 (12)	172	0	+87	0

Fig. 5.

To test the biological activity of post-microsomal RNA as an amino acid acceptor in the presence of the pH 5 fraction from the cell supernatant.

The pH 5 fraction from the 3 hr. cell sap, 1 hr. cell sap and post-microsomal pellet (each containing about 100 ug. RNA) were incubated with ATP (10 umoles) and ^{14}C-leucine (1 uC) in 1 ml. of the phosphate buffered medium of Rendi & Campbell (1959) for 10 mins. at 37°C. RNA (100 ug.) purified using phenol from the 3 hr. cell sap (PI) and from the post-microsomal pellet (PII) was added where noted.

Fig. 5.



ability to utilise post-microsomal RNA and even this was low, the RNA from this source being completely inert in the other two systems. Thus, the variations in amino acid incorporating ability per unit RNA of the individual pH 5 fractions may be related to the initial proportion of inert post-microsomal RNA in the system. Thus, the 5 hr. cell sap is highly active because little or no post-microsomal RNA is present; in 1 hr. cell sap there is 20-30%; in the 3 hr. cell sap-post-microsomal mixture the post-microsomal RNA amounts to 50%.

Therefore, although the total pellet is capable of incorporating amino acids by itself the post-microsomal RNA is not endowed with any amino acid accepting activity characteristic of sRNA.

To fractionate the RNA from the 3 hr. cell sap and post-microsomal pellet we next turned to the use of Ecteola cellulose resins which permits resolution of different species of RNA on the basis of their physico-chemical properties.

<u>Column chromatography of RNA prepared from sub-cellular fraction</u>

The column was eluted successively with (a) a gradient of phosphate buffer/sodium chloride pH 6.85. (b) normal sodium chloride. (c) a gradient of ammonium hydroxide in sodium chloride. and (d) sodium hydroxide. Fig. 6 shows a blank run in which these solvents were run through a column of Ecteola. Although the column had already been carefully prepared and washed according to Goldthwaite (1959a), a small area of ultraviolet absorbing material was eluted at the

start of the sodium chloride gradient and again at the start of the final elution with N sodium hydroxide. Even several alternate washings with acid and alkali did not remove this contamination. The NaOH was screened for ultraviolet absorbing material before application to the column; none was found. As a preliminary, a study was made of the elution patterns obtained when the RNA which is phenol extractable from each part of the cell cytoplasm was fractionated (Fig. 6).

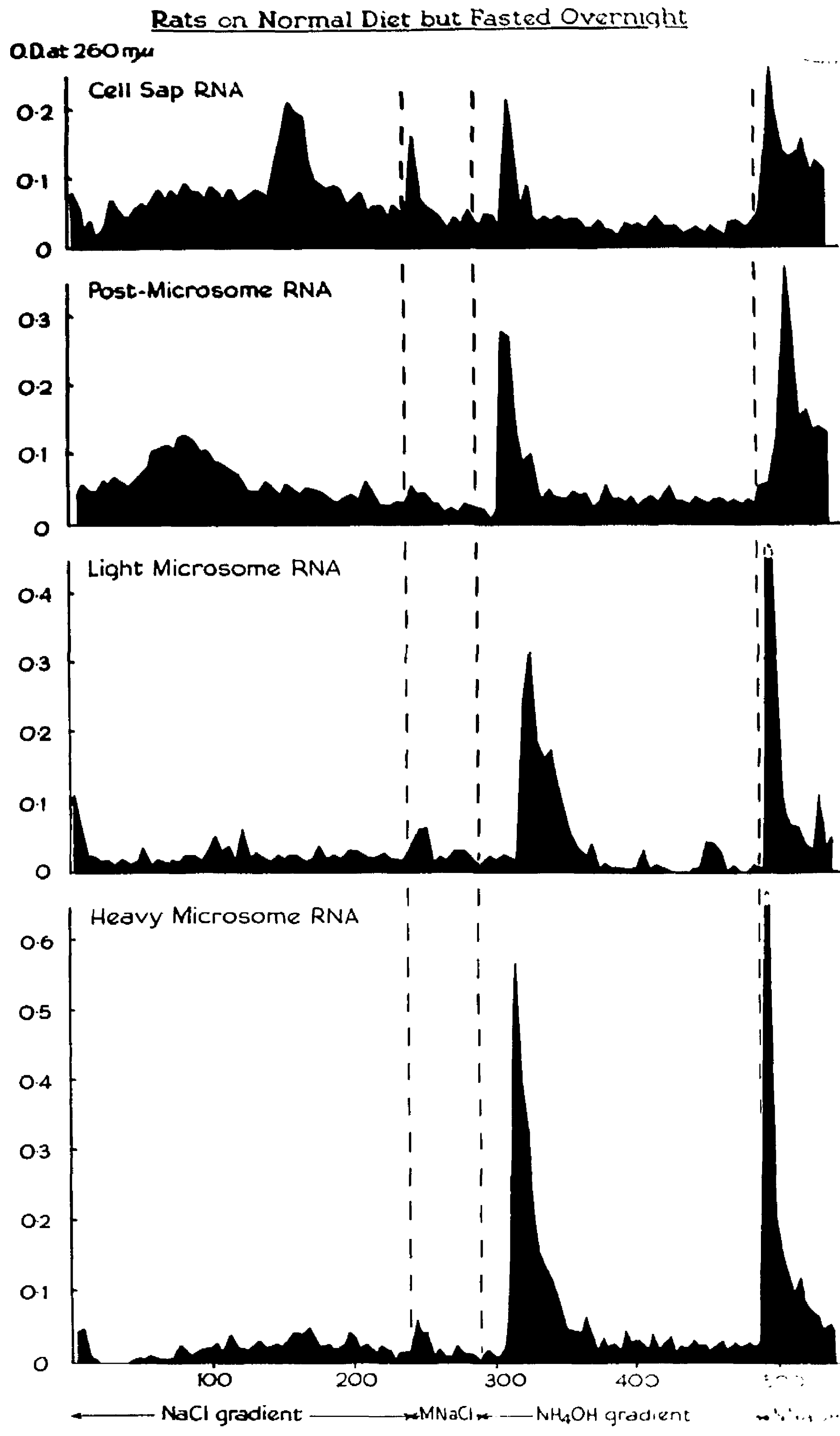
The profiles obtained from chromatography of the light and heavy microsomes are essentially similar. Two fractions of RNA are found, one obtained by an NH_4OH elution and one in the residual wash with N NaOH. The other areas of UV absorbing material eluted in the NaCl gradient do not exceed those found in the blank run and even elution with N NaCl produces no additional absorption. The peak found in NaOH is larger than that found in NH_4OH in both cases contrary to the findings of Goldthwaite (1959a) whose microsomal preparations presented a larger area of ultraviolet absorbing material in the NH_4OH peak. The presence of a large amount of absorption in the NaCl gradient is a very striking feature of the 3 hr. cell sap; which is most probably sRNA (Goldthwaite, 1959b). The remaining material which is appreciable in amount, may be composed of oligonucleotides chelated by metal ions (Bosch et al., 1960).

A small but distinct peak is observed also in the NaCl eluate which represents RNA incompletely washed off in the NaCl gradient. It may be noted that material eluted with NH_4OH and NaOH is not absent from this fraction.

Fig. 6.

RNA (about 600 ug.) was purified by phenol extraction from the cell sap, post-microsomal pellet and light and heavy microsomes and separated by chromatography on Ecteola resin into the components shown in the diagram.
Optical density of the eluate at 260 mu is shown as a function of volume.

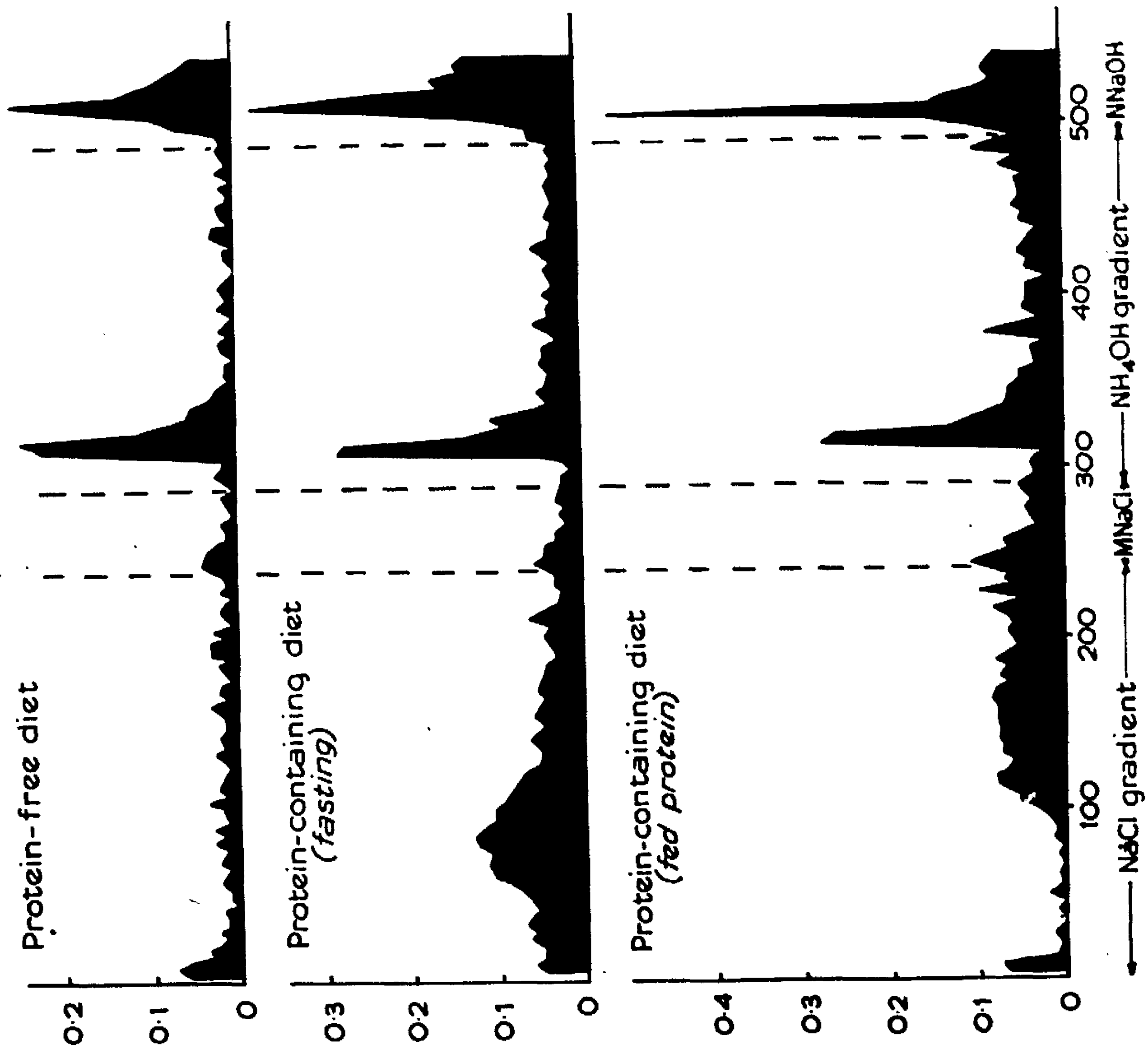
Fig. 6.



The presence of RNA other than sRNA in the 3 hr. cell sap indicates that cell sap, even when it is prepared by prolonged centrifugation at 105,000 g. is still contaminated by high molecular weight material. (Goldthwaite (1959a) also observed that cell sap RNA contains some high molecular weight RNA (RNA II in his nomenclature) even when centrifuged for a time equivalent to that used in our separation procedure.

The pattern of elution of ultraviolet absorbing material from RNA prepared from the post-microsomal pellet after various diets is shown in Fig. 7. This demonstrates that, in the case of animals on the protein free diet, there are only two peaks, one in the NH_4OH gradient and one in the NaOH eluate, just as was observed in the case of the microsome fractions. (Fig. 6.) However, pellet RNA prepared from rats which had received adequate protein in the diet before killing, whether fasted over-night (middle diagram) or fed protein just before death (lower diagram) exhibited a considerable amount of ultraviolet absorbing material eluted in the NaCl gradient. In addition, as well as the main peak in the NH_4OH gradient, a considerable amount of ultraviolet material can be seen extending along the base line of the latter chromatogram. The implication of this change in pattern with diet is that the RNA of the post-microsome fraction is more heterogeneous in molecular species when the animal has been receiving a diet containing protein (lower two diagrams, Fig. 7.) a finding in agreement with our conclusions from the study of uptake of ^{14}C-leucine by this pellet. It was therefore of some moment to confirm this finding by repetition of the

Fig. 7.



Post-microsomal RNA (about 600 ug.) was purified by phenol extraction and separated by chromatography on Ecteola resin into the components shown above.

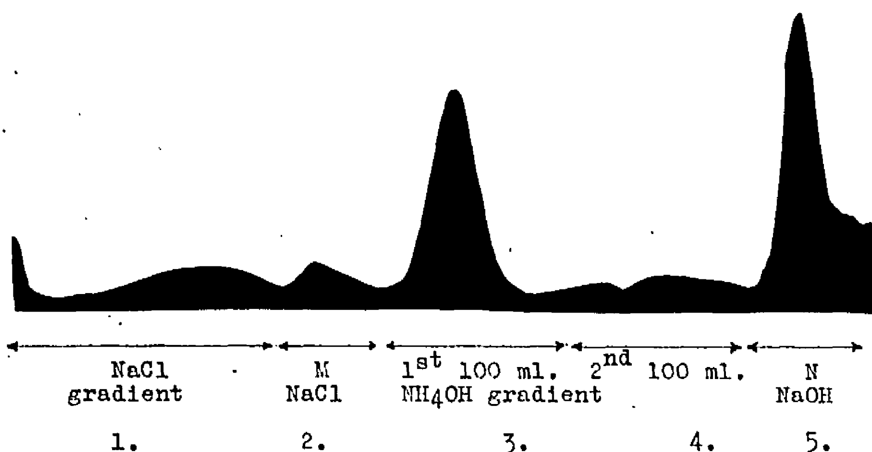
Optical density at 260 mu is shown as a function of volume.

column chromatography on further specimens obtained from animals on these different diets.

Three replications of the dietary experiment were therefore carried out, phenol RNA being prepared from the post-microsomal pellet and passed through the column. The data given in Fig. 8 are a summary of the average ultraviolet absorptions observed in the three experiments with each type of diet. They are presented in the form of the sum of mean ultraviolet absorptions at 260 mu of the RNA eluted in the sections of the solvent system stated less the absorption of a solvent blank from the same section. In each of these arbitrarily cut divisions except the wash with M NaCl, the amount of ultraviolet absorbing material was greater in the two protein-fed groups of animals than in the protein-depleted animals. Since these data were obtained with columns to which the same amount of phenol RNA was applied a larger amount of small-molecular-weight material is appearing in the post-microsomal RNA from the rats fed adequate protein This is reflected in the total ultraviolet absorption eluted from the column, the mean optical density per ml. of eluate being +0.024 for the protein-depleted group, +0.037 for the protein-fed group during fasting, and +0.035 for the protein-fed group after a meal of protein. Presumably, some of the applied RNA must have been retained on the column when the elution with NaOH was stopped; it is unfortunately not easy to compute with the necessary accuracy the amount of RNA eluted in relation to the amount applied, since (a) the native RNA has a lower UV absorption than degraded RNA

Fig. 8.

Elution pattern after applying 600 ug. RNA from the post-microsomal fraction to Ecteola columns.



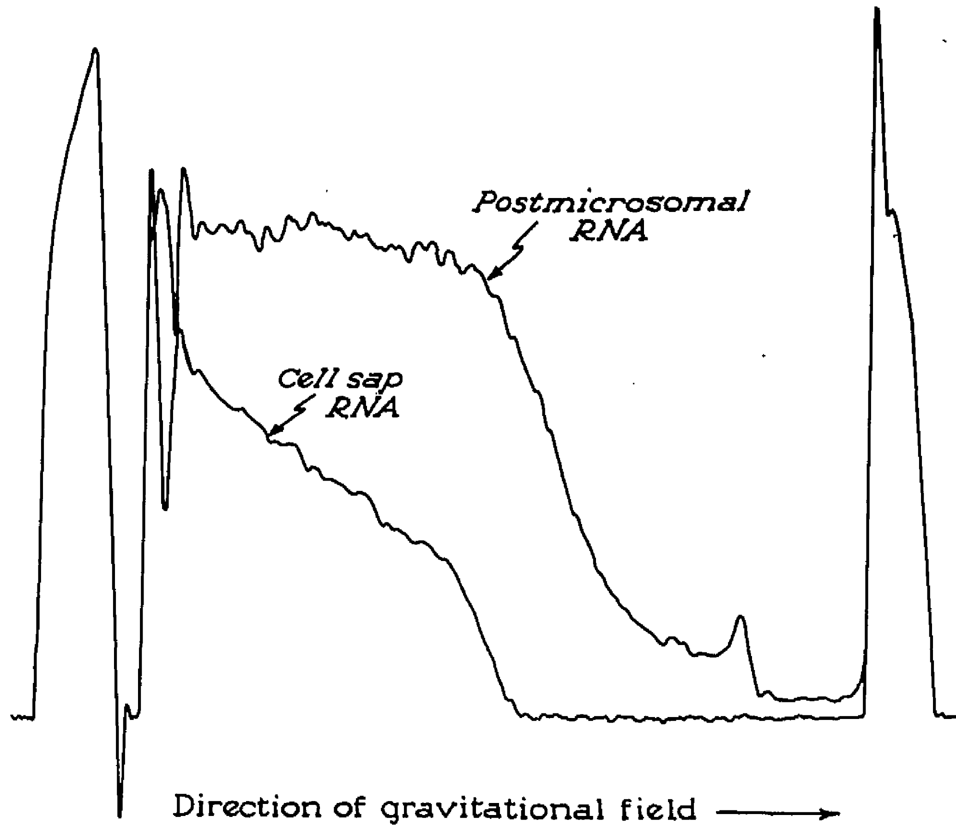
Dietary condition	Mean optical density at 260 mu 10^3					Mean optical density (Total) per ml. at 260 mu 10^3
	1	2	3	4	5	
Protein Free	3	24	67	5	75	24
Adequate Protein (fasted)	17	19	90	22	89	37
Adequate Protein (Fed protein)	11	37	75	26	94	35

(hyperchromic effect of a high degree of polymerisation), (b) the degree of degradation during elution with alkali cannot readily be assessed, and (c) minor variations in the base-line during elutions with the larger amounts of solvents lead to cumulative errors of considerable magnitude. Consequently, the computation of recoveries from such columns is not a profitable exercise.

A further attempt to confirm the heterogeneous nature of the pellet RNA and the effect of diet on the degree of heterogeneity was made by using the Spinco Model E analytical ultracentrifuge, with ultraviolet optical system, to assay the molecular sizes present in the post-microsomal pellet RNA samples. This has so far not lead to a sufficient degree of resolution to allow any useful comment to be made, but Fig. 9 shows that the molecular size of the pellet RNA is very much greater than that of the cell sap RNA, since the latter is clearly sedimented much more slowly.

A third approach to the question of heterogeneity in post-microsomal RNA varying with dietary conditions involved chromatographic separation of nucleotides in specimens of RNA from the post-microsomal pellet. Phenol RNA from animals receiving each of the diets was prepared on two occasions, hydrolised in alkali and the nucleotides separated in the solvent system of Lipshitz and Chargaff (1960). With this system up to 10 separate nucleotides can be identified in soluble RNA, an observation which has been confirmed in this laboratory. In the case of the pellet RNA, the usual 4 nucleo-

Fig. [illegible]



RNA, phenol-extracted from the [illegible] cell sap and the postmicrosomal pellet, was centrifuged at 5[illegible],7[illegible] r.p.m. on the Spinco Ultracentrifuge, Model E. The photograph was taken after a time-interval of 1[illegible] min.

tides were easily separable, and also a fifth area of ultra-violet absorption which was identified by its peculiar absorption shift in acid and alkali to be pseudo-uridylic acid. The concentrations of these five nucleotides in the specimens of phenol RNA are presented in Table 15, and show that diet did not significantly influence the proportions of the major nucleotides in this species of RNA, but there was a considerable change in the concentration of pseudo-uridylic acid; in the case of the animals fasting after the protein-containing diet, the concentration of pseudouridylic acid fell to about two thirds of that in the protein depleted animals and in the animals fed with protein a short time before killing. Thus, the data obtained from nucleotide analysis of RNA are consistent with a change in the composition of pellet RNA during the period of fasting after a protein containing diet; the change in pseudo-uridylic acid content would lead one to believe that the RNA added to the pellet at this time is deficient in pseudo-uridylic acid, an observation whose relevance will become apparent later on.

The base composition of the RNA from other cell fractions is included for comparison in Table 16.

A final observation regarding the origin of the RNA contained in the post-microsomal pellet is provided by the RNA/protein ratios after different diets. The mean data for three experiments gave ratios of 0.15, 0.12 and 0.15 respectively for the protein-free, protein-containing (fasting) and protein containing (Fed protein) groups. The RNA/protein ratios were

Table 15

<u>Influence of diet on the nucleotide composition of the Post-microsomal Pellet</u>

Dietary Group	Adenylic	Guanylic	Cytidylic	Uridylic	Pseudouridylic	Others
Protein	18.8	32.1	29.7	17.7	2.0	0
Adequate Protein (Fasting)	19.6	31.6	30.1	17.7	1.6	0
Adequate Protein (Fed Protein)	18.8	31.3	29.6	18.5	2.0	0

The values are a mean of 5 experiments

The changes in the level of pseudouridylic acid in the pm. pellet RNA are statistically significant (P 0.05).

Table 16.

<u>Influence of Diet on the Nucleotide Composition of Liver Cell Fractions.</u>

The values are a mean of 4 experiments.

From Goswami, Barr & Munro, (1962).

Fraction	Percentage					
	Adenylic	Guanylic	Cytidylic	Uridylic	Pseudouridylic	Others
Cell Sap	19.9	31.1	26.9	15.6	3.5	3.0
Microsomes Ribosomes	20.9	27.0	27.1	23.0	2.2	0
Membrane	17.0	34.1	32.3	16.8	0.3	0
Nuclei	18.7	31.8	31.3	18.4	0	0

also measured on two fractions of microsomes, namely the ribosomal components and on the membrane these fractions being separated by the pyrophosphate technique (Sachs, 1957). The RNA/protein ratios were 0.50 and 0.07 respectively on these two microsome fractions. It follows that the extra RNA found in the post-microsomal pellet of the animals fasted after a protein-containing diet cannot have arisen from break-down of ribosomes, because this would have raised the RNA/protein ratio of the pellet. The slight fall of the ratio suggests rather, an origin from membrane RNA.

Effect of preceding diet on the light and heavy microsome fractions.

Since the post-microsomal pellet showed considerable changes, both in composition and ability to accept labelled amino acids when the protein content of the diet was varied, it seemed desirable to explore the microsome fractions to determine whether they would also exhibit such variations or whether the post-microsomal response was peculiar to this cell fraction.

When microsomes prepared from animals on protein deficient diets and protein containing diets were incubated with ^{14}C-leucine and ATP under the conditions found to support incorporation into the pellet, it was observed that the uptakes were very much lower than with the post-microsomal pellet prepared from the corresponding animals, the amount of material incubated being adjusted to contain the same initial amount of RNA (Table 17). The customary effects of diet on the pellet RN

Table 17

The effect of Diet on the Uptake of ^{14}C-DL-leucine by Light, Heavy and Post-microsome Fractions of Rat Liver

Conditions of incubation are as in Table 14.

Dietary Group	Total cts./min./mg. RNA recovered		
	Heavy Microsomes	Light Microsomes	Post Microsomes
Protein Free	201	303	1120
Adequate Protein (Fasting)	188	456	422
Adequate Protein (Fed Protein)	205	359	930

uptake is evident in the data, but no such change is apparent in the case of either light or heavy microsomes. Indeed, the low-protein light microsome fraction appears to be less adept at taking up label. From these data, it can be concluded that the pellet uptake is unusual both in regard to its extent and in regard to its sensitivity to protein intake.

Survey of findings in Section I.

At this point, we may attempt to assess the conclusions reached as the result of these dietary studies on liver RNA metabolism.

(1) As pointed out in the introduction, protein depletion causes a pronounced loss of RNA from the liver cell which arises predominantly from loss of RNA associated with the endoplasmic reticulum. During the phase of acute loss of RNA, such as during a period of 18 hr. fasting after an adequate intake of protein, the RNA content increases in cell sap prepared by centrifuging sucrose homogenates for 1 hr. at 105,000 g. This increase in cell sap RNA may thus be due to an accumulation of breakdown products from the particulate components of the liver cell.

