ENDOPLASMIC RETICULUM AND PROTEIN SYNTHESIS IN THE LIVER CELL.

by Hazel J. Hird, B.Sc.

A study has been made of a particulate fraction obtained from the liver cell of the rat. This fraction was prepared from liver cell sap by prolonged centrifugation and thus belongs to the post-microsomal type of cell particle. Evidence previously obtained in this laboratory indicates that this fraction may be derived from breakdown products of the endoplasmic reticulum. The observation that this post-microsomal fraction has the capacity to incorporate amino acid in an *in vitro* system, by a reaction which is different from the currently accepted mechanism of protein biosynthesis, has initiated a detailed examination of the characteristics of the fraction.

It consists mainly of ribonucleoprotein material with very small amounts of phospholipid. However, it is not a homogeneous fraction, at least four separate sub-fractions being identifiable.

The characteristics of the reaction by which the post-microsomal fraction incorporates amino acids *in vitro* have been studied. On incubation in a medium of pH 7.8 containing $MgCl_2$ and $KHCO_3$ it will incorporate several amino acids into a form which is stable to extraction with hot perchloric acid. Of the amino acids which were tested, lysine and glycine did not require the presence of ATP for incorporation, leucine and methionine were only incorporated when ATP was added to the

medium and phenylalanine, alanine and glutamic acid were not incorporated to any extent in the presence or absence of ATP. The reaction by which leucine is incorporated has been studied in much greater detail. About 30% of this aminoacid is incorporated into an N-terminal position in the protein chain. Also, at least 30% of the ^{14}C-leucine incorporated in an initial period of incubation can be removed by a further period of incubation in the presence of a large excess of non-radioactive leucine. These observations, together with the finding that the addition of a complete amino acid mixture depresses incorporation, have led to the conclusion that true protein synthesis is not taking place.

The post-microsomal fraction has been found to contain enzymes which will activate amino acids in the presence of ATP. These enzymes can be used to replace the conventional pH 5 enzyme activating system in the common system of protein synthesis. The spectrum of activating ability of post-microsomal pellet for several amino acids is quite distinct from that of pH 5 enzyme; also, these activating enzymes are present in much higher concentration in the post-microsomal fraction. However, the activating enzymes of post-microsomal pellet can largely be removed by resuspending and resedimenting the fraction; presumably, therefore, these enzymes do not form an integral part of the structure of the post-microsomal fraction. Unlike pH 5 enzyme, post-microsomal pellet does not contain any active sRNA. It will, however, rapidly transfer

amino acids to RNA if isolated cell sap RNA is added to the incubation system. After a short period of transfer, there is a rapid decline in the labelling of the RNA; this is probably due to degradation of the terminal acceptor trinucleotide of the sRNA by post-microsomal degradative enzymes resulting in the release of the ^{14}C-leucine already incorporat

Several attempts have been made to obtain a fraction with post-microsomal pellet-like properties released on the breakdown of other sub-cellular components. These efforts have not, however, met with any success. It is, therefore, not certain what the relationship of this post-microsomal fraction to the breakdown of the endoplasmic reticulum is, despite evidence obtained previously in this laboratory which suggested that fragments from the reticulum may accumulate in the post-microsomal fraction under some conditions.

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by

Hazel J. Bird, B.Sc.

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GENERAL INTRODUCTION.

An Outline of the Presently Accepted Theories of Protein Biosynthesis.

It was in the early 19th century that the word 'protein' first came in to use. At this time the name was used to designate a class of nitrogen-containing organic compounds.

Since then, a very large amount of work has gone into the study of both the structure and function of these compounds and now, quoting Hoagland (1959), it may be said that "The blink of an eye, the beat of a heart, the ordered advance from embryo to adult - in short, all the simple and complex workings of living matter - are the result of the co-ordinated interplay of different protein molecules." Obviously, these molecules are of tantamount importance to the living cell for its substance, for the working of its machinery and for its individuality. Quoting Hoagland (1959) once again, "It is small wonder that the central problem of biochemistry today is: How do organisms make their protein?"

Since some knowledge had previously been obtained about the breakdown of protein by the digestive enzymes, it was natural that the first suggestion for the mechanism of the synthesis of proteins was by a reversal of these breakdown reactions. (See the review by Borsook and Wasteneys, 1930.) This mechanism proved to be unacceptable, however, as the products of the synthesis reaction were

obviously not tissue proteins. Fruton (1950) gave some cause for reappraisal of the function of these enzymes when he showed that certain of the proteolytic enzymes could transfer amino acids to and from peptides. By this means polypeptides could be formed but the inability of the mechanism to show any degree of specificity has made its importance seem very doubtful.