(2) The capacity of pH 5 enzyme preparations from the cell sap to incorporate labelled leucine was also significantly altered by diet. However the changes in amino acid incorporation were found to vary inversely with the RNA content, the least uptake being observed in animals undergoing acute loss of RNA from the endoplasmic reticulum due to overnight fasting. It would

thus appear that the RNA added to the cell sap under these nutritional conditions was diluting the capacity of the system to accept labelled amino acids.

(3) The cell sap was then divided into a particulate fraction (post-microsomal pellet) and a supernatant fraction (3 hr. cell sap by centrifuging for a further 3 hrs. at 105,000 g. The properties of these two subdivisions of the original cell sap were then studied in relation to diet. The dietary effects were confined to the post-microsomal fraction. The RNA content of the 3 hr. cell sap and its capacity to accept labelled amino acids was unaffected by protein intake. The pellet, on the other hand, exhibited a significant though reduced capacity to incorporate labelled amino acids which varied under different dietary conditions. Pellet prepared from animals during the phase of active loss of RNA from the cell contained the greatest quantity of RNA and had the least capacity to accept labelled amino acids. Surprisingly, the post-microsomal pellet prepared from animals protein depleted for several days showed the highest capacity to accept labelled amino acids, associated with the lowest amount of pellet RNA. The giving of a protein meal 1-2 hrs prior to sacrifice resulted in a post-microsomal preparation intermediate in its properties to the two preparations described above. These findings show that the amount of RNA in the post-microsomal pellet and its capacity to retain labelled amino acids under in vitro conditions vary inversely. When the cell is rapidly losing RNA, presumably from disintegration of the endoplasmic reticulum and released of some of its contained RNA

the RNA content of the pellet rises but its amino acid acceptor capacity falls. It is thus suggested that a different species of RNA produced by disintegration of the endoplasmic reticulum accumulates in the post-microsomal pellet. Administration of protein reverses this process by terminating the RNA breakdown occurring in the particulate portions of the cell. This dietary response is very rapid, since significant changes in the post-microsomal pellet can be demonstrated 1 hr. after protein adminstration.

(4) Since the RNA of the post-microsomal fraction appeared to become labelled during incubation with ^{14}C-leucine, we decided to extract the RNA from the post-microsomal pellets of animals receiving the various diets and add this material into a system which would transfer labelled leucine to sRNA. The pellet RNA did not act as an acceptor of ^{14}C-leucine when added to the enzyme system from cell sap, and thus is not a form of sRNA; preceding diet did not alter this absence of coupling with the activating enzymes of the cell sap.

(5) The nature and heterogeneity of RNA from the post-microsomal pellet was then explored by column chromatography on Ectoola. The RNA from the pellet of protein depleted animals showed only two peaks in the elution pattern, corresponding to similar elution peaks in RNA from microsomal preparations. However, the post-microsomal pellet RNA obtained from animals undergoing loss of endoplasmic reticulum as a result of withdrawal of protein from the diet contained additional RNA species of varying molecular size. An RNA type, which was identified with sRNA by

comparison with the work of Goldthwaite (1959b) was separated from the 3 hr. cell sap; no similar peak was recognised in the elution patterns from any post-microsomal pellet RNA preparation.

(6) Analysis of the nucleotide composition of RNA extracted from post-microsomal pellet showed that it contained significant amounts of pseudouridylic acid, which is a characteristic component of sRNA. The pseudouridylic acid content was significantly affected by diet, being reduced in RNA preparations obtained from animals undergoing loss of RNA following an 18 hr. fast. The composition of this pellet preparation was also explored by analysis of RNA/protein ratios and it was shown that a slight reduction of the ratio occurred in animals during the period of acute loss of RNA from the cell.

(7) Preparations of microsomes examined under the same dietary conditions as the post-microsomal pellet did not show the changes in uptake of labelled leucine which were observed with the post-microsomal pellet. It was therefore concluded that the properties of the post-microsomal pellet are distinct from those of either the cell sap or the microsomes.

SECTION II.

In the previous section we have described a fraction of liver cell homogenates, the post-microsomal pellet, which is capable of incorporating labelled leucine in the absence of added cell sap or pH 5 enzyme to a considerably greater extent than observed in parallel experiments with microsomal fractions. Examination of the RNA of this fraction for the presence of sRNA was negative, both by Ecteola chromatography and by the addition of the RNA to a system sensitive to the presence of sRNA. It therefore seemed of interest to explore the nature of the incorporation by the following scheme:

(1) A study of the conditions of incubation affecting incorporation, including the influence of the ions present, the pH of the medium, the need for an energy source, the effect of dialysis and the period over which incorporation continues to occur.

(2) Examination of the properties of the post-microsomal pellet system including comparison with uptake by other cell fractions incubated under the same conditions, effect of ribonuclease on incorporation , effect of adding pH 5 enzymes, and the effect of addition of other substances which are known to influence certain steps in protein synthesis (glutathione, chloramphenicol, nucleotide triphosphate mixtures and an amino acid mixture). Activating enzyme assays were also carried out.

(3) Attempts to purify the enzyme system involved in the incorporation.

(4) Attempts to identify the location of the label in the protein of the post-microsomal fraction.

In many of these experiments, the animals were given the protein free diet because the post-microsomal pellet prepared from such animals had been shown to have more activity.

EXPERIMENTAL METHODS.

The methods in this section are as for Section I with the undernoted changes and additions.

Animals and diets. In investigations carried out in Parts I & II animals which had been fed protein deficient diets as defined in Section I were used. In Parts III & IV rats in the same weight range were selected at random from stock and used without special feeding.

Materials. The triphosphates, guanosine triphosphate (GTP), cytidine triphpsphate (CTP), uridine triphosphate (UTP) were obtained as the disodium salt from Sigma: ribonuclease, five times crystallised from bovine pancreas from Sigma: glutathione and fluorodinitrobenzene from Light & Co.: pyruvate kinase and phosphoenolpyruvate (silver barium salt) from Boeringer: the L forms of the 18 common amino acids from B.D.H.:DL alanine-1-^{14}C (225umoles/uC), ^{14}C-1-DL-phenylalanine,(236umoles/uC) ^{14}C-1-DL-glutamic acid (320umoles/uC) and ^{14}C-2-DL-glycine (261umoles/uC) from the Radiochemical Centre, Amersham: ^{32}P sodium pyrophosphate from the same source: chloramphenicol from Parke-Davis. Visking dialysis tubing (½" diameter) was used.

Recrystallisation of the Ag Ba salt of phosphoenolpyruvate and conversion to the Na salt.

The Ag Ba salt of phosphoenolpyruvate (500 mg.) was dissolved in 0.2 N HNO_3 (about 16 ml) and centrifuged to remove a small brown insoluble impurity. The phosphoenolpyruvate (PEP) was precipitated from the supernatant by addition of 40% (w/v) barium

acetate (2 ml.). After 24 hrs. in the dark the crystalline PEP was collected in the bottom of the containing vessel by simple agitation. The impurities were left in suspension above the precipitate and were removed by decanting. Complete removal of impurities was obtained by repeating this separation process twice with 50% ethanol. The precipitate was washed with absolute alcohol (about 20 ml.) dried in a desiccator (P_2O_5) for 18 hrs.

The dry PEP was dissolved in 0.2 N HNO_3 and adsorbed on a Dowex 50 (Na form) column. The column was eluted with distilled water until E_{340} was equal to or less than 0.01. The eluant was freeze dried and the dry powder stored in the dark.

Buffers.

The homogenisation medium of Rendi and Campbell (1959) already described will be designated Campbell buffer in this section.

The other buffers used were:-

(a) sucrose 0.35M, potassium phosphate 0.02M, pH 7.8
(sucrose phosphate pH 7.8)

(b) same as (a) but adjusted to pH 3.4 with N HCl
(sucrose phosphate pH 3.4)

(c) sucrose 0.35M, ethylenediaminotetraacetic acid (EDTA) 0.001M, pH 3.4 (sucrose EDTA pH 3.4)

(d) same as (c) but adjusted to pH 7.8 with N NaOH
(sucrose EDTA pH 7.8)

(e) sucrose 0.35M, potassium phosphate 0.02M, EDTA 0.001M pH 3.4 (sucrose phosphate EDTA pH 3.4)

(f) same as (e) but adjusted to pH 7.8 with N NaOH
(sucrose phosphate EDTA pH 7.8)

(g) tris/HCl 0.1 M pH 7.8; $MgCl_2.6H_2O$ 0.01 M; KCl 0.025 M; $KHCO_3$ 0.03 M; sucrose 0.35 M.

(h) glycine/KOH 0.1 M pH 7.8; $MgCl_2.6H_2O$ 0.01 M; KCl 0.025 M; $KHCO_3$ 0.03 M; sucrose 0.35 M.

(i) veronal/acetate/HCl 0.029 M pH 7.8; $MgCl_2.6H_2O$ 0.01 M; KCl 0.025 M; $KHCO_3$ 0.03 M; sucrose 0.35 M.

Estimation of RNA.

In some experiments the modified procedure of Fleck and Munro (1962) was used. 0.5 volumes 2.1 N PCA were added to the sample and after 15 mins. standing the precipitate was centrifuged down and washed twice with 0.7 N PCA. The PCA was carefully drained off before addition of alkali. The samples were then digested in an incubator at 37°C in 0.3 N KOH for 1 hr. (3 ml. N KOH was used to allow for acid remaining in the precipitate). At the end of incubation, the chilled samples were neutralised with 10 N PCA and acidified with 1 vol. N PCA. The precipitate was separated centrifugally and washed twice with cold 0.5 N PCA. The combined supernatant fluid and washings were made up in 50 ml. 0.1 N PCA. The absorption was read at 260 mu on the Unicam SP 500 and the RNA content calculated using the following equation.

O.D. 1.0 = 32.87 ug. RNA/ml.

Estimation of Phosphorus.

The method used is a modified version of that described by Allen (1940). The total phosphorus is converted by digestion with sulphuric acid and hydrogen peroxide to inorganic orthophosphate from which a phosphomolybdate complex is formed by

reaction with ammonium molybdate. This complex is reduced to a blue pigment with the reducing agent amidol(2,4 diaminophenol-hydrochloride) the intensity of the blue colour is measured in the Unicam SP 600.

Reagents.

H_2SO_4 10 N (28 ml. conc. H_2SO_4 in 100 ml. H_2O)

H_2O_2 (M.A.R. 100 vols.)

Ammonium molybdate 8.3%

Amidol 1% in metabisulphite 20% (1 g. amidol dissolved in 100 ml. 20% Na or K metabisulphite solution and filtered. This solution was kept in a stoppered brown bottle and was only kept for 2-3 days)

Stock Standard (B.D.H.) 1 mg. P_i/ml.

Diluted phosphate standard. 1 mg. stock solution made up to 50 ml. with distilled water (1 ml. ≡ 20 ug. P_i

A sample of the solution to be assayed containing between 20 and 150 ug. P_i pipetted into a microkjeldahl digestion flask and 1.2 ml. 10 N H_2SO_4 and a glass bead were added. The flask was transferred to a digestion rack and digested over a small flame until the contents were dark brown. The flask was removed from the rack and two drops H_2O_2 added. Digestion was then continued until the contents were clear and fuming (If the solution failed to clear, the addition of H_2O_2 was repeated). The neck of the flask was washed down with a small amount of water and the contents once again evaporated to the fuming stage. The flask was then cooled and 21.65 ml. water, 1% amidol in 20%

Na or K metabisulphite 2.0 ml. and 8.3% ammonium molybdate 1 ml. added in that order with mixing between additions. After 10 mins. (but not more than 30 mins.) the intensity of the blue colour was read at the absorption maximum 725 mu in the Unicam SP 600 against a reagent blank. A standard phosphorus sample was assayed at the same time.

For smaller amounts of phosphorus the estimation was carried out using quantities scaled down as follows:-

Reagent	Full Allen ml.	1/2 Allen ml.	1/5 Allen ml.
H_2SO_4 10 N	1.2	0.6	0.24
Ammonium molybdate 8.3%	1.0	0.5	0.2
Amidol 1%	2.0	1.0	0.4
Water to	25	12.5	5.0

In most cases the estimations fell into the range of the 1/5 Allen (5 - 50 ug. P). A calibration curve for the method over this range was constructed using the B.D.H. phosphorus standard. For digestion of these smaller amounts of phosphorus, pyrex tubes graduated to 5 ml. containing an 'anti bump rod' were used.

No digestion is necessary for estimation of inorganic phosphate.

When pyrophosphate in the presence of inorganic phosphorus was measured the following procedure was used. An estimate of free inorganic phosphorus was carried out on one sample. A

second sample was first submitted to hydrolysis with an equal volume of 2N H_2SO_4 at 100°C for 15 mins. and then the total phosphorus was measured. (When this acid hydrolysis of samples was employed, adjustment was made to ensure that the final acid concentration in the final colour reaction was correct). The difference between these two estimations was equivalent to the pyrophosphate present. To check this procedure some tests were carried out (Table A) and it was found that estimations of inorganic phosphate by the Allen method were unaffected by the presence of unhydrolysed pyrophosphate and also that the method of hydrolysis outlined effectively converted the pyrophosphate to a form which is quantitatively measured by the Allen method.

Estimation of phospholipid phosphorus. (Folch et al., 1957)

Reagents.

Chloroform:methanol 2:1 by volume

Pure solvents, upper phase. Chloroform:methanol:0.58% NaCl (w/v) 3:4:3 by volume were shaken together, allowed to stand and the bottom layer discarded.

The cell fraction was shaken with 20 vols. of the 2:1 chloroform methanol mixture for 15 mins. After centrifugation at 1,500 r.p.m. for 15 mins., the solvent was quantitatively transferred into stoppered 10 ml. graduated test tubes. The crude extracts were then mixed thoroughly with 0.2 of their volume of 0.73% NaCl. The aqueous layer was separated by centrifugation at 1500 r.p.m. for 10 mins. and discarded. The removal of this aqueous phase was completed by rinsing the inter

Table A.

Different known concentrations of inorganic phosphate and pyrophosphate were assayed by the Allen method separately and together. In some cases prior hydrolysis with 2 vols. 2N H_2SO_4 at 100°C for 15 mins. was carried out.

P_1 = 1o ug. P_i PP_1 = 10 ug. PP

P_2 = 5 ug. P_i PP_2 = 5 ug. PP

Addition	Hydrolysis	Theoretical P_i value ug.	P_i found (Allen) ug.
P_1	-	10.0	10.7
$P_1 + PP_1$	-	10.0	10.7
$P_1 + PP_2$	-	10.0	10.5
$P_1 + PP_1$	+	30.0	29.5
$P_1 + PP_2$	+	20.0	20.0
PP_1	-	0.0	0.0
PP_1	+	20.0	19.0
P_2	-	5.0	5.0
$P_2 + PP_1$	-	5.0	5.3
$P_2 + PP_2$	-	5.0	5.1
$P_2 + PP_1$	+	25.0	24.5
$P_2 + PP_2$	+	15.0	15.0
PP_2	-	0.0	0.0
PP_2	+	10.0	9.7

face three times with small amounts of pure solvents, upper phase in such a way as to leave the lower phase undisturbed. The lower phase and residual rinsing fluid were then made into one phase by addition of methanol to a volume of 10 ml.

Aliquots of the extract were evaporated to dryness, digested and the phosphorus estimated by the method of Allen (1940).

<u>Estimation of Nitrogen.</u> (Paul, 1958)

This is a modification of the method of Nessler for NH_3.
(This method was only used in the <u>in vivo</u> experiment.)

Reagents.

Selenium dioxide 1% in 50% (w/v) sulphuric acid (nitrogen free - Analar)

Nessler reagent (Modified)

a KI 4 g.) in 25 ml. water.
HgI_2 4 g.)

b Gum acacia 3.5 g. in 750 ml. water.

Add together (a) And (b) and dilute to 1 l.

Sodium hydroxide 2N (8% w/v)

Stock standard $(NH_4)_2SO_4$ 0.471 g. in 100 ml. water.

Diluted nitrogen standard. 1 ml. of stock solution made up to 100 ml. with distilled water. (10 ug. N/ml).

Add 0.5 ml. of the selenium dioxide in sulphuric acid solution to the nitrogen containing sample (50-150 ug. N) in a pyrex tube graduated to 10 ml. with an 'anti bump rod' placed in the tube; digest until the water has evaporated and the solution is clear; cool and make up to 10 ml. with water.

To two ml. of the diluted digest add 2 ml. Nessler reagent, mix and add 3 ml. NaOH (2N). Allow to stand for 15 mins. and read within 20 mins. at 490 mu.

The method may be scaled down. The range 10 - 50 ug. N may be used for digestion by using 0.25 ml. selenium dioxide/H_2SO_4 for digestion, making up to 5 ml. and taking 2 ml. for colorimetry. Similarly 0.1 ml. selenium dioxide/H_2SO_4 may be used, made up to 2 ml. after digestion.

Ammonium sulphate fractionation.

The whole procedure was carried out at $0^{o}C$. A calculated volume of saturated ammonium sulphate was added to the solution to be fractionated in order to give a known percentage saturation with respect to the salt. The salt addition was made very slowly with constant steady stirring to minimise frothing. Meanwhile the pH which tended to fall was maintained at 7.8 by the addition of N NH_4OH. Thirty mins. after the final addition of ammonium sulphate the resulting precipitate was separated by centrifuging at 18,000 g. for 10 mins.. The supernatant was separated into further fractions by the addition of graded amounts of saturated ammonium sulphate in the manner described. Each resulting precipitate was dissolved in a small volume of sucrose phosphate buffer, pH 7.8, and dialysed against 4 l. of the same buffer for 18 hrs. with constant stirring.

Pyrophosphate treatment. (Sachs 1957)

The fraction in question was evenly suspended in 10 ml. ice-cold Na pyrophosphate ((B.D.H.) 0.11M in 0.25M sucrose,

pH 7.4) to provide a final protein content of 4-5 mg. per ml. 1.0 ml. of 0.25 M sucrose was added to adjust the pyrophosphate molarity to 0.10 M. After 30 mins. at 0°C the suspension was centrifuged at 105,000 g for 60 mins. In some cases, the separation at this speed was preceded by centrifugation at 2,000 r.p.m. for 10 mins.. The precipitates were suspended in sucrose phosphate buffer, pH 7.8, and dialysed at 0°C against 4 l. of the same buffer for 18 hrs. with constant stirring.

Preparation of dinitrophenyl derivatives of proteins and amino acids.

The preparation and treatment of these derivatives was carried out in the absence of strong light which tends to cause decomposition.

Preparation of Dinitrophenyl (DNP) protein. (Rhinesmith et al., 1956)

The dried, weighed protein (10-20 mg.) was washed into the reaction chamber of a radiometer titrigraph with about 10 ml. water. Once the pH was adjusted to 8.5 by titration with N NaOH fluorodinitrobenzene (100 ul.) was added. The reaction was carried out at 37°C with constant agitation and at a constant pH (8.5) which was maintained with NaOH. On completion, the reaction mixture was acidified with N HCl to pH 3. A flocculent precipitate of DNP protein formed and was separated by centrifugation. The yellow powder was washed 4 times with 10 ml. 0.1 N

HCl (to remove mineral salts) once with ethanol and three times with peroxide free ether (to remove excess fluorodinitrobenzene and dinitrophenol formed). The washings should be colourless at this stage. After evaporating to dryness over P_2O_5, the DNP protein was hydrolysed in 6N HCl (glass distilled) overnight in a sealed tube at 110°C.

The hydrolysate and washings from the tube were extracted five times with 3 ml. portions of ethyl acetate to obtain DNP-end group amino acids. Each extract was separately washed with the same 1 ml. of water before being combined and evaporated to dryness with an air blast. (The water washings remove any residual salt from the extracts. The same 1 ml. of water was used over and over again because it quickly became saturated with DNP-amino acids some of which are water soluble and therefore, there is a minimum loss of material.)

Preparation of DNP amino acids. (Heyns & Woeff, 1956)

The residual hydrolysate left after ethyl acetate extraction contains the individual amino acids excluding the N terminal amino acids and the DNP derivatives of a few amino acids which are insoluble in ethyl acetate e.g. DNP lysine. (These ethyl acetate insoluble DNP amino acids may be extracted with sec. butanol but since DNP leucine is ethyl acetate soluble, this second extraction step was omitted.)

After evaporation to dryness overnight in a vacuum desiccator the hydrolysate was dissolved in 1 ml. carbonate/bicarbonate buffer, pH 9 (M Na_2CO_3:M $NaHCO_3$ 1:9) to which was

added FDNB (50 ul.) in ethanol (2 ml.). After 2 hrs. incubation at 37°C, the reaction mixture was diluted with about 2 ml. of water and extracted with ether to remove unreacted FDNB. (The ether washing was continued until the extracts were colourless) The solution was acidified with N HCl and extracted 5 times with 3 ml. ethyl acetate, each extract being water washed as before. After the ethyl acetate, had been evaporated from the combined extracts, the excess DNP was sublimed off at 70°C. The DNP amino acids were redissolved in ethyl acetate, the solvent again evaporated and the sublimation repeated to remove the maximum amount of DNP.

Chromatography.

Immediately before application to the chromatogram, the dried DNPderivatives were dissolved in acid acetone. (250 ml. acetone + 2 ml. 2N HCl) Spots were applied to Whatman No. 1 filter paper sheets with a cold air blast and separated by descending chromatography in two dimensions each overnight in the dark at constant temperature using the following solvents.

First dimension: Butanol/NH_3 (Brauntzer,1955). Equal volumes of n-butanol and 0.1% NH_3 (1 vol. NH_3 + 300 vols. H_2O) were mixed thoroughly. After separation, the top layer was used. (Before addition of this solvent, the spotted chromatogram was allowed to equilibrate in a tank commonly used for butanol/NH_3 for a minimum of 30 mins.)

Second dimension: Phosphate buffer pH 6.0 (Levy, 1954) Na_2HPO_4 0.5 M, NaH_2PO_4 1.0 M. No equilibration in this solvent was necessary.

A control spot of authentic DNP leucine was run on a separate chromatogram and used as a marker.

The areas containing the separate DNP derivatives were located visually and under UV light. The spots were cut out into strips and eluted with 5 ml. water at $50^{o}C$ for 30 mins. After acidifying the extract with HCl, the DNP amino acids were extracted with 2 ml. ethyl acetate 3 times , plated and counted at infinite thinness. When the leucine spot was faint the control authentic DNP leucine spot was used to locate the test DNP leucine derivative. In addition, the surrounding area of the chromatogram was checked for radioactivity.

Measurement of activating enzymes.

A preliminary study was made on the adsorption of ATP using charcoal and the recovery of the β and γ phosphate groups of the ATP from the charcoal by acid hydrolysis.

The optical density of solutions of ATP containing from 25 to 125 mumoles of ATP were read at 260 mu on the Unicam SP 500. Optical density was plotted as a function of the molar concentration of ATP.

To 3.12 umoles ATP (calculated using a molar absorbancy for ATP in M/15 phosphate buffer of 15.3×10^{3} at pH 7 and read at 259mu) was added 50 mg. Norit A charcoal (Refluxed in 6N HCl) suspended in 5 ml. 0.4 N PCA. After 30 mins at $0^{o}C$, during which the tubes were agitated at regular intervals, the charcoal was sedimented by spinning at 2,000 r.p.m. at 0° for 10 mins. The supernatant was removed with a Pasteur pipette and the optical density measured at 260 mu using the calibration curve described.

the concentration of ATP unadsorbed by the charcoal was calculated. (Table B).

The charcoal sediment and parallel samples of ATP (3.12 mu) untreated by charcoal were hydrolysed in 2N H_2SO_4 (2 ml.) for 10 or 30 mins. at 100°C with periodic shaking of the tubes or totally digested in 10N H_2SO_4 as described in the method for Allen phosphorus estimation. (p 60). The charcoal was once more sedimented by spinning at 2,000r.p.m. for 10 mins. Phosphorus estimations by the Allen method were carried out on samples of the supernatant from the charcoal and on samples of the ATP hydrolysate untreated with charcoal. In addition aliquots of the charcoal were digested in absence of ATP to determine the total phosphorus content of the charcoal. Estimations were carried out also on the straight ATP solution to determine the level of free inorganic phosphorus in the ATP. The proportion of Norit A to ATP used was sufficient such that less than 0.15% of the ATP was unadsorbed by the charcoal. Hydrolysis of free ATP at 100°C in 2N H_2SO_4 was found to liberate 98% of the and phosphate groups in 10 mins. However charcoal adsorbed ATP proved more resistant to acid hydrolysis and after 10 mins. under the above conditions only 77% of the 2 terminal phosphate groups were released. Lengthening the hydrolysis period to 30 mins. released an average of 95% of the 2 terminal phosphate groups and thus in the activating

Table B.

ATP adsorption by and recovery from Norit A charcoal.

50 mg. Norit A (0.002 ug P_i/mg. charcoal) was used to adsorb 3.12 umoles ATP (3.5 ug free P_i/umole ATP) from an acid solution. The recovery of phosphorus from the charcoal by various methods in comparison with the recovery by the same methods from untreated ATP samples is shown. Correction is made for the level of free inorganic phosphorus present in the ATP used. These calculations are based on the assumption that acid hydrolysis of free ATP splits off the β and γ phosphate groups (although it is realised that a 30 min. hydrolysis may split a proportion of the α phosphate group also).

Mins. 100°C	% ATP unadsorbed by charcoal (by UV estimation)	Charcoal treated % theoretical P_i recovered	Untreated % theoretical P_i recovered.
10	0.13	77	98
"	0.02	79	98
30	0.08	96	100
"	0.14	93	105

enzyme assays the charcoal adsorbed ATP was submitted to acid hydrolysis for 30 mins. to ensure complete removal of the two terminal phosphate groups.

The amino acid activating enzymes may be assayed by measuring the ATP-^{32}P pyrophosphate exchange. (Hultin et al.1958).

The incubation mixture contained 4 umoles ^{32}PP; 6 umoles ATP; 10 umoles $MgCl_2.6H_2O$; KF 60 umoles; KCl 25 umoles; $KHCO_3$ 30 umoles; potassium phosphate 20 umoles, pH 7.8 and sucrose 350 umoles. The pH 5 fractions from the 1 hr. cell sap and the post-microsomal pellet (4 mg. protein in each case) were incubated under the above conditions for 12 mins. at 37°C. Duplicate samples were run containing L-leucine 3 umoles or an amino acid mixture containing the L forms of glycine, alanine, valine, leucine, isoleucine, serine, threonine, methionine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylalanine, tryptophan and proline, 3 umoles of each. The reaction was stopped by the addition of 6 vols. of 0.4N PCA at 0°C and after 30 mins. the protein was separated by centrifugation. In the supernatant, the ATP and pyrophosphate were separated using a modification of the method of Crane and Lipmann (1953). Norit A (100 mgs.) was uniformly suspended in 5 ml. portions of the PCA supernatant and left at 0°C for 30 mins. to ensure complete adsorption of the ATP present. The charcoal was separated by centrifugation and washed 5 times with 3 ml. water. The supernatant and washings were combined and made up to 25 ml. with distilled water. (Norit supernatant) The Norit was hydrolysed in 2N H_2SO_4 (2 ml.) for 30 mins. at

$100^{\circ}C$ to release the terminal ATP phosphates. The suspension was diluted to 6 ml. with distilled water and centrifuged. 5 ml. samples of the supernatant were taken. (ATP supernatant) Samples of the PCA supernatant and of the Norit supernatant were hydrolysed in 2N H_2SO_4 for 15 mins. at $0^{\circ}C$.

Phosphate determinations (Allen, 1940) and radioactivity estimations (liquid counters, Veall) were carried out on the ATP supernatant, the Norit supernatant and the PCA supernatant. Phosphate determinations were also carried out on the hydrolysed samples of the latter two supernatants. To control the efficiency of the adsorption process, the optical density of the Norit supernatant was measured at 260 mu and the ATP content read from a calibration curve. In addition, samples incubated without enzyme were included in all experiments. The percentage exchange was calculated using the following equation of Hoagland (1956)

$$\% \text{ Exchange} = \frac{\text{Cts./min./umole ATP}}{\text{Cts./min./umole (ATP+}^{32}\text{PP)}}$$

This gives the specific activity of the ATP as percent exchange of the value which would be obtained at equilibrium.

<u>In vivo incorporation of ^{14}C-leucine into subcellular fractions.</u>

Male albino rats were fed protein deficient diets as previously described for five days. On the sixth day ^{14}C-DL-leucine (12.5uC in 0.9%saline) was injected under ether (peroxide free) anaesthetic into the tail vein. After the time intervals stated, the animals were sacrificed, the livers removed and placed in ice cold distilled water. The exact time interval was

taken from the moment of injection until the chilling of the liver. The livers of two rats killed at each time interval were pooled and duplicate samples of the combined material taken.

The livers were blotted dry, weighed and homogenised as previously decribed in 2.5 vols. of ice cold Campbell buffer pH 7.8. A known volume of each homogenate was centrifuged at 18,000 g for 5 mins. at 0°C in the Spinco, rotor 40. The whole supernatant was topped up with Campbell buffer to fill a Spinco, rotor 40 tube and spun at 105,000 g for 60 mins. The precipitate (microsomes) was suspended by light homogenisation in Campbell buffer. The supernatant was topped up as before and separated by spinning at 105,000 g for 180 mins. into post-microsomes and 3 hr. cell sap. The latter was made up to a known volume with Campbell buffer and the former was suspended in a known volume of the same medium.

Aliquots of each fraction of approximately equal protein content were precipitated by the addition of 10 vols. 0.4N PCA (ice cold). After 30 mins. the precipitates were washed as described in Section I with 0.2N PCA followed by lipid solvents. After hot-acid extraction of the RNA the dry precipitate was dissolved in N NaOH (2 ml.) by incubating for 18 hrs. at 37°C. Estimations of nitrogen were carried out by the Nessler procedure (p63) and samples containing 1 mg. of protein were plated in each case.

<u>Preparation of post-microsomal pellet for incubation.</u>

Precipitation of the post-microsomal pellet at pH 5 was carried out as described in Section I before incubation in all

experiments except the following; firstly, the electron microscope studies described in Part I of the results and secondly, in the experiments in Part III (attempts at purificatio[n]) in which acid precipitation at pH 5 was carried out only where stated. This precipitation step was obviously used in the first instance because the pellet preparation was being compared with the pH 5 fraction of the 3 hr. cell sap. However, acid precipitation was continued in this section since it afforded a useful purification step and in addition provided a convenient method of transferring the pellet preparation from one buffer to another. In some experiments the post-microsomes were separated from a different medium than the Campbell buffer which was used for the incubation environment in the large majority of experiments. In such cases, the pellet newly separated from one buffer was superficially washed and re-suspended in a large volume of a second buffer from which it was precipitated at pH 5. The precipitate was then suspended and stored in a small volume of the second buffer.

Standard incubation system.

Aliquots of each fraction containing either equal amounts of RNA (around 225 ug.) or equal amounts of protein (in most cases 1 mg.) were incubated for periods up to 2 hrs. at 37°C with constant shaking in presence of ATP (1 or 10 umoles); ^{14}C-DL-leucine (1 uC); $MgCl_2.6H_2O$ 10 umoles; KCl 25 umoles; $KHCO_3$ 30 umoles; potassium phosphate 20 umoles (pH 7.8) and sucrose 350 umoles

Any alteration or addition to this system is noted in the legends to the tables.

(N.B. The optimal amount of ATP was shown to be 1 umole / ml. but only a small difference in activity was shown when amounts between 1 and 10 umoles ATP were added.)

Results.

Part I.

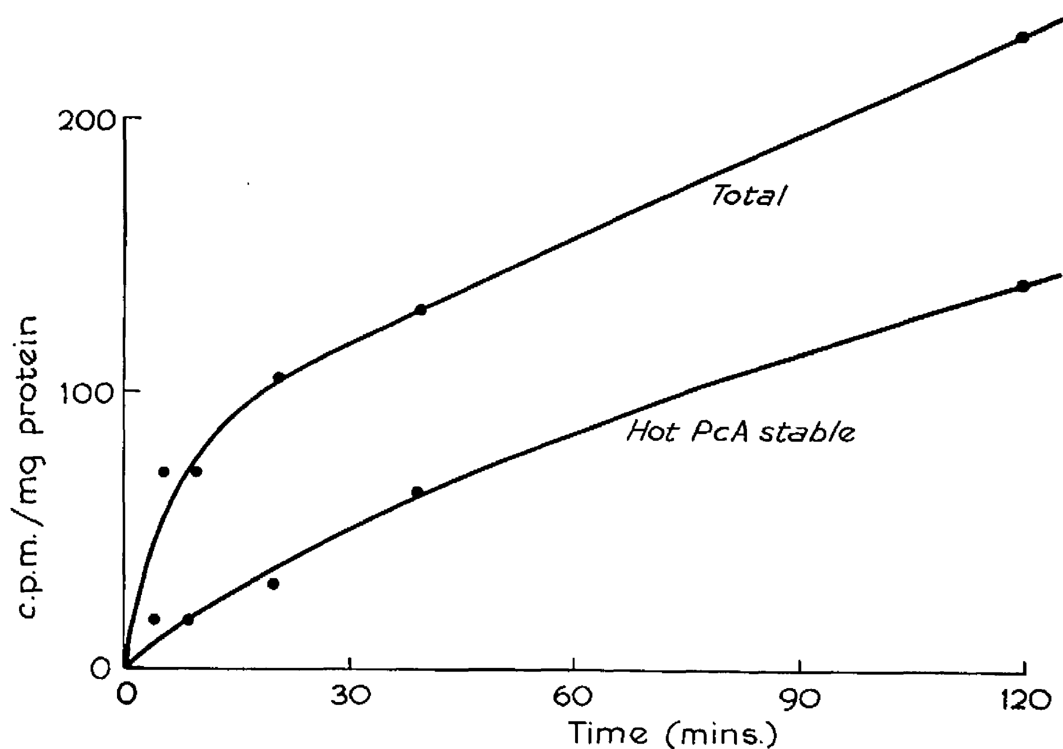
Incubation conditions affecting the uptake of ^{14}C-leucine by the post-microsomal pellet.

In the experiments described in the previous section, the incubation conditions used were those suitable for incorporation of amino acids by sRNA in the presence of activating enzymes, (Rendi and Campbell, 1959) and were not necessarily those optimum for the incorporation of amino acids by the post-microsomal pellet. Thus the factors affecting ^{14}C-leucine uptake by the post-microsomal pellet were explored. Pellet preparations from protein depleted animals were used for these investigations since they have been shown to be most active in amino acid uptake.

(a) Duration of incubation. The post-microsomal pellet was incubated under standard conditions with labelled leucine for periods of up to 2 hrs.. In the previous experiments with this fraction, the duration of incubation had been limited to 10 mins. on the grounds that sRNA becomes fully saturated with amino acids in this time. However, Fig. 10 shows that both the total uptake of leucine and the uptake into a hot-acid stable form, by the post-microsomal pellet continued linearly over the two hour period. In the case of the leucine incorporated into the hot-acid soluble form, (presumably attached to RNA) the uptake did not continue to increase beyond about 10 mins.; consequently, total uptake parallels

Fig. 10.

Time course of incorporation of ^{14}C-leucine by the post-microsomal pellet.



The post-microsomal pellet (about 1 mg. protein) was incubated for periods of up to 2 hrs. at 37°C with ATP (10 umoles) and ^{14}C-leucine (1 uC) in a total volume of 1 ml. Campbell buffer.

protein uptake after this time interval.

(b) Dependence upon ATP and Mg^{2+}. In the system used initially a large excess of ATP was added to promote labelling of sRNA by cell sap during a short period of incubation. This amount of ATP may bring disadvantages, notably its affect in combining with Mg, the latter being necessary for the stability of RNA in cell particles. In order to determine whether the concentration of ATP could be usefully reduced and whether the amount of Mg in the medium was critical, an experiment was carried out in which these two constituents of the reaction mixture were varied.

Table 18 demonstrates that the system is ATP-dependent. The counts incorporated into the pellet protein in the absence of ATP are about 10% of those obtained after 2 hrs. of incubation with any of the concentrations of ATP. The optimum uptake was achieved in the presence of the lowest concentration of ATP (1mM) used, but a tenfold increase in the ATP concentration produced only a 30% reduction in activity. The presence of a high concentration of Mg was also demonstrated to be of importance in achieving maximal amino acid uptake by the post-microsomal pellet: at 0.005M Mg level in the medium, activity was less at all levels of ATP than it was when the Mg concentration was increased to 0.01M. It will be noted that these effects of ATP concentration and Mg concentration on incorporation of labelled leucine into protein are also paralleled by similar changes in the amount of RNA remaining at the end of 2 hrs. incubation: more ATP or less Mg result in a

Table 18.

Influence of ATP and Mg^{2+} concentration on the uptake of ^{14}C-leucine by the post-microsomal pellet.

Post-microsomal pellet (0.99 mg. protein: 0.11 mg. RNA) was incubated for 2 hrs. at 37°C in a total volume of 1 ml. with ^{14}C-leucine (1 uC), sucrose 350 u moles, KCl 25 u moles, $KHCO_3$ 30 u moles, potassium phosphate, pH 7.8, 20 u moles and the concentrations of ATP and Mg^{2+} shown.

ATP u moles/ml.	0.005M $MgCl_2.6H_2O$		0.01M $MgCl_2.6H_2O$	
	ug. RNA recovered	PCA stable cts./min./mg. protein	ug. RNA recovered	PCA stable cts./min./mg. protein
10	65	172	73	285
5	57	181	64	290
1	74	225	93	355
0	80	24	98	17

reduced recovery of RNA at the end of the incubation period, a finding which is in accord with the known effects of Mg on stabilising the ribonucleoprotein particles of cells. It is notorious that ATP can interact with Mg and give rise to effects of this kind.

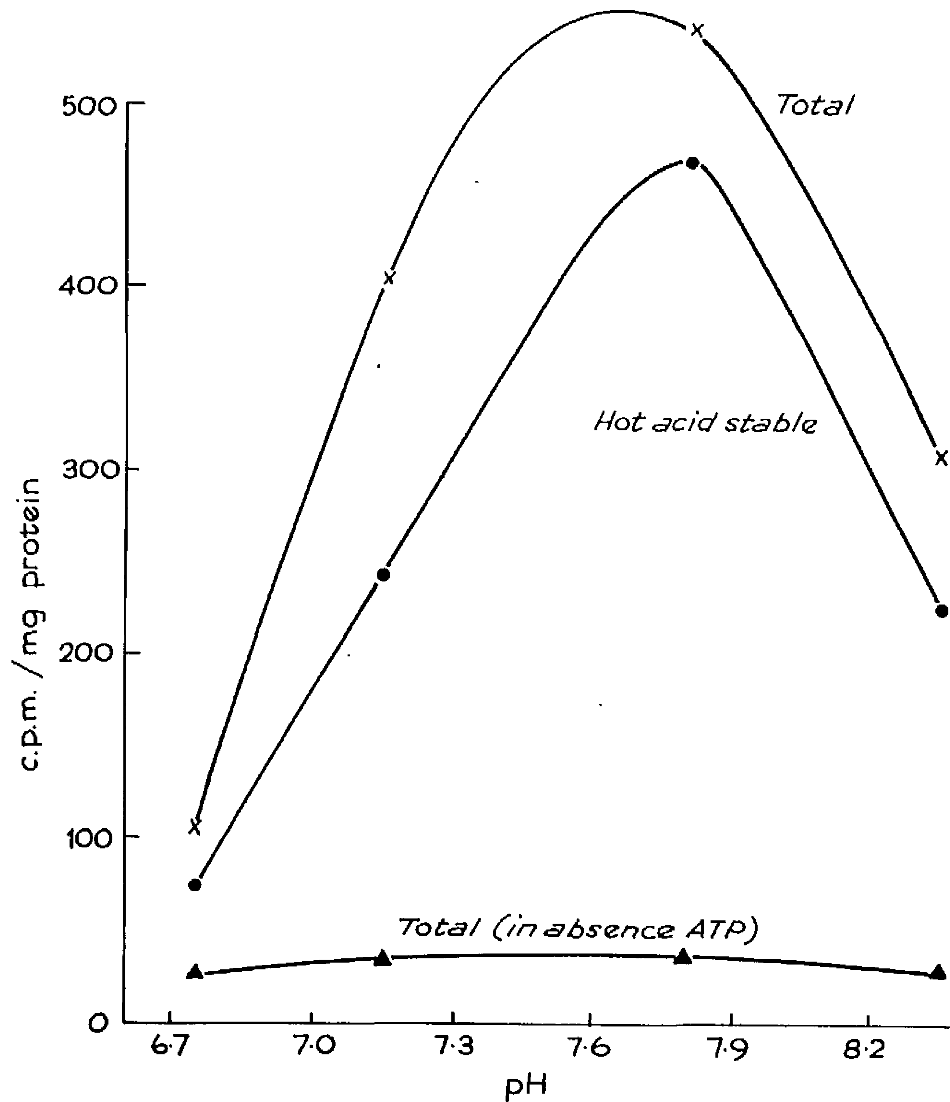
(c) Effect of pH. A single preparation of post-microsomal pellet was incubated under standard conditions in the phosphate buffered medium of Campbell which was adjusted to give pH levels varying from 6.7 to 8.4. Fig. 11 shows that the pH of the buffer (7.8) so far used for homogenisation and in the incubation medium gave a fairly sharp optimum for both total and hot-acid-stable radioactivity.

(d) Activity of post-microsomal pellet preparations suspended in other buffers. A system for amino acid incorporation into protein has been described in bacterial membranes by Beljanski and Ochoa (1958a&b), Nisman et al. (1960) and in mammalian cells by Zalta and Beljanski (1961). This system is associated with a series of enzymes which break down nucleoside triphosphates with liberation of inorganic phosphate and formation of peptides from amino acids present in the incubation medium. In order to test our pellet for the presence of such enzymes, it seemed desirable to transfer our preparation to a medium free of phosphate buffer so that the liberation of inorganic phosphorus during the course of incorporation of labelled amino acids into the protein might be examined.

The post-microsomal fraction, prepared as usual in Campbell medium, was resuspended at the end of the 3 hrs.

Fig. 11.

The effect of pH on leucine uptake by the post-microsomal pellet.



Post-microsomal pellet (about 1 mg. protein) was incubated for 2 hrs. at 37°C with ATP (1 umole) and ^{14}C-leucine (1 uC) in a total volume of 1 ml. Campbell buffer which was adjusted to provide a range of pH from 6.8 to 8.35.

centrifugation in various other buffered media which provided all the usual constituents of Campbell medium except the potassium phosphate.

Despite extensive testing of the system in various media, only Campbell medium provided any activity of note. Table 19 shows typical data obtained with several buffers. A higher activity was observed with phosphate than with tris, glycine, or veronal acetate buffer at the same pH; more significant is the fact that incorporation in the phosphate buffer was decidedly ATP-dependent whereas omission of ATP from the other buffer systems did not significantly affect the counts incorporated into the pellet protein.

Since the tris buffer has been the one of choice of the workers referred to above, who studied breakdown of nucleoside triphosphates in bacterial systems, it was studied more extensively by us in the expectation that an active system might eventually be evolved in a tris medium. Table 20 shows an experiment in which the pellet was dialysed against tris buffer and against Campbell buffer and its ability to incorporate labelled leucine was examined. It is apparent that dialysis did not diminish the capacity of the pellet suspended in Campbell medium to incorporate leucine. The pellet suspended in tris buffer was relatively inert whether dialysed or not. Furthermore, addition of other nucleoside triphosphates and of a complete amino acid mixture to this pellet failed to improve incorporation.

Since tris buffer by itself appears not to support active

Table 19.

Incubation of post-microsomal pellet in different buffered media.

Post-microsomal pellet (1 mg. protein) separated in Campbell buffer was incubated in 1 ml. of the buffer stated at pH 7.8 for 2 hrs. at 37°C with ^{14}C-leucine (1 uC), ATP (10 u moles), $MgCl_2.6H_2O$ 10 u moles, $KHCO_3$ 30 u moles, KCl 25 u moles and sucrose 350 u moles.

Buffer	ATP	ug. RNA recovered	PCA stable cts./ min./mg. protein
potassium phosphate 0.02M	+	167	126
	-	161	24
Tris/HCl 0.1M	+	138	58
	-	130	57
glycine/KOH 0.1M	+	172	58
	-	170	37
veronal/acetate/HCl 0.028M	+	105	33
	-	113	35

Table 20.

<u>The effect of dialysis on the capacity of post-microsomal preparations to incorporate amino acids in different buffers.</u>

Post-microsomal pellet prepared in Campbell medium was suspended either in this medium or in Tris buffer pH 7.8. Dialysis, where indicated, was against 2 l. of the containing medium with constant stirring overnight at 0°C. Incubation of samples with equal protein content was in 1 ml. of the containing medium with 1 uC ^{14}C-DL-leucine for 2 hrs. at 37°C. Additions were made where noted of ATP, CTP, GTP, UTP, 1umole each, a complete amino acid mixture 3 umoles each. Where the containing medium was Tris buffer the following additions were also made: $MgCl_2.6H_2O$ 10 umoles, KCl 25 umoles, $KHCO_3$ 30 umoles and 350 umoles sucrose. Only the hot-acid-stable cts./min./mg. protein are shown.

Dialysis	Additions to incubation	Campbell Buffer	Tris/HCl Buffer
-	None	-	10
	ATP	-	32
	ATP, CTP, GTP, UTP,	-	0
	ATP, CTP, GTP, UTP, amino acids.	-	32
-	None	38	19
	ATP	214	40
+	None	62	25
	ATP	256	99

uptake of labelled amino acids into post-microsomal pellet protein, it was conceivable that the presence of phosphate ions was a prerequisite of incorporation. To test this possibility, we therefore prepared pellet in Campbell buffer, resuspended it in tris buffer which contained all the constituents of Campbell medium except phosphate, and incubated it in presence of added phosphate sufficient to attain the concentration of the phosphate buffered medium of Campbell. Table 21 shows the effect of this phosphate on the ability of the pellet to incorporate leucine into the protein of the system. It will be seen that, in the Campbell buffer alone (phosphate in Table 21) the counts per mg. protein after incubation amount to,568, whereas in the tris medium alone the uptake is only 99 counts per mg. protein; addition of phosphate to the latter medium produced an activity at the end of incubation of some 71 counts per mg. protein incubated, indicating that addition of phosphate to the medium is not the essential factor in securing a highly active preparation. It may be therefore concluded that Campbell medium has some special property which permits activity in the system, or that tris has some inhibitory properties. Therefore an experiment was carried out to find which components of the Campbell buffer are necessary for incorporation of the labelled leucine by the post-microsomal pellet. In order to prevent carrying over traces of ions, the post-microsomal pellet was prepared from a homogenate of liver in sucrose phosphate buffer pH 7.8 which was free from the other constituents of Campbell buffer. The pellet so prepared.

Table 21

Influence of phosphate on leucine incorporation by the post-microsomal pellet.

Post-microsomal pellet (1 mg.protein) was incubated in 1 ml. of the buffer noted at pH 7.8 with ATP 1 umole, ^{14}-C-DL-leucine (1uC), $MgCl_2.6H_2O$ 10 umoles, KCl 25 umoles, $KHCO_3$ 30 umoles and sucrose 350 umoles for 2 hrs. at 37°C with shaking. Where noted 20 umoles potassium phosphate pH 7.8 was added.

Buffer	ATP	Additional phosphate 0.02M	ug. RNA recovered	PCA stable cts./min./mg. protein
potassium phosphate 0.02M	-	-	163	76
	+	-	173	568
tris/HCl 0.1M	-	-	166	77
	+	-	167	99
	+	+	83	71

was then incubated in the same sucrose phosphate medium and also with the additions, individually and in combination, of the various ions which make up Campbell buffer. Table 22 shows conclusively that the complete Campbell medium gives considerably greater activity than any of the deficient media. Also, Table 22 demonstrates that post-microsomal pellet prepared in the sucrose phosphate medium lacking the other ions is in no way inferior in amino acid incorporation to pellet prepared from the same liver samples using the complete Campbell medium; indeed there is less activity per mg. protein in the case of the latter pellet, a feature which will receive more consideration when we discuss the effect of preparatory media on the activity of the pellet.

(c) Summary of findings. It is apparent that the conditions for attaining active incorporation of amino acids into post-microsomal pellet are very exacting. The optimum conditions for incorporation of leucine are outlined below.

1. During incubations lasting up to 2 hrs., uptake of leucine increases both into the total pellet and into the hot-acid stable portion of the pellet.

2. An absolute dependence upon an energy source, ATP, is shown, the optimum amount being 1mM although a tenfold increase in ATP content reduces the activity by only 30%.