Much of the subsequent work has been based on the fundamental observation made by Caspersson (1941) and Brachet (1942) that there is a link between the ribonucleic acid (RNA) content of a cell and its capacity to synthesise proteins.

In the subsequent sections of this introduction we shall outline the presently held theories of protein synthesis. First, there is a short account of the morphology of the cell and the intracellular location of the processes involved in protein biosynthesis as envisaged at present. Following this is a description, in some detail, of the actual chemical reactions involved in the building of a protein molecule giving some idea of the methods used by the cell to achieve specificity in the formation of the protein. Lastly, the background to the present investigation of protein formation by a specific fraction of the liver cell is outlined.

Cell Structure.

As our interest lies mainly in animal tissues, we shall confine ourselves to studies on such cells. As early as 1839, Theodor Schwann propounded the theory that the living cell was the fundamental unit of all living organisms. Since then, investigations have followed two paths, one developing the microscopic and submicroscopic anatomy of the cell, the other the biochemical reactions that underlie the processes of life. Now these two pathways have met and cell structure and function must be studied in conjunction.

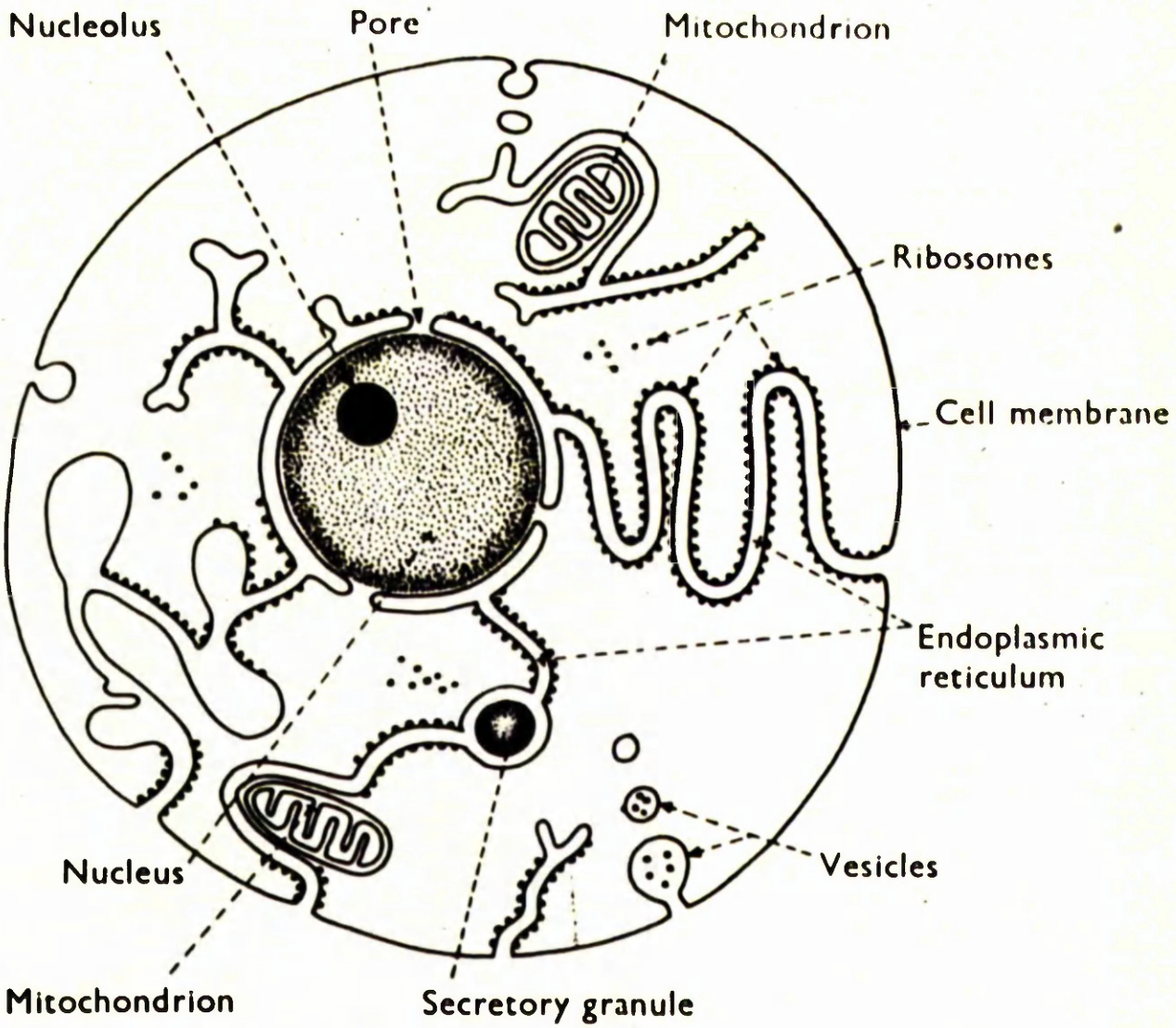
Each cell is surrounded by a cell membrane of complex structure, although only 100 Å thick (Holter, 1961). Within the membrane lies the cytoplasm containing a variety of organelles; the nucleus, mitochondria and endoplasmic reticulum (Porter and Kallman, 1952; Porter, 1953) otherwise known as ergastoplasm (Chantrenne, 1961) or α-cytomembranes (Sjostrand, 1956) being the most obvious of these structures. (See Fig. 1.)