3. Magnesium concentrations of less than 10mM produced diminution of activity. Concentrations above this level were not tested.

4. An incubation environment of pH 7.5-7.8 provides an optimum

Table 22.

Requirement for constituents of incubation medium.

Post-microsomal pellet (1 mg. protein) separated either in Campbell buffer or 0.35M sucrose containing 0.02M potassium phosphate pH 7.8 (sucrose phosphate, pH 7.8) was incubated with ATP (1 umole) and ^{14}C-DL-leucine (1 uC) for 2 hrs. at 37°C with constant shaking. The total volume was 1 ml. and contained the additions shown.

Values in parenthesis represent radioactivity in absence of ATP.

Preparation medium	Incubation medium total volume, 1 ml.	PCA stable cts./min./mg. protein.
Campbell Buffer	Campbell Buffer	408 (36)
sucrose/ phosphate pH 7.8	sucrose/phosphate pH 7.8	35 (26)
	" + 10 umoles $MgCl_2.6H_2O$	33 (26)
	" + 30 umoles $KHCO_3$	147 (53)
	" + 25 umoles KCl	39 (26)
	" + 10 umoles $MgCl_2.6H_2O$, 30 umoles $KHCO_3$ and 25 umoles KCl	621 (36)
	(i.e. Campbell Buffer.)	

activity.

5. The presence of $KHCO_3$ and KCl was found to be necessary. The levels used were 30mM and 25mM respectively.

6. It thus appears that the complete phosphate buffer of Rendi and Campbell (1959) is necessary for activity. No alternative medium which supports amino acid uptake by the post-microsomal pellet has so far been found.

7. No leucine incorporation is observed when incubation is conducted in a tris buffered medium even in the presence of all constituents of Campbell medium including potassium phosphate. Tris either produces a specific inhibition of leucine uptake or only Campbell medium itself supports leucine uptake.

8. Separation of pellet from homogenates prepared in incomplete Campbell buffer (sucrose phosphate pH 7.8) and notably in absence of Mg produced an active preparation which retained its amino acid incorporating capacity only when the incubation was conducted in Campbell buffer.

9. No change in activity was noted if the pellet was dialysed previous to incubation. It was therefore assumed that the superficial washing and acid precipitation steps at pH 5 carried out were sufficient to remove any residual contamination by low molecular weight material.

Part II.

Properties of the post-microsomal pellet.

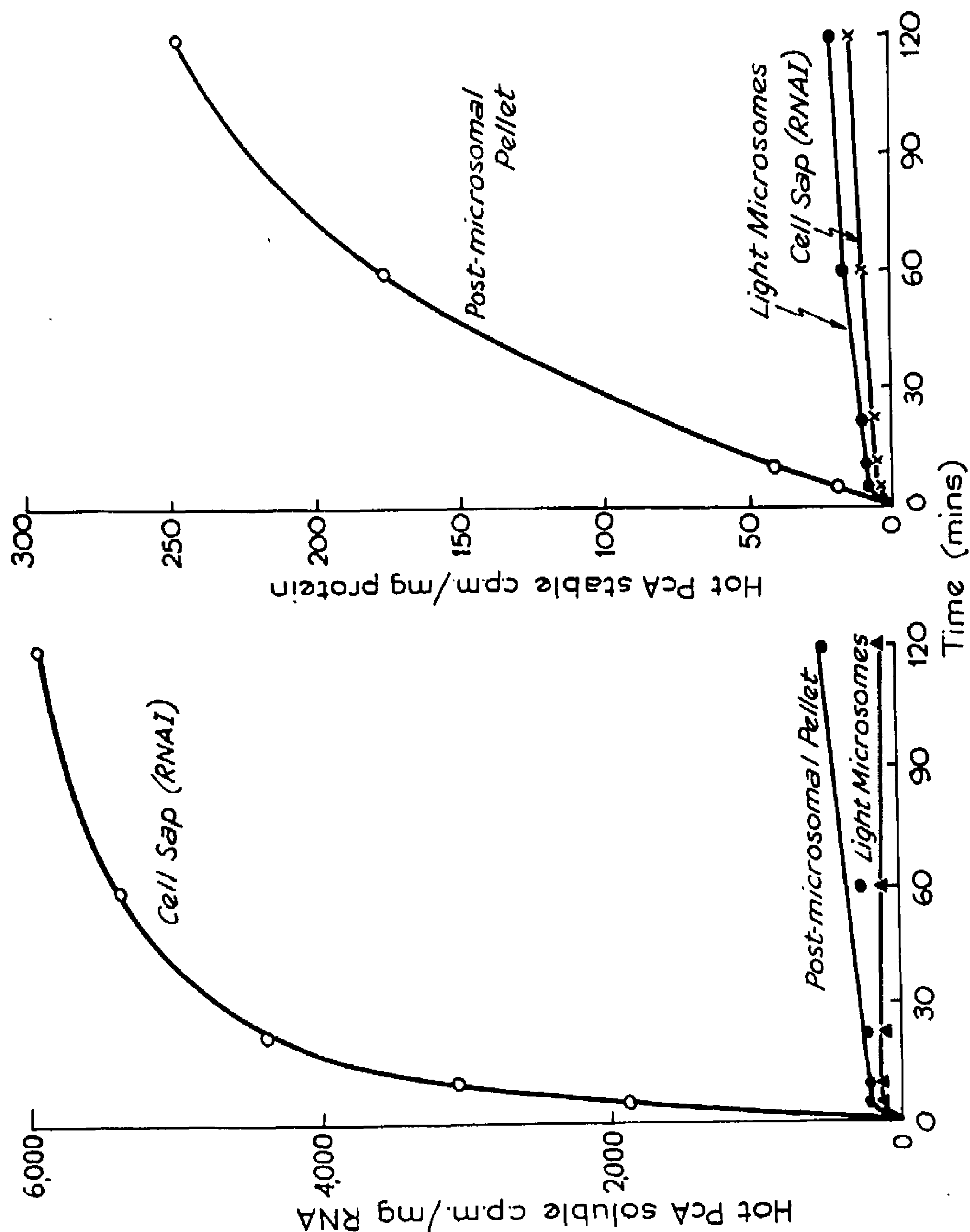
The post-microsomal pellet can incorporate amino acids into a hot-acid-stable form, protein, in the presence of ATP and yet the RNA from this fraction exhibits no soluble RNA activity. Other cell fractions have been shown to be very active sites of amino acid incorporation, but the conditions required are more complicated. The microsomes are only active in the presence of numerous factors including sRNA, activating enzymes, ATP, GTP and an energy generating system. The cell sap can incorporate amino acids in the presence of ATP into a hot-acid-labile form i.e. into sRNA. Thus it was of interest to compare the activity of these fractions under the conditions found in Part I to be optimum for amino acid incorporation by the post-microsomal pellet.

(a) Examination of RNA and protein labelling in different cell fractions at different time intervals.

Cell sap, post-microsomal pellet and the light microsome fraction were individually incubated with ATP and ^{14}C-leucine for periods up to 2 hrs.. The uptake of label into the hot-PCA-soluble fraction, RNA, of cell sap was the most extensive of these three fractions, and fell off after about 10 mins., a period comparable to that observed by Rendi and Campbell (1959) using a similar incubation system. Labelling of post-microsomal pellet RNA also fell off after about 10 mins., and was, as found before, much less than that of cell sap RNA. However, the RNA fraction of the pellet still showed considerably more labell-

Fig. 12.

In vitro incorporation of ^{14}C-leucine into sub-cellular fractions of rat liver.



Portions of the 3 hr. cell sap, the post-microsomal pellet and the light microsomes of equal protein content were incubated in 1 ml Campbell buffer with ATP (10 umoles) and ^{14}C-leucine (1 uC) at $37^{\circ}C$ for the time intervals noted.

Each point is a mean of 2 identical experiments.

ing than that of the light microsome fraction during incubation.

On examining the hot-PCA-stable activity (i.e. protein) it will be seen from Fig. 12 that the activity of this protein from cell sap was slight even after 2 hrs. of incubation, and similarly the protein of the light microsomes was only slightly labelled, but the protein of the pellet was steadily and quite extensively labelled as incubation proceeded. It must be emphasised that the conditions employed in these experiments are not those under which protein normally accepts labelled amino acids from activating enzymes and sRNA, in which the presence of GTP is a prerequisite for transfer. We shall, in fact, record in a later experiment the observation that GTP is inhibitory to post-microsomal pellet activity.

The post-microsomal pellet is capable of accomplishing considerable labelling of its own protein in vitro under conditions which preclude amino acid uptake into the protein of other fractions.

(b) Incorporation of amino acids by subcellular fractions of the liver in vivo.

In view of the higher labelling in vitro of the post-microsomal pellet protein in comparison with other cell fractions it was thought desirable to examine the uptake in vivo of ^{14}C-leucine by the protein of this cell fraction.

Microsomes, post-microsomes and 3 hr. cell sap were prepared as described in the methods section from animals previously injected with ^{14}C-leucine. The hot-acid-stable activity in these fractions is shown as a function of time in

after injection in Fig. 13. Each point is a mean of 2 experiments.

The microsomes, as befits the subcellular fraction most important in protein synthesis, exhibits the highest specific activity which rises very sharply during the first 10 mins. and then slows down and falls off quite markedly 30 mins. after exposure to the labelled amino acid. The post-microsomal pellet contains about 30% of the specific activity exhibited by the microsomes and is at all time intervals more highly labelled than the cell sap protein. The activity rises sharply during the first 10 mins. and then increases very slowly during the following 2 hrs.

(c) Uptake of ^{14}C-leucine by the protein of the post-microsomal pellet in relation to previous diet.

In section I diet was shown to have a significant effect on amino acid incorporation by the post-microsomal pellet, the material from the protein deficient animals having a higher activity than that from those an an adequate protein diet whether fasting or in the absorptive state. These results were expressed as counts per min. per mg. RNA recovered at the end of a 10 min. incubation. The finding that the pellet protein becomes more highly labelled over longer time intervals prompted us to investigate dietary effects on the amino acid uptake of the post-microsomal pellet over a 2 hr. incubation period. The same variation in response to diet was found (Table 23) whether the results in terms of radioactivity were related to protein or RNA. The post-microsomal pellet from animals on the

Fig. 13.

In vivo incorporation of ^{14}C-leucine into sub-cellular fractions of rat liver.

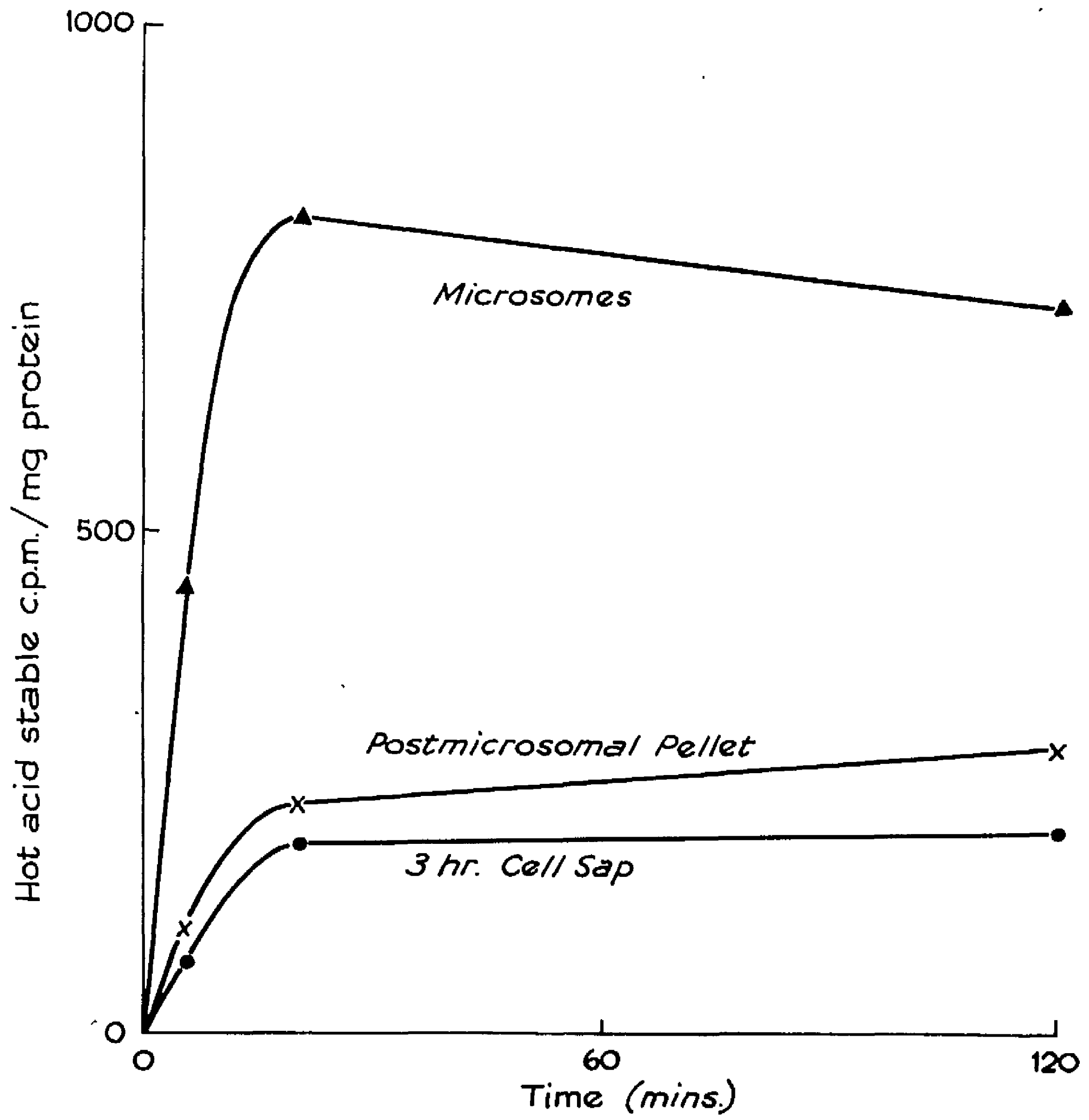


Table 23

Effect of Diet on the Post-microsomal Pellet

Portions of the post-microsomal pellet (1 mg. protein) prepared from dieted animals were incubated for 2 hrs. at 37°C with ATP (1 μm) and ^{14}C-leucine (1 μc). Values in brackets represents the uptake in absence of ATP. The data are a mean of 2 experiments.

	Total cts./min./mg.		PCA stable cts./min./mg.		PCA soluble cts./min./mg.	
	RNA	Protein	RNA	Protein	RNA	Protein
Protein deficient	1680 (430)	316(81)	217(24)	1130(166)	105(39)	637(290)
High Protein (fasting)	720 (266)	168(62)	146(40)	597(131)	24(23)	223(110)
High Protein (fed)	1070 (455)	155(66)	109(40)	832(295)	46(32)	329(249)

protein deficient diet showed the highest activity, which persists in the total, acid-soluble and acid-stable activity at the end of a 2 hr. incubation.

(d) Examination of the effect of RNAase on labelling of post-microsomal pellet protein.

It is apparent from the results shown in Fig. 10 that the post-microsomal pellet is capable of accomplishing a considerable labelling of its own protein when incubated with labelled amino acids. Labelled amino acids also appear in the fraction extractable with hot-PCA, and presumably the label in this case is attached to RNA. It follows that the RNA of the pellet may act as an obligatory intermediate in the labelling of the protein of the pellet, just as sRNA is needed before the amino acids, activated by the activating enzymes, can become attached to the protein of the ribosomes in the now classical system of protein synthesis. If this thesis is correct, then obliteration of the RNA with RNAase will destroy the capacity of the post-microsomal pellet to label its own protein with ^{14}C-leucine.

Fig. 14 shows the results obtained in an experiment in which the labelling of the protein fraction and the RNA fraction of the post-microsomal pellet was followed for a 2 hr. period in the presence of RNAase. Control tubes were run in which RNAase was omitted. The measurement of amounts of RNA present at different periods during the incubation shows that the enzyme added was effective in removing the RNA. In the control specimen, the RNA drops sharply during the first 20-30 mins., and thereafter plateaus. With addition of RNAase, the

Fig. 14.

<u>Leucine uptake into the post-microsomal pellet in the presence of ribonuclease.</u>

Post-microsomal pellet (about 1 mg. protein) was incubated in a total volume of 1 ml. of Campbell buffer with ATP (10 umoles) ^{14}C-leucine (1 uC) and RNAase (300 ug) at 37°C for the time intervals noted. Control samples to which no RNAase was added were included.

Each point is the mean of 2 identical experiments.

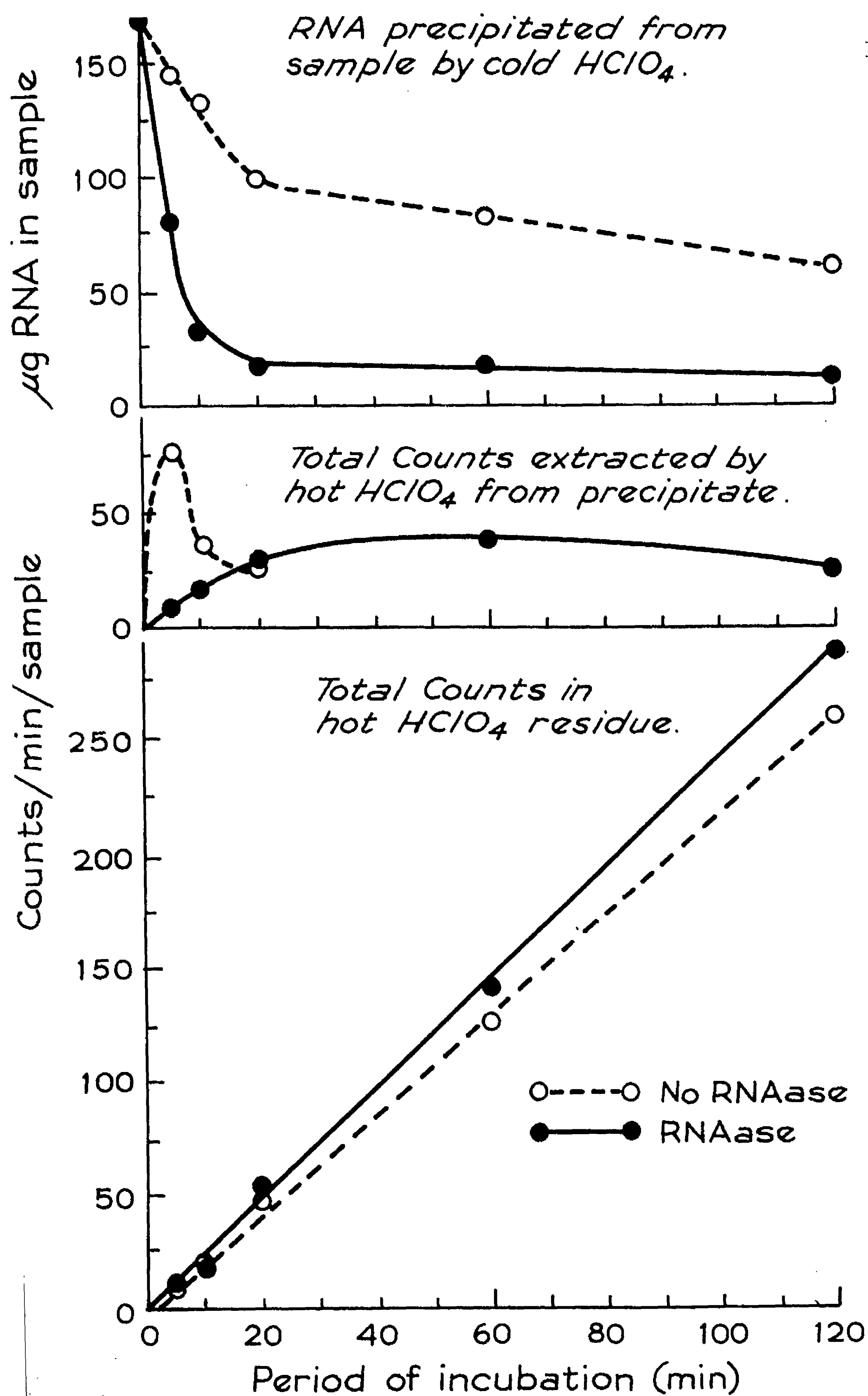


Fig. 14.

drop during thefirst 10 mins. is precipitous, and from 20 min. onwards the RNA remaining represents only about 8% of the initial RNA value and about 50% of the control specimen content at this time interval.

The effect of this removal of the RNA on the level of ^{14}C-leucine incorporated by the post-microsomal pellet can be seen from the lower 2 graphs of Fig. 14. The activity extractable with hot-PCA ('RNA') rises initially in the control specimen to a much higher level than in the RNAase-treated specimen, suggesting that at 10 mins. of incubation these counts are in fact connected with the presence of RNA in this extract. However, at later time intervals, the hot-acid-labile activity of the control and RNAase treated samples remained at approximately the same low level even although the RNA had virtually disappeared from the RNAase treated samples. No correlation was therefore found between hot-acid-labile activity and RNA content.

The lowest graph in Fig. 14 exhibits the counts which remained in the residue after extraction with hot PCA, and are assumed to be incorporated into the protein of the post-microsomal pellet. The scale of the activity is the same as that of the graph of activity extractable with hot PCA, and it will be seen that most of the activity taken up by the pellet at the end of 2 hrs. incubation is located in the protein fraction. Furthermore, the presence of RNAase has, if anything, stimulated uptake into this protein.

These findings indicate that the integrity of the RNA of

Table 24.

Effect of ribonuclease on incorporation of amino acids by subcellular fractions.

Amounts of each cell fraction were taken to provide approximately 225 ug. RNA per tube initially; the initial protein content per tube averaged 4.56 mg. for cell sap, 0.87 mg. for post-microsomes and 1.01 mg. for light microsomes. The samples were incubated for 10 mins and 60 mins. at 37°C in 1 ml. Campbell buffer to which were added ATP 10 umoles, and ^{14}C-DL-leucine 1 uC. RNAase when added provided 300 ug./tube. The data are the average of 2 identical experiments.

Cell fraction	RNAase	% RNA/tube after incubation		Hot PCA extractable cts/min/tube		PCA stable cts/min/tube	
		10'	60'	10'	60'	10'	60'
Cell sap	-	100	71	687	978	7	21
	+	6	4	4	9	9	19
Post-microsomal pellet	-	72	48	351	53	29	119
	+	20	10	6	10	18	147
Light Microsomes	-	94	59	31	13	3	9
	+	24	10	4	4	8	13

the post-microsomal pellet is not a necessary condition for the labelling of the protein of this fraction.

(c) Examination of the action of RNAase on labelling in other cell fractions.

The occurrence of a RNAase resistant system of protein labelling in the post-microsomal pellet was so surprising that it was deemed advisable to examine the action of RNAase on protein labelling in other cell fractions. In particular, it was thought that the light microsomal fraction, which also contains high molecular weight RNA, might show a similar degree of activity in its protein after removal of the RNA with RNAase.

Table 24 shows the effect of RNAase on the activity of cell sap, the light microsome fraction and post-microsomes after 10 mins. and 60 mins. of incubation with ATP and labelled leucine. The greatest activity extractable with hot PCA was obtained with the cell sap preparations; this activity presumably associated with the RNA, was wholly suppressed by adding RNAase at the start of incubation. A smaller but still significant amount of radioactivity obtained in the hot PCA extracts of the post-microsomal preparations was also RNAase sensitive. The microsomes showed little uptake of label which could be extracted with hot PCA.

Incorporation of leucine by the protein (hot-PCA extracted residues) of the cell sap and the microsomes was slight. However, incorporation into the protein of the post-microsomal pellet was considerable and addition of RNAase at the start of

incubation, slightly stimulated this uptake confirming the results shown in Fig. 14. Because the incubation mixtures for each fraction had been adjusted to provide the same initial RNA content, the post-microsomal preparations contained less protein per tube than the other 2 fractions; consequently, the difference in uptake of the post-microsomal pellet and the other 2 fractions is accentuated when related to the amount of protein incubated.

The RNA content at the end of incubation showed that the RNAase was indeed effective; especially sensitive was the RNA of the cell sap which was reduced to negligible amounts within the first 10 mins. of incubation. With this disappearance of RNA from the cell sap fraction goes the capacity to accept labelled amino acids.

(f) Addition of a pH 5 enzyme preparation to the post-microsomal pellet.

The post-microsomal pellet appears to be unique among the fractions considered in that its ability to incorporate amino acids is independent of its RNA content. If this is in fact correct, then addition of pH 5 enzyme should not augment the incorporation by the post-microsomal pellet, since the pH 5 enzyme provides a system in which sRNA plays an integral part. Thus, a pH 5 fraction prepared from the 3 hr. cell sap was incubated alone and with the post-microsomal fraction under standard conditions (Table 25). The presence of sRNA and activating enzymes from the 3 hr. cell sap far from stimulating amino acid incorporation by the post-microsomal pellet produced

Table 25.

Addition of pH 5 enzyme to post-microsomes.

The cell fractions were separated from sucrose phosphate EDTA pH 3.4. Aliquots of the post-microsomal pellet (1 mg. protein) and of the pH 5 enzyme fraction from the 3 hr. cell sap (1 mg. protein) were incubated in 1 ml. Campbell buffer with ATP (1 umole) and ^{14}C-leucine (1 uC) for 2 hrs. at 37°C with shaking. (The values in parenthesis were obtained in absence of ATP)

Fraction incubated	Total uptake cts./min./mg. protein	PCA stable cts./min./mg. protein	PCA soluble cts./min./mg. protein
post-microsomes	2,480 (117)	1,375	1,105
pH 5 enzyme	305 (43)	80	225
post-microsomes+ pH 5 enzyme	515 (225)	156	259

an 80% reduction in activity.

(g) Effect of preincubation in presence and absence of RNAase and the effect of phosphoenolpyruvate, pyruvate kinase and GTP, on amino acid uptake of the post-microsomal pellet.

Even although cell sap RNA is extremely vulnerable to the effects of RNAase it was thought possible that the RNA present at the beginning of incubation, could function sufficiently as an amino acid carrier before it was reduced to a low level. If this is so, incubation with RNAase before the addition of an energy source and the radioactive amino acid would inhibit sRNA dependent leucine incorporation.

Preincubation of the pellet in presence of RNAase (Table 26) has no effect on the hot-acid-stable counts and increases the hot-acid-soluble counts even although the RNA is reduced to a low level. In absence of RNAase, preincubation caused an increase in the hot-acid-stable and labile counts.

Table 26 also provides data on the effects of adding pyruvate kinase, phosphoenolpyruvate and GTP which are necessary for incorporation into microsomes. A slight stimulation in the protein counts may have occurred but no outstanding effects were found, and it was not considered sufficiently promising to justify repetition.

(h) Effects on amino acid incorporation by post-microsomal pellet due to the addition of various factors involved in protein synthesis.

Using detergents to disrupt liver microsomes Zalta (1960) obtained evidence of a RNAase resistant system for amino acid

Table 26.

Influence of preincubation and of the addition of GTP, PEP and pyruvate kinase on the amino acid incorporation of the post-microsomal pellet.

Aliquots of the post-microsomal pellet (1 mg. protein) were incubated for 1 hr. in 0.5 ml. Campbell buffer with shaking in the presence or absence of 300 ug. RNAase. The tubes were then chilled and 10 umoles ATP and 1 uC ^{14}C-leucine added. In some cases PEP (100 umoles), GTP (5 umoles) and pyruvate kinase (300 ug.) were also added. These samples and a control sample which had not been preincubated were incubated at 37°C for a further 1 hr. in a total volume of 1 ml. Campbell buffer.

Addition before preincub -ation	Preincub -ation	Additions before final incubat- ion	ug.RNA/tube in relation to final incubation		Hot acid soluble Cts./min. /mg. protein	Hot acid stable Cts./min. /mg. protein
			Before	After		
None	-	None	146	79	70	130
None	+	None	79	62	225	338
RNAase	+	None	16	7	230	102
None	+	PEP GTP PK	79	62	117	80
RNAase	+	PEP GTP PK	16	7	56	64

incorporation. In some of his preparations the combined addition of all four nucleoside triphosphates, an amino acid mixture and RNA resulted in a considerable increase in incorporation. In our system, the inclusion of the nucleotide mixture increased total uptake of ^{14}C-leucine to the same extent in the absence and presence of added amino acids (Table 27). However, the presence of amino acids in the reaction mixture resulted in a redistribution of radioactivity when the triphosphates were added; the hot-PCA-soluble activity fell and the activity in the protein rose.

Addition of individual nucleotides to the post-microsomal pellet pinpointed ATP as the only nucleotide which would act as an energy source; no other single nucleotide would support the incorporation of ^{14}C-leucine into the post-microsomal pellet. In the presence of ATP, other nucleotides caused a distinct inhibition of leucine incorporation. (Table 28).

The activity of the S protein of Sachs (1957) which stimulates transfer of amino acids into microsomal protein apparently in the absence of sRNA (Kendi & Campbell, 1959) is significantly increased in the presence of -SH groups in the form of glutathione.

However the post-microsomal pellet whether dialysed or undialysed, shows a distinctly reduced ability to incorporate amino acids in the presence of glutathione. (Table 29)

Chloramphenicol, a suppressor of protein synthesis in many systems, has no effect on the amino acid incorporating ability of post-microsomal pellet. (Table 30)

Table 27.

Effect of addition of amino acids and nucleotides to the post-microsomal pellet.

Aliquots of post-microsomal pellet with equal RNA content were incubated at 37°C with shaking in a total volume of 1 ml. Campbell buffer with 1 umole ATP and 1 uC ^{14}C-leucine for 2 hrs. Nucleoside triphosphates were added to give a final concentration of 60 mumoles/tube and also an amino acid mixture of 17 L-amino acids (leucine omitted) 5 mumoles/tube of each. The data are the average of 2 identical experiments.

Additions	RNA at end of incubation ug.	Total activity Cts./tube	Hot PCA soluble activity Cts./tube	Hot PCA residue activity Cts./tube
0	80	1,283	322	961
GTP CTP, UTP	59	1,479	402 (+80)	1077 (+116)
Amino acids	61	1,113	379	734
Amino acids + GTP, CTP, UTP	58	1,440	145 (-234)	1,295 (+561

Table 28.

Uptake of amino acids by post-microsomal pellet in the presence of individual nucleotides.

Post-microsomal pellet (1 mg protein) was incubated in a total volume of 1 ml. Campbell buffer for 2 hrs. at 37°C with 1 uC ^{14}C-DL-leucine, 1 umole ATP and 60 mumoles GTP, CTP, and UTP where noted.

Additions	PCA stable cts./min./mg. protein.
0	85
ATP	588
GTP	33
CTP	38
UTP	41
ATP+GTP	382
ATP+CTP	302
ATP+UTP	264

Table 29.

Effect of glutathione on the incorporation of amino acids by dialysed and untreated post-microsomal pellet.

Aliquots of post-microsomal pellet of equal protein content were incubated for 2 hrs. at $37^{o}C$ in 1 ml. Campbell buffer with ATP (1 umole) and ^{14}C-DL-leucine (1 uC). Glutathione (1.2 umoles) was added where indicated.

(The values in brackets were obtained in the absence of ATP)

Treatment	GSH	Total cts./min./mg. protein	PCA stable cts./min./mg. protein
Dialysed	-	2,265 (71)	713
Dialysed	+	835 (64)	416
None	-	1,175 (73)	1,076
None	+	655 (116)	497

Table 30.

Influence of chloramphenicol on the uptake of amino acids.

by post-microsomal pellet.

Post-microsomal pellet (1.45 mg. protein) was incubated in 1 ml. Campbell buffer for 2 hrs. at 37°C with ATP (10umoles) and ^{14}C-DL-leucine (1 uC). Graded amounts of chloramphenicol were added as shown.

Chloramphenicol ug.	Total cts./min./tube	Hot PCA stable cts./min./tube
0	716	370
5	562	331
50	445	362
500	534	430

(i) Activating enzymes in post-microsomal pellet.

The level of activating enzymes in the post-microsomal pellet was assayed by measuring the rate of isotope equilibration between ^{32}P-pyrophosphate and ATP in the presence of added amino acids. As a control, estimation of the ATP-PP exchange in the pH 5 fraction prepared from 1 hr. cell sap from the same animals was measured simultaneously.

The soluble fraction of the liver is known to contain enzymes which activate substrates other than amino acids with the release of pyrophosphate from ATP (Lipmann, 1954). An active ATPase is also present. To avoid this non-specific exchange which takes place in the absence of added amino acids, both the 1 hr. cell sap and the post-microsomal pellet were submitted, as in the previous experiments to acid precipitation at pH 5 from a large volume of Campbell buffer before the assays were carried out. To suppress the activity of the ATPase potassium fluoride was added to the incubation system. These precautions were successful, especially in the case of the post-microsomal pellet where only 10% of the total exchange was observed in the absence of added amino acids. A higher endogenous exchange was observed in the cell sap pH 5 enzyme fraction (about 30% of the total). However, Hultin et al. (1958) have obtained this level of non-specific exchange even when the pH 5 enzyme fraction was dialysed before use to remove endogenous amino acids. Table 31 shows that the total activating enzyme activity in the cell sap was in good agreement with that found by Hultin et al. (1958) (recalculated by the equation

Table 31.

Amino acid activating enzymes in the post-microsomal pellet and 1 hr. cell sap.

The activating enzyme level was assayed in the pH 5 fractions of the post-microsomal pellet and 1 hr. cell sap by measurement of the amino acid dependent ^{32}P exchange between ATP and PP as described in detail in the text.

The values given were calculated by the equation of Hoagland et al. (1956).

Additions	% exchange*	
	Post-microsomal	1 hr. cell sap
None	2.5	9.0
16 L-amino acids	24.4	26.3
L-leucine	20.0	16.5

* % exchange of ATP and pyrophosphate in absence of added enzyme was 0.4

of Davie *et al.*,(1956)) and the activating enzyme activity obtained for leucine was the same as described by Hoagland *et al.* (1956).

No concentration curve for activating enzymes was made and comparison is made of the percentage exchange caused by equal amounts of protein from these two fractions. Thus, the total activating enzyme activity found in the post-microsomal pellet was almost equal to that found in the 1 hr. cell sap, and was even greater than that in the 1 hr. cell sap when the endogenous exchange was subtracted before comparison. Leucine activating enzyme appears to have been concentrated in the post-microsomal pellet since leucine alone produces a greater percentage exchange between ATP and pyrophosphate in this fraction than in the 1 hr. cell sap, although the two fractions were adjusted to provide the same amount of protein in the incubation mixture.

(j) Uptake of amino acids other than leucine by the post-microsomal pellet.

In bacterial systems nucleoside triphosphates other than ATP have been shown to catalyse amino acid incorporation into peptides (Beljanski, 1960). The amino acids display a limited specificity for one of the four common nucleotides and are not incorporated into peptides unless a correct energy source is provided. In our system incorporation of leucine by the post-microsomal pellet is dependent upon ATP but the characteristics of the system are at variance with sRNA dependent protein synthesis in many respects. For this reason the uptake of

Table 32.

Incorporation of different amino acids by the post-microsomal pellet.

Post-microsomal pellet (1 mg. protein) was incubated in a total volume of 1 ml. of Campbell buffer for 2 hrs. at 37°C with DL-leucine-1-^{14}C (1 uC), DL-alanine-1-^{14}C (1 uC), DL-phenylalanine-1-^{14}C, (1 uC), DL-glutamic acid-1-^{14}C (1 uC) or glycine-2-^{14}C (1 uC ATP, GTP, CTP, or UTP each 1 u mole were added where noted. These figures represent amino acid incorporated into a hot PCA stable form.

Additions	muM amino acids incorporated per mg. protein 10^{-2}.				
	leucine	phenyl-alanine	glutamic acid	alanine	glycine
None	16	26	19	53	78
ATP	97	26	22	39	72
GTP	14	21	23	51	67
CTP	14	21	26	61	63
UTP	17	21	18	57	79
ATP+GTP+ CTP+UTP.	33	24	26	39	61

several amino acids by the post-microsomal pellet was studied in the presence of different nucleoside triphosphates.

Table 32, shows, in agreement with the earlier experiments, that leucine uptake is supported only by ATP. In contrast, phenylalanine and glutamic acid are not incorporated under any of the conditions tested. Alanine and glycine are incorporated at a rate which is approximately 50% and 70% respectively of that observed with leucine. This incorporation is not however dependent on the addition of nucleotides and is notably independent of ATP. Zero time controls were not included in this experiment mainly because of restrictions in available pellet material and partly because zero time controls for leucine incorporation have been shown to have negligible activity. The activity due to alanine and glycine could therefore be caused by contamination, but this seems unlikely since with leucine in an ATP-free medium the level of incorporation is very much lower (Table 32).

(k) Morphological and chemical characteristics of the post-microsomal pellet.

The electron microscope pictures showed the post-microsomal pellet to consist of small particles uniformly distributed (Plate 1) with no visible vesicular structures as shown in a similar photograph of microsomes (Plate 2). This difference is borne out by the variations in chemical content noted.

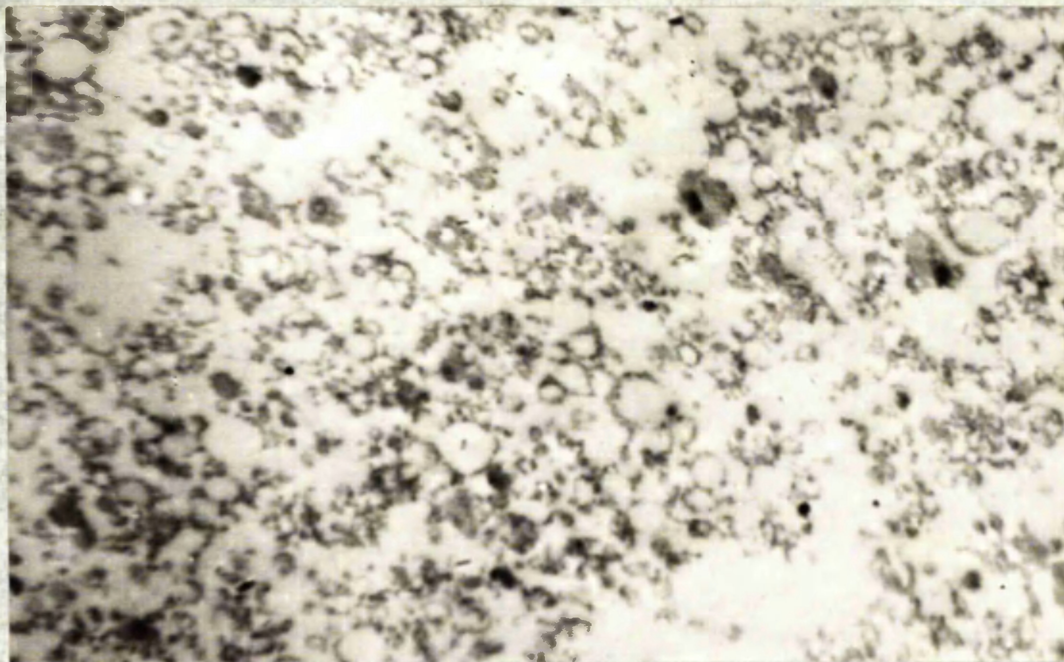
(l) Summary of findings.

(i) Among the fractions studied under our incubation conditions the post-microsomal pellet was unique in being able to incorporate labelled leucine in an

Plate 1. Microsomes.

Immediately after separation from Campbell medium, a small portion of the microsome fraction was taken and placed in osmic acid buffered to pH 7.4. After dehydration, the material was embedded in n-butyl methacrylate and sections cut.

Magnification; 13,300.



$\frac{\text{RNA}}{\text{Protein}} = 0.179$ $\frac{\text{Lipid P}}{\text{Protein}} = 0.284$

Plate 2. Post-microsomal pellet.

The sections were prepared as described for Plate 1.

Magnification: 13,300.

$\frac{\text{RNA}}{\text{Protein}} = 0.239$ $\frac{\text{Lipid P}}{\text{protein}} = 0.118$

These analyses were carried out on pH 5 precipitated material.

ATP as sole energy source. The system has considerable stability since incorporation continues unabated in intensity over at least a 2 hr. period of incubation. A small proportion of the total amino acid incorporation is soluble in hot acid, suggesting that the RNA of the post-microsomal pellet plays some part in incorporetion of labelled leucine, though the evidence presented in Section I of this thesis shows that sRNA is not likely to be present.

(ii) When radioactive leucine was injected in vivo into rats, it was found that the protein of the post-microsomal fraction of the liver was labelled to a level approaching 1/3 of the activity in the microsomal proteins. This radioactive uptake is most intense during the first 10 mins. after injection, and thereafter increases very slowly.

(iii) Under in vitro labelling conditions, uptake of ^{14}C-leucin by the hot-acid-stable protein portion of the post-microsomal pellet showed the same effects of dietary conditions as those noted with The RNA of this cell fraction in the preceding section of the thesis. The highest capacity to incorporate label into the protein of the fraction was observed in animals previously fed on a protein free diet.

(iv) Treatment of the post-microsomal pellet with RNAase caused a reduction of RNA content to 8% of its original value without affecting its capacity to incorporate labelled leucine into the pellet protein in a form stable to hot acid. In some instances the incorporation appeared to be augmented by RNAase treatment. At 10 mins. of incubation, specimens of pellet not

treated with RNAase showed a maximum acid-labile uptake (presumably associated with RNA), but this peak was absent in the RNAase-treated specimens; it is possible that this RNAase labile uptake represents traces of sRNA in the pellet. The loss of this sRNA does not have any detrimental effect on incorporation into the protein. Amino acid uptake by cell sap preparations was highly sensitive to the presence of RNAase; the uptake into the hot-acid-labile portion, representing sRNA uptake, was the main incorporation and was abolished by enzyme treatment. This is consonant with the main uptake by cell sap being into sRNA, a picture which is completely different from that found in the post-microsomal pellet. Similarly, the effect of incubation of microsomes with ^{14}C-leucine in the presence of RNAase differed from the results observed with post-microsomal preparations; the microsomes took up little labelled amino acid during incubation and were unchanged by the presence of RNAase.

(v) Addition of amino acid activating enzymes together with sRNA, in the form of a pH 5 enzyme preparation, failed to stimulate amino acid uptake by the post-microsomal pellet; on the contrary, these caused a marked inhibition of incorporation.

(vi) Glutathione causes inhibition of amino acid incorporation into the protein of the post-microsomal pellet, but chloramphenicol over a wide range of concentrations produced no consistent action on uptake of amino acids.

(vii) Preincubation of the post-microsomal pellet in Campbell buffer resulted in a pronounced stimulation in

incorporation both in hot-acid-labile and hot-acid-stable forms. In the presence of RNAase, the increase noted in the hot-acid-stable counts was abolished.

(viii) The addition to the post-microsomal pellet of GTP, pyruvate kinase and phosphoenolpyruvate in proportions sufficient to support amino acid incorporation by microsomes failed to increase uptake of ^{14}C-leucine by the post-microsomes.

(ix) Incorporation is slightly reduced by addition of a complete amino acid mixture. Uptake was not supported by nucleoside triphosphates other than ATP, and in the presence of ATP these nucleotides had an inhibitor action. Addition of the amino acid mixture with all four nucleotides caused no greater stimulation of total uptake into the pellet preparation than that produced by the nucleotide mixture alone, but a redistribution took place resulting in more of the labelled amino acid being incorporated into the hot -acid-stable fraction (protein) and less into the hot-acid-labile fraction (RNA).

(x) The total activating enzyme activity in the post-microsomal pellet is almost as high as that found in the 1 hr. cell sap. The leucine activating enzyme is more active in the post-microsomal pellet since the pyrophosphate exchange caused by leucine alone is greater than that found in the 1 hr. cell sap and represents a large proportion of the total amino acid dependent exchange in the post-microsomal pellet.

(xi) Individual amino acids are incorporated into the post-microsomal pellet at different rates. Leucine is incorporated

at the highest rate whereas phenylalanine and glutamic acid are not incorporated. The significance of the uptakes of alanine and glycine, which are smaller than that of leucine, is not understood since no nucleotide dependence was found.

Apart from leucine, the specific activity of the amino acids remained unchanged in the presence of nucleoside triphosphates other than ATP. Leucine uptake took place only in the presence of ATP.

Part III.

Attempts to attain larger yields of post-microsomal pellet.

One of the difficulties so far encountered was the small yield of post-microsomal pellet obtained from the livers of large batches of animals by a long separation procedure. The situation was complicated by the instability of the pellet material even when stored in the cold. Purification on such a small scale proved a profitless exercise and so attempts were made to prepare larger amounts of post-microsomal pellet and incidentally, to throw some light on the source of this material.

(a) Treatment of microsomes with deoxycholate or pyrophosphate.

For the following reasons it was supposed that the post-microsomal pellet was derived from some part of the endoplasmic reticulum. The RNA from these fractions has been shown by column chromatography to be very similar. The data presented in Section I suggest that part of the pellet material arises from the endoplasmic reticulum when certain changes occur in the diet. However, the intact microsomes do not display any capacity for taking up amino acids in the way observed with post-microsomal preparations and therefore, attempts were made to obtain active preparations by disrupting microsomes with deoxycholate and pyrophosphate; one to solubilise the membrane and the other to disrupt the ribosomes.

Microsomes were prepared from the livers of stock animals and treated with Na deoxycholate by the method of Littlefield <u>et al.</u> (1955). The limiting membrane and content of the vesicles

Table 33.

The uptake of labelled leucine by microsomes.

Aliquots of the post-microsomal pellet and of microsomes, treated as listed below, containing 1 mg. protein were incubated in 1 ml. Campbell buffer with ATP (10 umoles) and ^{14}C-DL-leucine (1 uC) for 2 hrs. at 37°C.

(Values in brackets were obtained in absence of ATP.)

Fraction	Treatment	Total cts./min./mg. protein
Post-microsomal pellet	none	531 (181)
Microsomes	none	45 (30)
Microsomes	sucrose washed	80 (38)
Microsomes	pyrophosphate treated	54 (49)
Microsomes	deoxycholate Treated	24 (17)

is solubilised leaving ribonucleoprotein particles which were easily separated by centrifugation and examined for post-microsomal pellet activity under the standard conditions. No active preparation was found (Table 33). This finding was not unexpected since Kirsch _et al._,(1960) have shown that the ribonucleoprotein particles similarly prepared from pig liver incorporate amino acids only in the presence of PEP, pyruvate kinase, GTP and an energy source. Alternatively the ribosomes were solubilised using Na pyrophosphate (Sachs, 1957) and the membranous component of the microsomes was recovered. By testing under the standard incubation conditions a highly active preparation was obtained on one occasion but this finding could not be duplicated on several subsequent attempts. Thus, the membrane portion of the endoplasmic reticulum did not exhibit the behaviour characteristic of post-microsomes.

In view of the complete failure of these attempts, a table including only one set of typical results is included (Table 34).

(b) Seperation of post-microsomal pellet from liver homogenised in different buffers.

Since attempts to fractionate the microsomes failed to provide a preparation with activity similar to that observed in the post-microsomal pellet, we proceeded to prepare homogenates of liver in different media in the hope that the yield or the activity of the preparation might be augmented. It may be noted at this time that in the preceding experiments the incubation environment of the pellet was varied, and the medium

used to prepare the homogenate for separation of the pellet was always that of Campbell. In this series the procedure was reversed, various preparatory media were used but Campbell buffer was used in each case as the incubation environment.

The pellet was prepared in the buffer noted (Table 35), then resuspended and incubated in Campbell buffer. The activity of these pellet preparations are shown in relation to the activity of that prepared in Campbell buffer. The first finding of note is that separation of the post-microsome pellet in a phosphate free medium containing only sucrose provided an active preparation in contrast to the requirement for phosphate during incubation. Addition of phosphate to the sucrose augments the pellet activity only slightly. Preparation in tris buffer results also in an active post-microsomal pellet although the hot-acid-stable counts are low. (This is interesting since the reverse procedure i.e. preparation in Campbell buffer and incubation in a phosphate free tris buffer results in a pellet preparation which fails to incorporate leucine.) The post-microsomal pellet separated from a sucrose EDTA homogenate possesses a very high activity, a surprising finding in view of the low initial pH of the medium used. Preparation of post-microsomal pellets in a medium containing pyrophosphate in the presence or absence of EDTA produced a large yield of an RNA rich pellet. Unfortunately this material possessed little ability to incorporate amino acids, indicating that the extra bulk is due to material inert in this system and also supports the concept that microsomal fragments released from the reticulum by pyrophosphate treatment do not contribute to the activity of

Table 35.

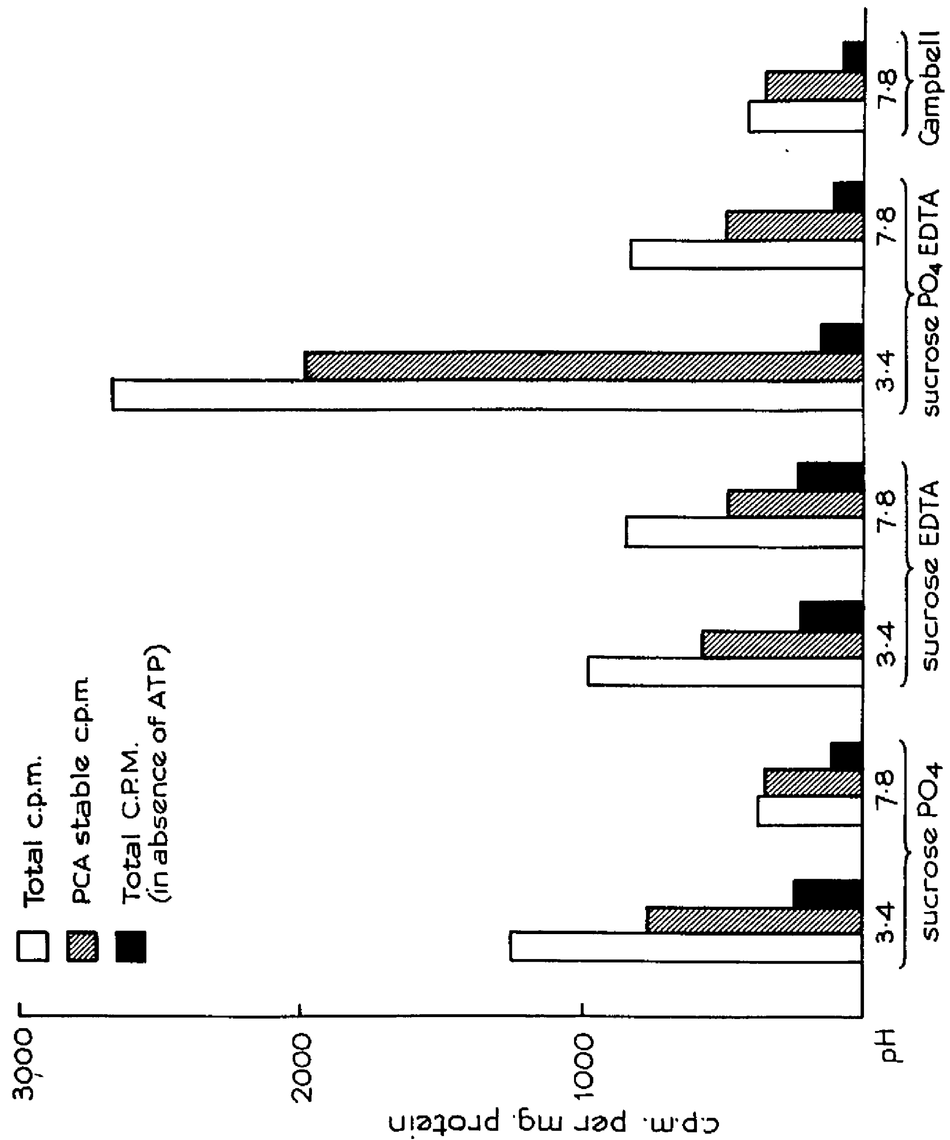
Effect of preparation medium on the activity of the post-microsomal pellet.

Post-microsomal pellet prepared in the buffers listed was suspended and reprecipitated at pH 5 in Campbell buffer. Aliquots of the precipitated material of equal protein content were incubated in 1 ml. Campbell buffer with 1 umole ATP and 1 uC ^{14}C-leucine for 2 hrs. at 37°C.

Homogenisation medium	RNA/Protein in pellet	Total cts./min./mg. protein		TCA stable cts./min./mg. protein		No. of experiment
		+ATP	-ATP	+ATP	-ATP	
Campbell	0.191	140	24	102	11	3
Sucrose 0.35M	0.173	163	-	130	-	2
Sucrose phosphate pH 7.8	0.160	195	22	124	11	1
Sucrose 0.35M Tris 0.1M pH 7.8	0.232	150	-	87	-	1
Sucrose 0.35M EDTA 0.001 M pH 3.4	0.167	408	-	240	-	2
Sucrose 0.35M Pyrophosphate 0.11M pH 7.4	0.502	69	-	33	15	2
Sucrose 0.35M Pyrophosphate 0.11M EDTA 0.001M pH 7.4	0.527	-	-	28	54	1

Fig. 15.

Activity of post-microsomal pellet prepared in various media.



Portions of post-microsomal pellet (about 1 mg. protein) prepared in the media noted below the diagram were incubated for 2 hrs. at 37°C with ATP (1 umole) and ^{14}C-leucine (1 uC) in a total volume of 1 ml. Campbell buffer at pH 7.8.

the post-microsomal pellet.

When initial homogenisation is carried out in a Mg free buffer e.g. sucrose phosphate pH 7.8 (Tables 27 & 35), the activity of the resulting pellet did not decline as was anticipated due to dilution with ribosomal breakdown products and was often stimulated. Therefore the complete removal of the Mg from the preparatory medium by inclusion of the metal binding agent EDTA was tested (Table 35). The post-microsomal pellets prepared in this way showed a pronounced increase in total and PCA stable counts. It was noted that this action of EDTA was usually accompanied by a considerable reduction in the pH of the medium and accordingly further exploration of post-microsomal activity was made in relation to Mg content, EDTA content and pH of the preparatory medium.

At pH 7.8, the sucrose phosphate pellet and the Campbell pellet had about equal activities whereas the sucrose EDTA and sucrose phosphate EDTA pellets had activity higher than either of these (Fig. 15). The finding of activity in the post-microsomal fraction after preparation in sucrose EDTA confirms that the phosphate content of the homogenisation buffer is not essential for separation of active post-microsomes. At pH 3.4, the pellets prepared in the various buffers showed higher activities than that prepared in Campbell medium, as regards both total and hot-PCA-stable counts. The fraction containing very high levels of radioactivity was, however, prepared in sucrose phosphate EDTA pH 3.4, and was found to retain all the characteristics of pellets prepared in Campbell medium so that incorporation of amino acids remained ATP dependent. ~~RNAase insensitive and~~

the level of radioactive amino acid incorporated into the total and hot acid stable material increased for 2 hrs.

Consequently, since higher activity was obtained, post-microsomal pellets prepared in sucrose phosphate EDTA pH 3.4 were used in the subsequent attempts to purify the enzyme system from the post-microsomes.

Attempts to purify the incorporation system of post-microsomal pellet.

In all experiments discussed in this part the post-microsomal pellet was prepared from the livers of animals on stock diet using a homogenising medium consisting of sucrose 0.35M, potassium phosphate 0.02M and EDTA 0.001M pH 3.4 (pH of the homogenate 6.0 -6.2). All steps were carried out at 0°C.

(a) By ammonium sulphate fractionation.

In the first experiment (table 36) the post-microsomal pellet was suspended in sucrose phosphate pH 7.8 and, omitting the acid precipitation step, centrifuged at 2,000 r.p.m. for 10 mins. to remove any sedimentable material which was discarded. This suspension was made up to a known volume with sucrose phosphate pH 7.8, and separated into four fractions 0-25%, 25-50%, 50-75% and 75-100% saturation of ammonium sulphate as described in the methods section. A control sample of the untreated suspension was retained.

The major part of the original activity was found in the first two fractions, 0-25 and 25-50 percent saturation, although no significant increase in specific activity was obtained. The majority of the protein was found in the 25-50%

Table 36.

Aliquots of each fraction (with equal protein content) as defined in the text were incubated in a total volume of 1 ml. Campbell buffer with ATP (1 umole) and ^{14}C-leucine (1 uC) for 2 hrs. at $37^{o}C$ with constant shaking.

Values in brackets were obtained in absence of ATP.

Fraction	% of total protein in each fraction	Total Cts./ min./mg. protein	PCA stable Cts./min./ mg./protein	ug. RNA recovered.
Control	-	780 (132)	624	62
0-25%	8	886 (330)	950	31
25-50%	82	580 (78)	544	18
50-75%	7	110 (110)	68	33
75-100%	4	170 (----)	90	64

fraction (Table 36) and so this experiment was repeated, sub-dividing this fraction and including acid precipitation at pH 5 as the first step. The post-microsomal pellet was suspended in sucrose phosphate pH 7.8 and spun at 2,000 r.p.m. for 10 mins.. A small amount of sedimentable material obtained in this way was suspended in sucrose phosphate pH 7.8, (Fraction 1, Table 37). The supernatant fluid was adjusted to pH 5.2 with acetic acid and centrifuged at 2,000 r.p.m. for 10 mins.. The supernatant after this acid preparation step was retained (Fraction 2, Table 37) and the precipitate was suspended in sucrose phosphate pH 7.8 (Fraction 4, Table 37). After centrifuging at 18,000 g for 10 mins. to remove sedimentable material (Fraction 3, Table 37) the supernatant fluid was divided into precipitates obtained with 0-25, 25-40, 40-57, and 57-100% saturation with ammonium sulphate (Fractions 5,6,7 and 8 respectively, Table 37) and resuspended in sucrose phosphate pH 7.8. All fractions were dialysed for 18 hrs. with stirring against 4 l. sucrose phosphate pH 7.8.

The activity of the pellet suspension after acid precipitation (Fraction 4, Table 37) is higher than any of the ammonium sulphate fractions.

No purification has been obtained by salt fractionation, the activity seems merely to have been distributed among the fractions. The most unexpected finding was that suspension of pellet in sucrose phosphate pH 7.8 without any further treatment produces a very small fraction (Fraction 1., Table 37) which sediments at the low centrifugation speed of 2,000 r.p.m.. This fraction may be an aggregation of ribonucleoprotein particles which exist _in vivo_

Table 37.

Conditions were as given in Table 36. The different fractions are described in the text.

(Values in parenthesis were obtained in absence of ATP).

See text for description of fractions		mg. RNA / mg. protein	ug. RNA recovered	Total cts./min./mg protein	PCA stable cts./min./ mg. protein
Ppt. before pH 5	1	0.093	53	- (-)	3550
Supernat. after pH 5	2	0.038	40	136 (27)	53
Ppt. after pH 5 (18000g)	3	0.061	51	815 (356)	416
pH 5 ppt. (2,000r.p.m.)	4	0.084	53	1030 (47)	648
0-25% satn. $(NH_4)_2SO_4$	5	0.022	10	360 (55)	344
25-40% satn. $(NH_4)SO_4$	6	0.156	68	515 (42)	310
40-57% satn. $(NH_4)_2SO_4$	7	0.110	62	435 (39)	325
57-100% satn. $(NH_4)_2SO_4$	8	0.350	314	- (--)	255

caused by the prevailing conditions. The preliminary centrifugation at 18,000 g would certainly have removed these particles if they had been present in the original homogenate. An extremely high level of amino acid incorporation into a hot-acid-stable form, five times the activity of any other fraction, was shown by this insoluble fraction.

The particulate nature of the starting material proved an obstacle in purification studies. Preliminary centrifugation was employed to remove any sedimentable material before each stage and as shown (Table 37), the fraction which separates initially contains the highest amino acid incorporating ability. In some experiments this fraction was discarded. We thus attempted to solubilise the original post-microsomal pellets or at least the active component, by the pyrophosphate method of Sachs (1957). This method was chosen since pyrophosphate solubilises the ribonucleoprotein particles associated with the endoplasmic reticulum.

(b) Fractionation by centrifugation with or without pyrophosphate treatment.

A portion of the pellet (Fraction 1, Table 38) was suspended in sucrose phosphate pH 7.8. The remainder was suspended in 0.11M pyrophosphate as described in the methods section and separated by centrifuging at 105,000g for 60 mins.. The resulting pellet was suspended in sucrose phosphate pH 7.8 (Fraction 6, Table 38). A portion of the supernatant fluid was retained (Fraction 2, Table 38). The remainder was treated in two ways.

Table 38.

Conditions as described Table 36. Preparation of fractions is described in the text.

Fraction		mg.RNA / mg.protein	ug.RNA recover-ed	Total cts./min./mg. protein	PCA stable cts./min./mg. protein
Control untreated	1	0.105	47	307 (56)	249
PP supernat. untreated	2	0.092	53	538 (84)	258
" 0-40%	3	0.048	31	619 (51)	1050
" 40-100%	4	0.121	63	501 (34)	113
" pH 5 ppt.	5	0.131	81	1090 (38)	828
PP pellet untreated	6	0.098	54	3855 (132)	3185

(i) Adjustment to pH 5 with N acetic acid with separation of the precipitate (Fraction 5, Table 38) by centrifugation.
(ii) Salt fractionation with ammonium sulphate. Due to the small amount of material available only two fractions were made, 0-40% (Fraction 3, Table 38) and 40 - 100% saturation (Fraction 4, Table 38).

All precipitates were dissolved in a small volume of sucrose phosphate pH 7.8, and dialysed against 4 l. sucrose phosphate pH 7.8 with stirring for 18 hrs.

The results of this experiment are shown in Table 38.

On this occasion the activity shown by the control specimen was very low which makes evaluation of the results more difficult. A tenfold increase in amino acid incorporating capacity was noted in the material still sedimentable after the treatment of the post-microsomal pellet with pyrophosphate (Fraction 6, Table 38). In the previous experiment Fraction 1 (Table 37) sedimentable at 2,000r.p.m. with no pretreatment, showed high activity and would, if unaffected by pyrophosphate, be present in Fraction 6 (Table 38).

An attempt to differentiate between these two fractions and to show whether pyrophosphate treatment produces any specific effect is shown in Table 39. Post-microsomal pellet prepared as described was divided into three parts and treated as follows.

(i) The first part was suspended in sucrose phosphate pH 7.8
(ii) The second part was suspended in the same medium and centrifuged at 2,000 r.p.m. for 10 mins. followed by 105,000 *g* for 60 mins.

(iii) The third portion was treated with pyrophosphate as described and separated into 2 fractions by centrifugation at 2,000 r.p.m. for 10 mins. and 105,000 g for 60 mins.

The precipitates in each case were suspended in a small vo volume of sucrose phosphate pH 7.8. All suspensions and the supernatants were dialysed for 18 hrs. against 4 l. sucrose phosphate pH 7.8 with stirring.

The results from this experiment are shown in Table 39. The most active preparation so far obtained is the insoluble precipitate formed when post-microsomal pellet, suspended in sucrose phosphate buffer pH 7.8, is spun for 10 mins. at 2,000 r.p.m. The pellet material which is not solubilised by pyrophosphate also shows high activity but this treatment provides no greater activity than that obtained on simple centrifugation.

(c) Fractionation by centrifugation after RNAase treatment.

In earlier experiments (Part II, section (d)) treatment of the post-microsomal pellet with RNAase had either no effect or even improved the ability of the pellet to incorporate amino acids. The RNAase and the products of RNAase action remained in the incubation mixture. In order to show whether these breakdown products of RNA had any influence on the incorporation powers of the post-microsomal preparation the following experiment was carried out.

Post-microsomal pellet was prepared in sucrose phosphate EDTA pH 3.4, and suspended in Campbell buffer. A sample was taken as a control and the remainder treated in the following ways. The protein content was adjusted to 1 mg./ml. suspension

Table 39.

Conditions were as for Table 36.

Fraction		mg.RNA mg.protein	ug.RNA recovered	Total cts./min./mg. protein	PCA stable cts./min./mg. protein
Total post-microsomal pellet suspended in sucrose phosphate pH 7.8		0.090	53	1033 (44)	650
Suspend in sucrose phosphate pH 7.8	2000 rpm ppt.	0.080	-	740 (--)	992
	105000 g	0.135	57	925 (102)	798
	supernatant	0.063	77	400 (42)	317
Suspend in 0.1 M sodium pyrophosphate pH 7.4	2000 rpm ppt.	0.141	45	626 (--)	-
	105000 g	0.077	49	1055 (196)	1220
	supernatant	0.082	52	665 (49)	398

and portions were incubated in the presence and absence of RNAase, sufficient to provide 300ug./ml., for 30 mins. at 37°C. Another portion of the suspension was adjusted to pH 5.2 with N acetic acid, centrifuged at 2,000 r.p.m. for 10 mins. and resuspended in Campbell buffer to give a protein content of 1 mg./ml. In the presence of enough RNAase to provide 300 ug. /ml., this suspension was incubated for 30 mins. at 37°C. Each of these 3 fractions was centrifuged first at 18,000 g for 10 mins. and subsequently at 105,000 g for 60 mins. The precipitates thus formed were suspended in Campbell buffer and incubated under the standard conditions for 2 hrs. at 37°C. The results are shown in Table 40.

In every case the hot-acid-stable counts run parallel to and represent a large proportion of the total counts. Incubation with or without RNAase followed by reisolation of post-microsomal material does not denature the post-microsomal pellet; higher or at least equal amino acid incorporation was noted in each incubated fraction compared with the control (unincubated) specimen. As could be forecast from the previous experiments, the more rapidly sedimentable material had a very high specific activity and also showed a very low RNA/protein ratio. Treatment with RNAase, whether or not preceded by precipitation at pH 5 produced an appreciable diminution of the counts but still did not reduce them to the level of the control The more slowly sedimenting fractions had about the same specific activity as the control (unincubated) specimens and higher RNA/protein ratios. Of the two 105,000 g fractions which had

Table 40.

The post-microsomal pellet was suspended in sucrose phosphate pH 7.8, treated, as described briefly here and fully in the text, and the precipitates obtained by centrifugation at the speeds noted were incubated under the conditions described in Table 36.

Pre-treatment	Centrifugation speed g	Total cts./min./ mg.protein	PCA stable cts./min./ mg.protein	mg.RNA mg.protein
None	None	423	332	0.043
Incubation alone	18,000	6,710	6,200	0.002
	105,000	558	530	0.078
Incubation in presence of RNAase	18,000	2,140	2,020	0.003
	105,000	405	348	0.077
Acid ppt. at pH 5 followed by incubation in presence of RNAase	18,000	1,570	1,470	0.003
	105,000	300	283	0.025

been treated with RNAase, the one which had been submitted to acid precipitation showed the largest reduction in RNA content which confirmed a fact which we already suspected - acid precipitation makes the pellet RNA more susceptible to RNAase action.

Preincubation thus provides a post-microsomal pellet with a greatly enhanced capacity to incorporate amino acids in the form of a rapidly sedimentable fraction. The presence of RNAase reduced the activity found in the precipitable fraction but not to the control level.

Summary of findings.

In every attempt to purify and solubilise the post-microsomal pellet, the same result was obtained. Simple suspension of the newly prepared post-microsomal pellet material in buffer, causes the production of a granular precipitate which contains a large proportion of the amino acid incorporating ability. Preincubation of the suspension increases the production of this easily sedimentable material and increases its activity. The aggregation of material observed is not due to increase in the Mg concentration, since in some cases the pellet, prepared in sucrose phosphate EDTA was resuspended in Mg free sucrose phosphate pH 7.8. Even post-microsomal pellet prepared in Campbell buffer and resuspended in Campbell buffer produced this granular material if left for several hours; it seems therefore that the aggregation occurs spontaneously.

Part IV.

The nature of amino acid incorporation by post-microsomal pellet.

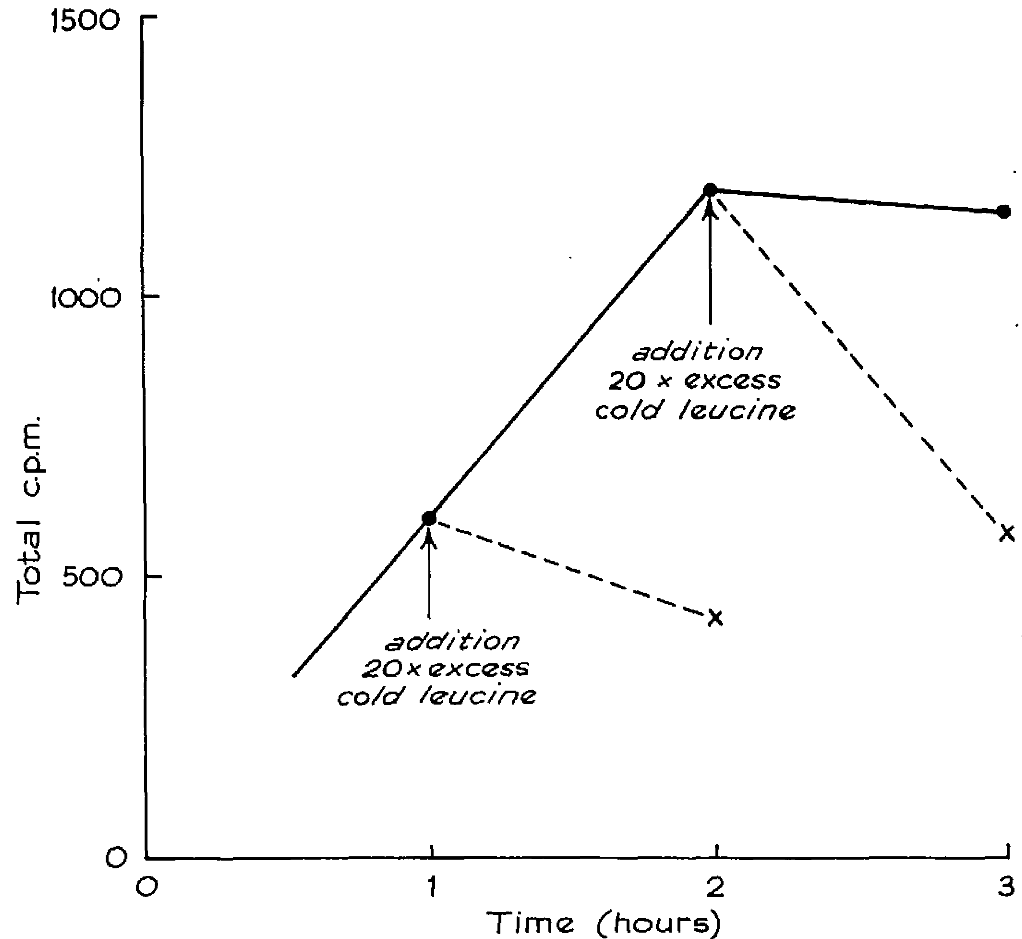
If an amino acid is incorporated by an energy-dependent reaction into a peptide chain and joined there by a true α-peptide linkage, addition of a large excess of the same amino acid to the system should cause no displacement of the amino acid units already forming part of the peptide. By using a radioactive amino acid for the initial incorporation and later flooding the system with an excess of the same amino acid, unlabelled, it is possible to detect any radioactive amino acid which has been incorporated and subsequently displaced by an unlabelled molecule.

This principle was applied to the post-microsomal pellet. Under standard conditions, a pellet preparation was incubated with radioactive leucine. After the time intervals stated in Fig. 16, a 20-fold excess of unlabelled leucine was added to the system and the incubation continued. The distribution of the total counts remaining is shown in Fig. 16. The unlabelled leucine produced a distinct fall in radioactivity at both time intervals examined, a 28% reduction after 1 hr. and a 50% reduction after 2 hrs. The leucine uptake into the post-microsomal pellet therefore, was not into the interior of a peptide chain as the leucine could readily be displaced from its position by exchange. The leucine appears to be attached in a readily accessible position, perhaps in the end group, and so a way of distinguishing between the internally and externally bound leucine was sought.

Fluorodinitrobenzene (FDNB) reacts with free amino groups of a protein molecule which include the free amino groups of the

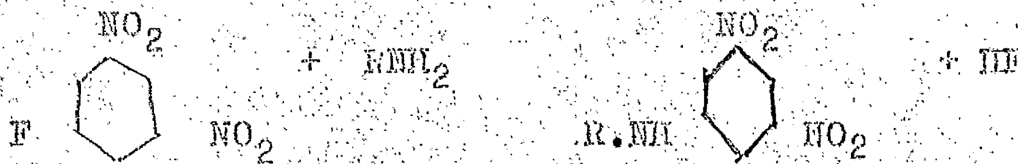
Fig. 16.

Effect of isotope dilution on leucine uptake by post-microsomal pellet.



The post-microsomal pellet (about 1 mg. protein) was incubated with ATP (1 umole) and ^{14}C-leucine (1 uC) in 1 ml. Campbell buffer for 3 hrs. at 37°C. A 20-fold excess of ^{12}C-leucine was added where indicated and incubation was continued. The broken line represents the activity remaining after addition of the unlabelled leucine. The continuous line represents leucine uptake without dilution.

N-terminal amino acid and the ϵ-NH_2 group of lysine. (Porter and Sanger, 1948)



The linkage of the FDNB to the amino group is more stable to acid hydrolysis than the peptide bonds of the protein. Thus, after preparation and hydrolysis of a DNPprotein, isolation and characterisation of the DNP amino acids formed provides a means of identifying the N-terminal amino acids. To show whether the leucine newly incorporated by the post-microsomal pellet is in the N-terminal position or is bound in protein the following experiment was carried out.

Post-microsomal pellet was incubated with radioactive leucine under the standard conditions, washed and extracted with hot PCA as described, so that only the hot-acid-stable activity need be considered. Dinitrophenyl derivatives of, first the end group amino acids, then the internally linked amino acids were made from the same post-microsomal preparation. The individual DNP amino acids were separated by paper chromatography; the spots were identified, cut out, eluted and counted. Samples of the total hydrolysate were also counted.

Background levels of radioactivity were found in all areas of the chromatogram apart from the region corresponding to DNP-leucine and the origin which displayed a low level of radioactivity only slightly above background. The radioactivity found in the N-terminal DNP-leucine has been expressed as a percentage of

Fig. 17.

Map of a chromatogram of the ethyl acetate soluble DNP-amino acids (excluding the N-terminal amino acids) of the residual protein hydrolysate of the post-microsomal pellet.

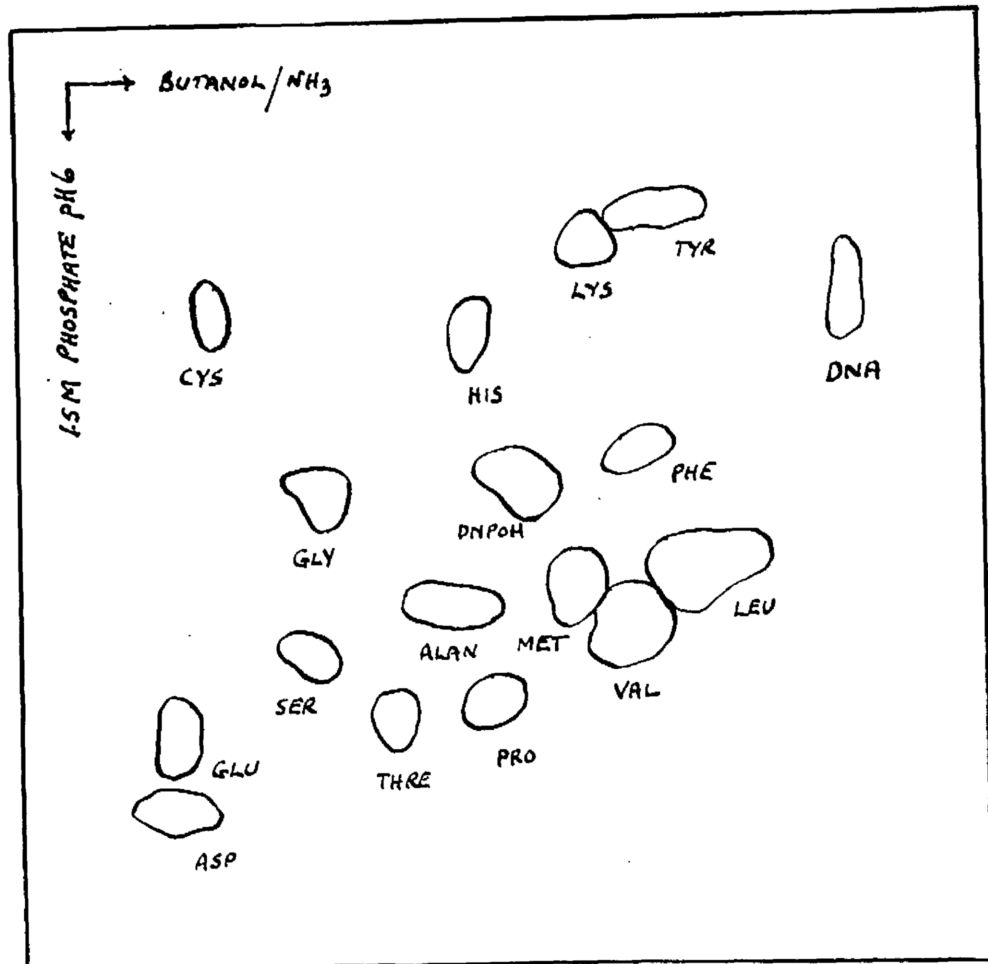
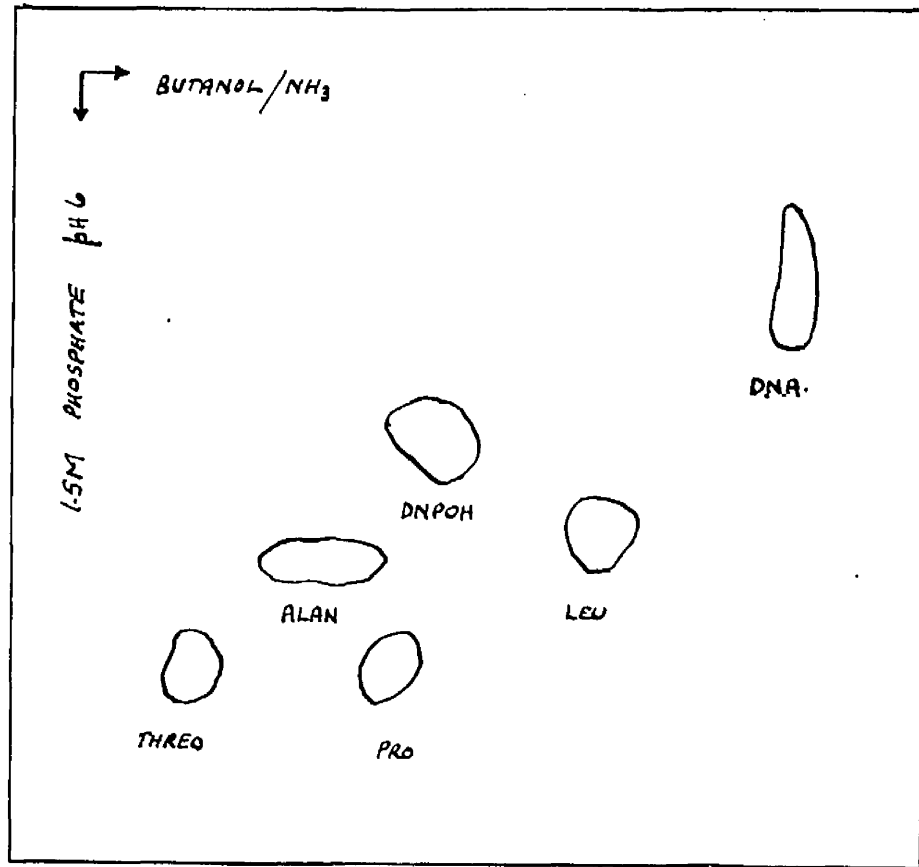


Fig. 18.

Map of a chromatogram of the ethyl acetate soluble N-terminal DNP-amino acids from the post-microsomal pellet.



the counts found in the total hydrolysate. Correction was made for loss of material on the chromatogram.

	Post-microsomal pellet	% of counts in total hydrolysate
Total hydrolysate	10,730	100
End group leucine	8,420	79
Internally bound leucine	735	7
Total recovered as DNP-leucine	9,173	86

The data show significant labelling only in the N-terminal position. Thus the bulk of the leucine incorporated into the hot-acid-stable portion of the post-microsomal pellet is not incorporated into a protein molecule but is found in the N-terminal position. The small amount of leucine found in the total hydrolysate after removal of the terminal leucine is compatible with the known incompleteness of the reaction of FDNB with protein.

GENERAL DISCUSSION.

The appeal of the Hoagland-Zamecnik system for activation of amino acids and their incorporation into protein via sRNA has caused some workers in this field to overlook the evidence of alternative pathways of protein fabrication. Yet the case for the existence of such other means of amino acid uptake into protein is quite persuasive. Our data on incorporation into post-microsomal pellet have compelled us to examine the nature of these alternative mechanisms, since we cannot reconcile our findings with the classical Hoagland mechanism.

Alternative pathways of protein formation.

The Hoagland mechanism involves activation of amino acids with the splitting of ATP to AMP and pyrophosphate. However, it has been known for some time that in the synthesis of glutathione (Snoke & Bloch, 1955; Snoke, 1955), the products of ATP breakdown are ADP and inorganic phosphate. The formation of this tripeptide from γ-glutamyl-cysteine and glycine by an enzyme purified from a yeast extract involves an exchange between ATP and ADP. Although an activated derivative of glutamyl-cysteine is possible, no phosphorylated intermediate has been shown to be present. At the time of description this system was not thought to have relevance in the mechanism of protein synthesis because of the special nature of the tripeptide formed. However other peptide forming systems have been described in which ATP is utilised as an energy source with the formation of ADP and inorganic phosphate.

The system of Beljanski arose from studies on fractions of the organism Alcaligenes faecalis. After disrupting the cells the amino acid incorporating capacity of different fragments was

examined. It was found that parts of the cell membrane were capable of incorporating amino acids in the presence of an energy-generating system and that this incorporation was much augmented by addition of a partially purified enzyme from the cell supernatant fraction (Beljanski & Ochoa, 1958b). The most important observation, however, was that neither fraction exhibited amino acid activating enzymes, as judged by ATP-^{32}PP exchange in the presence of amino acids, even when the particulate fraction was solubilised with perfluorooctanoate (Beljanski, 1960a). This uptake of labelled amino acids was equated to protein synthesis since the activity was hot-acid-stable and was not reduced by the addition of an excess of unlabelled amino acids.

Beljanski extended his studies of the enzyme system involved. He found that the partially purified enzyme present in the cell supernatant which stimulated amino acid incorporation by the particles, also included a mixture of four separate kinases, each of which was capable of catalysing the magnesium dependent exchange of phosphate groups between corresponding nucleoside triphosphates and diphosphates (Beljanski, 1960a; 1960b). This reaction which he names "reaction of exchange", was unaffected by chloramphenicol and, in absence of amino acids, no release of inorganic phosphate takes place (Beljanski, 1959). A different sequence of events takes place when amino acids are present in the incubation mixture. The amino acids are converted into peptides and at the same time inorganic phosphate is liberated from the triphosphate. This reaction is dependent on nucleotides as an energy source but ATP is not unique in this respect. All nucleoside

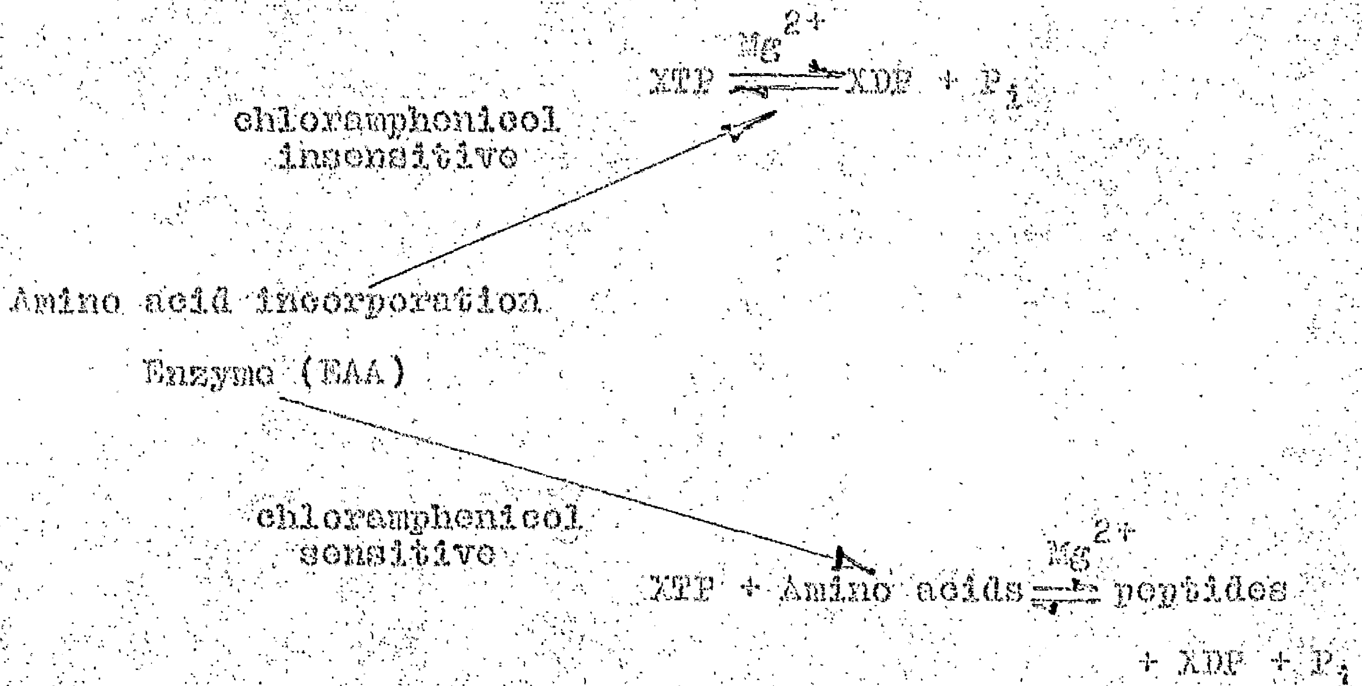
triphosphates can participate, and there is some degree of specificity of certain amino acids for individual nucleotides. The mechanism is thus currently considered to be as follows:-

Using a specific nucleoside triphosphate as an energy source, the carboxyl group of the amino acid becomes activated and joins onto the N-terminal position of the peptide molecule (Beljanski, Beljanski & Lovigny, 1962); the triphosphate is split to form a diphosphate and free inorganic phosphate is released into the medium. This is named by Beljanski the "reaction of liberation" (Beljanski, 1960c) and it is, unlike the reaction of exchange, chloramphenicol-sensitive. The two reactions are shown diagrammatically in Fig. 19. The peptides or polypeptides formed by this enzyme system have, when studied by ^{14}C-valine incorporation, exhibited no C-terminal valine activity and only 15% was found in the N-terminal position. (Beljanski, 1960a). Internal labelling was shown to be present by the partial hydrolysis technique. This can be accepted as evidence of a genuine incorporation of amino acids by formation of peptide bonds.

The occurrence of this system in mammalian cells has also been explored by the same group of workers (Beljanski & Ochoa, 1958b). The purified amino acid incorporation enzyme from A. faecal preparations can stimulate incorporation of amino acids into rat liver microsomes in the absence of a source of conventional activating enzymes. However, others (Campbell, 1960) have not been able to replicate this. The occurrence of "exchange" and "liberation" enzymes in rat liver cell sap has recently been investigated by Zalta & Beljanski, (1961). In the pH 5 fraction

Fig. 19.

The amino acid incorporation system of Beljanski.



XTP = Any of the four nucleotides: ATP, GTP, CTP, UTP.

obtained from the cell sap, both the characteristic formation of peptides with liberation of orthophosphate (liberation reaction) and the exchange of phosphate between tri- and diphosphates was demonstrated in the mammalian system; both of these reactions were insensitive to chloramphenicol.

Particles and enzymes similar to those described by Beljanski have been reported by Nisman and his colleagues, in preparations from E. coli and B. megatherium. Using particulate fractions separated from disrupted cells, they were able to show the incorporation of labelled amino acids into a defined protein, the enzyme β-galactosidase (Nisman & Fukuhara,1959), in the absence of activating enzymes. The synthesis of this protein was stimulated by a mixture of nucleotides and by nucleic acid extracted from membrane fraction sedimented at low speeds of centrifugation (Nisman & Fukuhara, 1960a). The combined use of ribonuclease and deoxyribonuclease has demonstrated that the stimulant factor in these extracts is DNA. RNA isolated from the membranes has no effect but sRNA does stimulate amino acid uptake (Wachman et al., 1960), due apparently to its capacity to liberate nucleotides, since its effectiveness was enhanced by addition of RNAase; addition of amino acid mixtures depressed rather than stimulated the incorporation.

In addition to studies on the particulate fraction of the disintegrated bacteria, these authors examined the cell supernatant remaining after removal of the membranes. This they divide into a final pellet, P_3 (Nisman, Fukuhara & Hirsch, 1960) and a supernatant fraction, S_3 . Both of these cell preparations show

activityof the type described by Beljanski, namely formation of peptides from amino acids with simultaneous release of inorganic phosphate. It is claimed that this occurs with net synthesis of β-galactosidase in fraction P_3. Protein synthesis in both fractions is stimulated by phenol extracted DNA from the cell, polynucleotide phosphorylase, nucleotide mixtures and by amino acid mixtures. The S_3 fraction differed from the other cell fractions examined in that it showed enhanced incorporation activity when phenol-extracted RNA from the cell was added to the mixture, Further purification of S_3 (Nisman & Fukuhara, 1960b) demonstrated that for incorporation each amino acid requires as an energy source, a nucleoside triphosphate which is split with the formation of inorganic phosphate and the nucleoside diphosphate. Each amino acid shows a limited specificity for one nucleotide though most amino acids can utilise other nucleotides to a lesser extent. Feeble or no activating enzyme activity has been noted in these fractions as judged by ATP-pyrophosphate exchange. These findings are in close agreement with those observed by Beljanski and presumably are expressions of a similar mechanism.

A cell-free particulate fraction which resembles these systems to some extent has been isolated by Chakravorty & Burma (1962) from A. vinelandii. This fraction is able to incorporate amino acids into a hot-acid-stable fraction which they equate to protein, in the absence of activating enzymes. No ATP-dependence of this reaction was shown but since no incorporation takes place under anaerobic conditions, the energy is probably derived from oxidative phosphorylation. The amino acids incorporated were shown to be

internally bound in protein and no stimulation in the presence of nucleotide and amino acid mixtures was noted.

Unconventional protein-synthesising or amino acid incorporating systems are not confined to bacterial metabolism. A system very similar to those described above has been investigated by Zalta (1958; Zalta & Khouvine, 1959; Zalta & Lachurie & Osono, 1960 a & b) in the case of rat liver microsome preparations. Particles isolated with the use of detergents were shown to retain their ability to incorporate amino acids despite the loss of amino acid activating enzymes during the preparation stages. The same pattern emerges in Zalta's system as in those described for the bacterial system of Beljanski and Nisman. Incorporation of amino acids into the ribonucleoprotein particles continues for periods up to 2 hrs. by a mechanism which differs from the conventional one in a number of ways:-

The amino acid uptake is insensitive to RNAase and is stimulated by the addition of amino acid mixtures in the presence of all four nucleoside triphosphates. A variable response to the addition of pH 5 enzymes was noted but in most cases the amino acid uptake was unaffected by the added material. The reaction is dependent on ATP which is broken down with the formation of ADP and inorganic phosphate, just as in Beljanski's bacterial system.

Another mammalian particulate fraction which apparently allows incorporation of amino acids in the absence of activating enzymes has been prepared by Cohn (1959) from rat liver microsomes with the combined use of perfluorooctanoate and lubrol-W. This preparation has no amino acid activating enzyme activity and a low RNA/protein

ratio (0.4) in comparison with ribonucleoprotein particles prepared with the use of deoxycholate, which have a RNA/protein ratio of about 1.0 (Korner, 1959). Incorporation of amino acids into protein by this system is depressed by addition of soluble enzymes present in the cell sap (pH 5 enzyme). The relationship of this system to the Zalta and other mechanisms is not clear and no further data seem to have been published to determine the effects of nucleotide and amino acid additions or the resistance of the incorporation to RNAase treatment.

A somewhat different system has been prepared from rat liver in which incorporation of amino acids into microsomal particles is enhanced by an essential protein from the cell sap (Sachs, 1957). The cell sap factor, termed S-protein, does not contain sRNA (Rendi & Campbell, 1959) but is able to catalyse incorporation of amino acids into microsomes with an efficiency almost equalling that of pH 5 enzyme which, of course, contains sRNA; inferiority of the S-protein system compared with the conventional pH 5 enzyme system has been attributed to the lengthy preparative procedure necessary for isolation of the S-protein fraction (Rendi & Campbell, 1959). The presence of an S-protein -amino acid intermediate has not however been demonstrated and no incorporation of amino acids by S-protein itself has been found. Nevertheless, a separate enzyme system seems to be present, because S-protein activity is stimulated by the addition of glutathione whereas the pH 5 enzyme activity is inhibited by this reducing agent. S-protein also exhibits some special properties in preserving the activity of the microsomes against the detrimental effects of heat, ATP, RNAase

and high salt concentrations. This stabilising action has been attributed by Sachs to preservation of the essential microsomal ribonucleoprotein structure.

In the presence of pH 5 enzyme, S-protein catalyses amino acid incorporation into sRNA and was thus assumed at first to contain activating enzymes(Campbell, 1960). However, Rendi and Hultin (1959) have shown that S-protein is practically free from this enzymic activity and, using 2-mercaptoethylamine (MEA) and dichlorophenolindophenol (DPIP), they have been able to differentiate between incorporation of amino acids into sRNA catalysed by S-protein and incorporation catalysed by pH 5 enzyme. The activity of the pH 5 enzyme is unaffected by either of these compounds; the activity of the S-protein was inhibited by DPIP and enhanced by MEA. They suggest that in the presence of S-protein MEA stimulates the incorporation of amino acids into sRNA by a mechanism which does not involve formation of amino acid adenylates.

On further purification of S-protein, Hultin (Rendi & Hultin, 1960) isolated a fraction, S_{50}, in which the activating enzymes are reduced to 2% of the original concentration, and the remaining amount was mainly tyrosine activating enzyme. No sRNA was observed in the S_{50}-proteinfraction even by use of radioactivity measurements after prelabelling of RNA with ^{32}P in vivo. (von der Decken & Hultin, 1960a). This is a sensitive procedure for detecting traces of RNA, and none was observed. The S_{50} fraction catalyses incorporation of labelled amino acids into ribonucleoprotein particles in the presence of ATP, GTP, PEP and pyruvate kinase, the

latter two being generators of high energy phosphate bonds. Labelled amino acids already bound to sRNA also become transferred to microsomal particles in presence of the purified S_{50} preparation. This transferring activity is weak, but is considerably enhanced by addition of glutathione.

At this point, it is desirable to consider the similarities and differences between labelling of microsomes by the S-protein system and the mechanism involving the activating enzymes and sRNA. The systems have been compared in a diagram by Rendi and Campbell (1959). The early work with the pH 5 fraction did not distinguish between the activating enzymes and the enzymes responsible for the subsequent transfer of the amino acid to the microsome template However, it has been shown that the enzyme carrying out transfer of the amino acids from sRNA to microsomes can be separated from the activating enzymes, as discussed earlier. Moreover, this glutathione stimulated transfer reaction can function independently of activating enzymes. Consequently, the action of the S_{50} protein is identical in this respect with the isolated transfer enzyme of the conventional pathway of amino acid incorporation;
(a) it can transfer amino acids from sRNA to microsomes; and
(b) this transfer is seemingly glutathione dependent.
In addition however, the S_{50} protein has the property of transferring free amino acids to sRNA or directly to microsomes in the absence of activating enzymes.

There is also some indirect evidence to suggest that pathways other than the activating enzyme-sRNA mechanism may operate in protein synthesis in the liver. In normal and regenerating liver,

Rendi (1959) found that labelling of the sRNA with amino acids was similar, whereas the pH 5 fraction from the regenerating liver had a greater capacity to promote incorporation of amino acids into microsomes. The ratio of RNA/protein was the same in pH 5 enzymes from both sources; consequently, either the binding of amino acids to sRNA is not a rate-limiting step, or a pathway other than that involving sRNA is present in the pH 5 enzyme preparation. Rychlik <u>et al.</u> (1959) has provided evidence of a similar dissociation of sRNA activity from rate of incorporation into microsomes. In his system, incorporation of labelled amino acids into the microsomal protein of mouse pancreas was only partially reduced by a concentration of 6-azaguanine diphosphate sufficient to produce a 98% inhibition of amino acid-acyl-sRNA formation. Rychlik suggests either that the system may be saturated by a small amount of sRNA still actively present or that another route is available for amino acid incorporation which does not involve sRNA.

The main feature of these systems is that amino acid uptake into particles derived from bacteria and from rat liver microsomes takes place in the absence or in the presence of very low amounts of amino acid activating enzymes. It is extremely difficult to free these particulate fractions from gross contamination with activating enzymes but in each of the systems described (Beljanski & Ochoa, 1958a &b; Zalta <u>et al.</u>, 1960b; von der Decken & Hultin, 1960b; Chakravorty & Burma, 1962), the particles, and in some cases the soluble fraction, were submitted to extensive testing for

activating enzymes and either none or a low level, sometimes confined to one amino acid was found. Therefore, unless activating enzymes are present and are in fact effective in amounts too small to be detected, amino acid incorporation and genuine protein synthesis can take place in the absence of this enzyme. In addition because the action of sRNA depends on a supply of activated amino acids, the exclusive role of sRNA in protein synthesis has been made less certain by this evidence.

Support for this argument was obtained from another source when it was observed that the S-protein of Sachs (1957) could transfer free amino acids to microsomes and yet contained no detectable RNA (Rendi & Campbell, 1959; von der Decken & Hultin, 1960a). In this case, however, as the presence of trace amounts of sRNA in the particulate components used was not excluded, it is possible that sRNA is available in amounts sufficient to provide the observed transfer. This explanation of S-protein action is more feasible on consideration of the fact that only 50 ug. sRNA are required to transfer amino acids to 8 mgs. microsomal protein (Zamecnik, 1960). The S_{50} protein (von der Decken & Hultin, 1960b) a more highly purified preparation of S-protein is active in catalysing the transfer of sRNA bound amino acids to ribonucleoprotein particles, a function which is completely independent of amino acid activating enzymes. It would thus appear that the procedure for preparation of S-protein had partly separated the activating enzymes from the enzyme system responsible for transferring sRNA bound amino acids to the microsomes. Thus, these studies do not finally exclude sRNA as part of the underlying mechanism in amino

acid incorporation by the S-protein and suggest that S-protein is a transferring enzyme.

During studies on the effects of protein intake on the livers of rats a particulate fraction was isolated from the 1 hr. cell sap which could incorporate amino acids into a form which was stable to hot acid extraction. This fraction was called post-microsomal pellet, a name chosen by Palade and Siekevitz (1956) to designate two fractions which they separated from a high density medium by centrifuging for 3 and 15 hrs. The name was used subsequently by Goldthwaite (1959a) for the pellet spun out of 0.25 M sucrose at 105,000 g. for 3 hours. The properties of amino acid incorporation by the post-microsomal pellet cannot at once be reconciled with the Hoagland system of protein synthesis involving sRNA and would appear to belong to the category of alternative mechanisms.

Conclusions concerning the nature of amino acid uptake by the post-microsomal pellet.

Although the study of this cell fraction is not yet complete, enough data have been accumulated to warrant a place in the catalogue of incorporation systems so far described.

In view of the activating enzymes present (Table 31) the most obvious solution is that the post-microsomal pellet is some variant of the Hoagland system. It is conceivable that, under the high gravitational forces applied to the cell supernatant, the leucine activating enzyme sediments and is found in high concentra-

tion in the pellet. The case for and against attributing the amino acid uptake observed in the post-microsomal pellet to the retention of leucine in the form of the leucine-adenylate-activating enzyme complex is as follows:-

1. For the reasons given in detail below, leucine uptake by the post-microsomal pellet does not depend upon the presence of sRNA. This is consistent with the binding of the leucine as the adenylate-enzyme complex which is formed as a preliminary to transferring the leucine to sRNA.

2. Amino acid uptake by the post-microsomal pellet is insensitive to RNAase treatment (Fig. 14). No inhibition of the reaction catalysed by activating enzymes is found in the presence of RNAase (Hoagland et al., 1957; von der Decken & Hultin, 1959b) although amino acid uptake in the pH 5 enzyme by sRNA is abolished by similar treatment (Hoagland et al.,1958).

3. The leucine is not incorporated into inter-peptide linkages and may easily be displaced on exposure to high concentrations of the same amino acid (Fig.16). Again this is consistent with binding of the leucine to the activating enzyme as in that case leucine would be located in a reactive position.

4. The chemical characteristics of the amino acid-adenylate-enzyme complex would appear to make it a suitable candidate to be the labelled material present in the post-microsomal pellet. Although the free leucyl-adenylate is extremely reactive (DeMoss et al., 1956), the enzyme bound adenylate is very stable and in the absence of a suitable acceptor for the activated amino acid dissociates only to a small extent. In our system, we have obtained

leucine incorporation into the hot-acid-stable portion of the pellet. In evaluating this, it should therefore be noted that during attempts to prove that amino acid adenylates were true intermediates in protein synthesis, Kingdon _et al._,(1958) have shown that the free tryptophan adenylate is _released_ into the soluble fraction following the denaturation of the activating enzyme with _cold_ acid. Therefore, treatment of the post-microsomal pellet with cold perchloric acid to terminate the reaction after incubation will release any leucine attached to the activating enzymes in the form of a leucyl-adenylate.

5. The leucine uptake by the post-microsomal pellet is strictly dependent on the type of buffer used for the incubation medium (Table 19). No such stringent buffer requirements have been demonstrated for the action of the amino acid activating enzymes from the 1 hr. cell sap. It would be of some interest to know if the post-microsomal activating enzymes remain active in tris buffer, a medium which inhibits leucine incorporation into the post-microsomal pellet. If this is true, the incorporation must be at least partially independent of activating enzymes. This is under investigation.

6. In the post-microsomal pellet, the leucine containing component, whatever its nature, is unable to donate the attached leucine to sRNA. No increment in hot-acid-soluble counts was observed when the 3 hr. cell sap was incubated with post-microsomal pellet and ^{14}C-leucine (Table 25) even when additional purified sRNA was present (Fig. 5). Since amino acid-adenylate-enzyme complexes are able to transfer the attached amino acid to sRNA in the absence

of additional enzymes (Berg & Ofengand, 1958), the activated complex would appear either to be absent from the post-microsomal pellet or to be unable to donate its attached amino acids to sRNA.
7. If, as suggested, in the absence of an acceptor for amino acids the leucyl-AMP-activating enzyme complex is formed in large amounts in the post-microsomal pellet, no such sequence of events occurs in the 3 hr. cell sap. Thus, after removal of the 3 hr. cell sap RNA with RNAase, leucine incorporation into RNA was completely abolished but no increase in the hot-acid-stable counts was observed (Table 24). Furthermore, even when the 3 hr. cell sap was incubated with ^{14}C-leucine in the presence of additional activating enzymes in the form of post-microsomal pellet only a small increase in the hot-acid-stable fraction (equal to 6% of the original pellet activity) was observed. (Table 25).

There is thus no evidence of the formation of an amino acid complex which is stable to hot acid extraction in the 3 hr. cell sap whether sRNA is present or absent.

In the face of this evidence the activated leucyl-adenylate, as found in the pH 5 enzyme, was excluded as the reason for the leucine uptake by the post-microsomal pellet.

Alternatively, the post-microsomal pellet may consist of particulate matter derived from the endoplasmic reticulum which contains enough adsorbed activating enzymes and sRNA in combination with low molecular weight components which would permit amino acid incorporation into ribonucleoprotein similar to microsomal protein synthesis. This possibility has also been excluded for the following reson s:-

1. There is little evidence for, and some strong evidence against, the presence of sRNA in the post-microsomal pellet.

(a) Purified post-microsomal RNA was found to be inert as an amino acid acceptor in the presence of pH 5 enzymes (Fig. 5), thus indicating that the sRNA content of the post-microsomal pellet was low or zero. (Hoagland <u>et al</u>.(1958) have suggested that the RNA sedimentable from the 1 hr. cell sap by centrifuging for a further 3 hrs. is inert as an amino acid acceptor because, although the pH 5 fraction from the 3 hr. cell sap contains only 50% of the RNA present in the same fraction from the 1 hr. cell sap, the amount of leucine incorporated into the RNA of these two fractions remained the same. Contrary to these findings Goldthwaite (1959b) has shown that RNA II, an RNA fraction which he separates from the 1 hr. cell sap and which closely corresponds to post-microsomal RNA, is utilised by the pH 5 enzyme as an amino acid acceptor with about 50% of the activity shown by sRNA.

(b) Examination of the post-microsomal RNA by column chromatography failed to demonstrate the presence of sRNA (Fig. 6).

(c) Treatment with RNAase confirmed that leucine uptake into the hot-acid-stable fraction of the post-microsomal pellet was independent of the RNA present (Fig. 14).

(d) Pseudouridylic acid, a characteristic component of sRNA, was found in small amounts in post-microsomal RNA (Table 15) although none of the other unusual bases detected in sRNA was present. Thus, examination for the rarer nucleotides does not remove the doubt that the post-microsomal fraction is significantly contaminated by cell sap.

2. It is to be observed that the microsomes which are undoubtedly contaminated with cell sap do not exhibit the behaviour characteristic of the post-microsomal pellet.
3. Low molecular weight cofactors which are necessary for amino a acid incorporation into microsomal protein are not necessary for post-microsomal incorporation. In fact, GTP does not enhance but rather inhibits incorporation of leucine by the post-microsomal pellet.(Table 28)

It was concluded that the post-microsomal pellet incorporates leucine by a mechanism which is incompatible with microsomal protei synthesis.

As a constituent of the 1 hr. cell sap, the post-microsomal pellet might represent an aspect of the S-protein system described by Sachs (1957) and von der Decken and Hultin (1960b). This S-protein occupies a dubious position in the system of Hoagland because, although several features of the S-protein activity are unusual, recent work by von der Decken and Hultin (1960b) has suggested that the true role of this protein fraction is to catalyse the transfer of sRNA bound amino acids to protein.
In any case post-microsomal pellet was differentiated from S-protei in the following ways:-
1. sRNA is capable of accepting amino acids from S-protein (Rendi & Campbell, 1959) but not from the post-microsomal pellet (Table 25.
2. The post-microsomal pellet becomes labelled on exposure to ^{14}C-leucine in the presence of an energy source, whereas, under similar circumstances no evidence of a labelled S-protein intermediate has been found (Rendi & Campbell, 1959).

3. The activity of S-protein is stimulated by glutathione which inhibits amino acid uptake by the post-microsomal pellet (Table 29)

Although the capacity of the post-microsomal pellet to transfer amino acids to microsomal protein has not been tested, present evidence suggests that the post-microsomal pellet cannot be identified with S-protein.

No satisfactory answer to the problem of the mechanism involved in the leucine uptake by the post-microsomal pellet was supplied by this comparison with the Hoagland system and so we turned to consider the alternative pathways of amino acid incorporation described in the literature. A growing body of evidence has been presented supporting the contention that uptake of amino acids may take place without the participation of an sRNA bound amino acid intermediate formed by activating enzymes. Several investigators (Beljanski & Ochoa 1958a; Wachman _et al._, 1960; Chakravorty, 1962) have reported that particulate fractions derived from bacterial cell walls are capable of incorporating amino acids in the absence of activating enzymes. Although the post-microsomal pellet exhibits a high level of these enzymes, some resemblance between the two systems was noted. In both cases the amino acid uptake was found to be unaffected or even stimulated by the presence of RNAase (Fig. 14). As found in the system of Chakravorty and Burma (1962) we have observed that, in the post-microsomal pellet, the rate of amino acid uptake is very dependent on the buffer system employed for incubation (Table 19) and, over the range tested, each amino acid is incorporated to a different

extent (Table 32).

Comparison of the post-microsomal pellet with a similar anomalous system (Zalta _et al._, 1960a) derived from rat liver microsomes was also made. In both of these fractions amino acid uptake was insensitive to RNAase (Fig. 14) and was stimulated by amino acid mixtures in the presence of a nucleotide mixture (Table 27). However, Zalta's particles possess an amino acid incorporating capacity which is unaffected by additional pH 5 enzymes from liver cell sap and like the bacterial systems described above are free from activating enzymes.

Thus the major discrepancy between the particulate systems described above and the post-microsomal pellet is that the former have proved to be devoid of amino acid activating enzymes. A second difference is that Zalta _et al._, (1960b) and Chakravorty & Burma (1962) have shown that 80% and 95% respectively of the incorporated amino acid is internally linked in a peptide chain. It is obvious that the post-microsomal pellet cannot claim identity with these systems since in our fraction,

(a) amino acid activating enzymes for leucine are present in large amounts (Table 31) and

(b) 80% of the leucine is incorporated terminally.

The only remaining possibility to be considered is that post-microsomal amino acid uptake is mediated by the peptide synthesising amino acid incorporation enzyme of Beljanski (1960b) (Fig.19). This is a soluble enzyme which was originally isolated from _A. faecalis_ and has now been shown to be present in the soluble fraction of rat liver by Zalta and Beljanski (1961). It catalyses

amino acid incorporation into soluble peptides utilising different nucleoside triphosphates as energy sources. Each nucleotide is to some extent specific for a range of amino acids. Although it was realised that, using our procedure of protein isolation, no acid-soluble peptide formation would be detected, the ability of individual nucleoside triphosphates to enhance the uptake of a series of amino acids by the post-microsomal pellet was tested. Leucine uptake which was distinct from the other amino acids tested, was supported only in the presence of ATP. Background levels of incorporation were observed in presence of the other nucleotides. Independent of whether nucleoside triphosphates were absent or present the amino acids glutamic acid, phenylalanine, alanine and glycine were incorporated to an extent which was always less and sometimes negligible in comparison with leucine uptake. Therefore, no evidence of the soluble amino acid incorporation enzyme was obtained by testing the post-microsomal pellet in this way, although the method used was inconclusive.

From the data presented the exact role and relation of the post-microsomal pellet to the cell economy has not been clarified. This fraction can incorporate radioactive leucine by an energy dependent reaction and yet the properties of the system are not in agreement with any of the existing protein synthesising mechanisms described. The salient points are summarised as follows:-

1. Leucine activating enzyme is a component of the post-microsomal pellet but the presence of the complete range, comprising similar enzymes for all amino acids, is doubtful.
2. The leucine activating enzyme present in this fraction appears

to be incapable of activating and donating ^{14}C-leucine to sRNA.

3. The bulk of the leucine once incorporated is stable to hot-acid extraction, the remainder being solubilised by this treatment.

4. The leucine incorporated into the hot-acid-stable portion is located in the N-terminal position and is easily displaced by the addition of excess quantities of the same amino acid.

5. The rate of leucine uptake was unaffected by RNAase and was stimulated by amino acid mixtures in the presence of all four nucleoside triphosphates.

In must be remembered that there is evidence to show that the RNA of the post-microsomal pellet becomes labelled. The final assessment of the significance in cell metabolism of the post-microsomal pellet system must therefore include consideration of the nature and function of the RNA in the pellet.

Post-microsomal pellet RNA.

In the current stages of this problem, the post-microsomal RNA, which by weight represents 10-20 percent of the post-microsomal protein, presents something of an enigma.

On incubation of the post-microsomal pellet with labelled leucine, a significant proportion of the radioactivity incorporated was solubilised by extraction with hot acid and was assumed to be associated with RNA. The properties of the post-microsomal RNA in relation to leucine uptake are as follows:-

1. When leucine incorporation was related to the RNA content, the specific activity of the pellet was found to vary depending on the

previous dietary protein intake. The overall activity expressed as the product of the total counts and the RNA per unit body weight remained constant however and it was assumed that two types of RNA were present.

(a) A part of the RNA remained constant and was concerned with the uptakeof amino acids.

(b) A second part varied in amount according to protein intake becoming greater when protein was removed from the diet, and was inert in amino acid incorporation.

2. Column chromatography of the RNA confirmed that the heterogeneity of the post-microsomal RNA varied according to protein intake as above.

3. As described, the native RNA appears to be associated with a proportion of the leucine uptake and yet RNA, phenol-extracted from the post-microsomal pellet, irrespective of dietary condition is unable to act as an amino acid acceptor in the presence of pH 5 enzymes.

Variable properties of RNA of a similar nature have been reported by Goldthwaite (1959b) who studied the distribution of leucine uptake between two types of RNA which he isolated from the cell sap; RNA I which corresponds to sRNA and RNA II which closely corresponds to our post-microsomal RNA. After incubation of the 1 hr. cell sap with ^{14}C-leucine he found that 80% of the activity was associated with RNA II. The accepting capacity of the RNA II remains in doubt however, because if RNA purified from the cell sap with phenol is added to the cell sap and the incubation repeated, 90% of the labelling is found in RNA I.

4. After 10 mins. of incubation a large proportion of the radioactivity is found in the hot-acid-soluble fraction (Table 12 and Fig. 14). If the incubation is continued further the activity in this fraction declines during the next 10 mins. and remains at this reduced level during the subsequent 2 hrs. (Fig. 14) Inclusion of ribonuclease in the incubation mixture abolishes the 10 min peak of hot-acid-soluble activity but surprisingly has no effect on the lower level of counts which persist during the 2 hr. period tested. This observation suggests that all of the radioactivity found in the hot-acid-soluble fraction is not due to RNA-linked ^{14}C-leucine. The experiments in which the post-microsomal pellet was preincubated before examination of amino acid uptake support this contention. Preincubation of the post-microsomal pellet produces an equal stimulus in the hot- acid-stable counts whether the integrity of the RNA is maintained or destroyed by RNAase treatment (Table 26). An RNA bound ^{14}C-leucine intermediate has not been isolated from post-microsomal pellet.

5. Nucleotide analysis of post-microsomal RNA has revealed the presence of the unusual base pseudouridylic acid in amounts which are characteristic of the ribosomal RNA (Tables 15 & 16) and which are considerably less than the amounts found in sRNA. The percentage composition of the post-microsomal RNA with respect to pseudouridylate varies in response to protein intake. When the RNA content of the post-microsomal pellet increases in response to protein depletion the percentage of pseudouridylate present falls due presumably to the influx of RNA which contains less of this unusual base. It is thus suggested that the source of the extra

RNA in the post-microsomal pellet is the membrane part of the endoplasmic reticulum.

6. In the presence of amino acid and nucleotide mixtures, leucine incorporation into the hot-acid soluble fraction is reduced.

From these points it can be concluded that the initial (hot-acid-soluble) activity observed during incubation with ^{14}C-leucine is probably associated with the RNA of the pellet which is consequently an acceptor under these circumstances. Furthermore it seems likely that this acceptor RNA is provided with ^{14}C-leucine by the activating enzymes of the pellet. This RNA however, seems to be unnecessary for incorporation into the hot-acid-stable fraction of the post-microsomal pellet.

The biological significance of the post-microsomal pellet.

We have isolated from rat liver cells, a particle which exhibits unusual properties of leucine incorporation. The contribution of post-microsomal pellet to protein synthesis is unclear and only very tentative deductions concerning the true function of this fraction can be offered. These particles represent a cross section of the cell population which sediment from the cell sap more slowly than free ribosomes. Although the RNA present in the post-microsomal pellet and the ribosomes are both characterised by the same amount of the rare nucleotide pseudouridylic acid, these fractions may be distinguished from each other by the following properties.

1. The RNA/protein ratio of the post-microsomal pellet varies from 0.1 to 0.2 while that of purified ribosomes is essentially 1.

2. The ribosomes are the main site of protein synthesis (Littlefield *et al.*, 1955; Littlefield & Keller, 1957) whereas the post-microsomal pellet does not appear to show the same capacity to synthesise protein.

However, the ribosomes have not been excluded as the intracellular origin of the post-microsomal pellet. In bacteria it has been shown that only the "active 70S" ribosomes are capable of taking part in protein synthesis.(Tissieres *et al.*,1960; Tal & Elson, 1961). The 70S ribosomes can be dissociated into two smaller fragments with different sedimentation coefficients usually 50S and 30S (Tissieres *et al.*, 1959). These smaller particles are biologically dissimilar (Elson & Tal, 1959) and both have lost the capacity to incorporate amino acids. *In vivo* Tissieres *et al.*(1960) envisage a process whereby completed protein molecules are released from the "active 70S" ribosome with the simultaneous formation of a 50S and a 30S particle. They suggest that the 50S and 30S ribosome must be reactivated in some way before participating once more in protein synthesis. Elson (1961) has also put forward the theory that the protein synthesising apparatus is unequally divided among different types of ribosomes.

We therefore suggest that the post-microsomal pellet may consist of fragments which are derived from ribosomes and which have a lower molecular weight than "active 70S" ribosomes, because:

1. The post-microsomal pellet lacks the full mechanism for protein synthesis.

2. Preparation of post-microsomal pellet in the presence of the metal-chelating agent EDTA, which induces disaggregation of the

ribosomes, increases the activity and to some extent the amount of the post-microsomal pellet. (Note, however, that direct treatment of the microsomes with pyrophosphate does not produce an active post-microsomal pellet.)

3. Electron microscope pictures show the post-microsomal pellet as a uniform granular material in which no vesicular structures are apparent, and thus no complete microsomes would appear to be present.

4. <u>In vivo</u> the pellet protein became labelled with ^{14}C-leucine to an extent which never exceeded 30% of that found in whole microsomes. It is thus impossible to differentiate between independent incorporation into the post-microsomal pellet and transfer to this fraction of protein prelabelled in the microsomes.

The amino acid leucine appears to have some special significance in this system and is incorporated by the post-microsomal pellet in the presence of an active RNAase. The uptake of leucine may be involved in some mechanism which reactivates ribosomal sub-units for protein synthesis, even if a latent RNAase is present. The post-microsomal pellet, or at least part of it, would therefore be concerned in the reconstitution of active ribosomes for protein synthesis.

Finally it has been suggested from time to time that more than one method exists for protein synthesis and that perhaps structural protein is synthesised by a mechanism differing from that responsible for the making of enzymes and other proteins. No real advances have been made either to prove this hypothesis or to show that only one mechanism exists. It may be that the post-

microsomal pellet is indeed a member of the category embracing alternative mechanisms of protein synthesis as this particulate material cannot be readily reconciled with the available evidence concerning protein synthesis.

SUMMARY.

Summary.

1. Protein depletion causes pronounced loss of RNA from the liver cell, a loss which is associated predominantly with the endoplasmic reticulum. We have shown that, during this period of acute RNA breakdown, the RNA content of the cell sap is increased. Evidence has been presented suggesting that the RNA breakdown products from the endoplasmic reticulum accumulate in the cell sap.

2. The capacity of the cell sap RNA to bind amino acids is also altered by diet but varies inversely with the RNA present. When, in response to protein depletion, the RNA content increases, the incorporation of leucine is correspondingly diminished. However, although the amount of cell sap RNA changes with protein intake, the <u>total</u> capacity to accept amino acids is unchanged. Thus the additional RNA found in the cell sap appears to be inert as an amino acid acceptor and merely dilutes leucine incorporation by the sRNA present.

3. The effects produced on the cell sap RNA by the dietary conditions are due to changes in the post-microsomal pellet, a particulate fraction which is sedimented by centrifuging the cell sap for 3 hrs. at 105,000 g. As in the cell sap, the capacity of the post-microsomal pellet to accept amino acids and the amount of RNA present vary inversely with diet.

4. RNA purified from the post-microsomal pellet was inert as an amino acid acceptor, even in the presence of rat liver pH 5 enzyme. sRNA was therefore presumed to be absent from the post-microsomal pellet; this was confirmed by column chromatography.

5. In the presence of an energy source, the post-microsomal pellet is capable of incorporating leucine into a form which is stable to hot-acid extraction and not therefore associated with RNA. Only a small proportion of the total leucine incorporated was solubilised by this treatment.

6. The apparent existence of an sRNA free mechanism for amino acid incorporation prompted an investigation of the amino acid uptake observed in the post-microsomal pellet.

7. The leucine uptake into the hot-acid-stable fraction of the post-microsomal pellet is

a. dependent on ATP as an energy source.

b. inhibited by GTP, CTP and UTP.

c. stimulated by amino acid mixtures in the presence of all four nucleoside triphosphates.

d. inhibited by glutathione.

e. unaffected by chloramphenicol.

f. unaffected by ribonuclease (possibly even augmented) in sufficient concentration to reduce the RNA present to 8% of its original value.

g. inhibited by addition of pH 5 enzyme from the cell sap.

8. The leucine uptake cannot be equated to complete protein synthesis since the incorporated amino acid is located in a terminal position from which it may easily be displaced in the presence of excess leucine. Other amino acids are incorporated to a reduced extent or not at all.

9. The total activating enzyme activity in the post-microsomal pellet as judged by amino acid dependent ATP-^{32}P pyrophosphate exchange is almost equal to the level of these enzymes found in

the cell sap. A large proportion of the enzyme present is specific for leucine. However, if the activated acyl-adenylate complex for leucine is formed, in the post-microsomal pellet, it appears to be unable to donate the leucine to sRNA, the natural acceptor.

10. The behaviour of the post-microsomal pellet is not in accordance with the mechanism described by Hoagland for protein synthesis from free amino acids and indeed cannot be reconciled with any step in the complicated pathway involved in amino acid activation and formation into peptide chains. The relationship of this system to various protein synthetic mechanisms proposed in the literature is discussed.

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