All cells capable of self reproduction have a nucleus. The membrane surrounding the nucleus forms the interior boundary of the cytoplasm. It is a double membrane and it has been suggested that it is continuous with the endoplasmic reticulum, thus forming an extremely convoluted channel throughout the cell (Hartmann, 1953; Watson, 1955; Gay, 1960). Base analyses of the RNA of these membranes and the nuclear RNA have been carried out showing that these

Fig. 1.

A Schematic Representation of a "Typical" Animal Cell.

(From Davidson, 1960.)



RNAs are very similar (Goswami et al., 1962) but different from that of other subcellular fractions giving some weight to this hypothesis.

Within the nucleus are the filaments of chromatin in which the cell's genetic material, deoxyribonucleic acid (DNA), is localised and also the nucleolus, a tightly packed mass of tiny granules with a very high RNA content. In each nucleus there may be more than one nucleolus. Bathing these structures is the proteinaceous "nuclear sap".

The function of the nucleus in protein synthesis has not been completely clarified. The DNA of the nucleus must control the mechanism of synthesis but it is not likely that it exerts this control directly (Chantrenne, 1961). The present evidence suggests that an RNA molecule is formed on the DNA template and is secreted into the cytoplasm where it directs protein synthesis (Caspersson, 1950; Berg, 1961). The nucleolus apparently functions directly in protein synthesis but whether the protein synthesised there is purely nuclear protein or whether it is secreted into the cytoplasm is not clear at present (Allfrey et al., 1957; Fieq and Errera, 1959; Rees et al., 1961; Sirlin et al., 1962).

The second largest structures present in the cytoplasm of animal cells are the mitochondria which occur in abundance in each cell. They have a complex structure with a double

membrane, the internal one being involuted to form many lamellae. This structure allows the mitochondrion to transform the energy contained in cell nutrients to high-energy bonds in adenosine triphosphate (ATP) in a very efficient manner. Thus, the function of the mitochondria in a cell is to provide energy for all the cell processes. They have been shown to synthesise protein to some extent also, but the significance of this has not, as yet, been determined (McLean et al., 1958; Roodyn et al., 1962; Truman and Korner, 1962).

We come now to the endoplasmic reticulum, an extremely complex system of membranes within the cytoplasm, some of which have smooth surfaces (Porter and Yamada, 1960; Moulé et al., 1960; Porter, 1961), and some are covered with tiny granules of ribonucleoprotein (Palade and Porter, 1954). The degree to which this membrane system is developed depends on the type of cell, being most abundant in cells specialising in secreting proteins such as liver and pancreas (Birbeck and Mercer, 1961). This membrane system is probably continuous with the cell membrane and the nuclear membrane (Barer et al., 1960) thus presenting a very large surface area for communication to the fluid bathing the cell.

The function of the smooth membrane, β-cytomembranes, is not known. Porter and Yamada (1960) have shown that they have no affinity for unattached granules. The smooth membranes have been noted to occur in close relationship

to stored glycogen granules in liver cells, however (Porter, 1961).

As far as the rough membranes are concerned, Siekevitz and Palade (1958) have good evidence that in the pancreas at least, the finished proteins arise in the granules attached to the outer surface of the membranes and that these molecules pile up within the reticulum in the form of zymogen granules(Siekevitz, 1959; Hirsch, 1960).

Intracellular Sites of Protein Synthesis.

The granules covering the vesicles of the endoplasmic reticulum have been shown to be ribonucleoprotein particles. In cells producing large amounts of protein there are a very large number of these particles either attached to the membrane or free in the cytoplasm (Chauveau et al., 1962) suggesting that they may be the site of protein synthesis.

Kinetic studies on the incorporation of labelled amino acids into the protein of fractions isolated by centrifugation showed all fractions to incorporate amino acids, but that microsomes (i.e. the homogenisation product of the endoplasmic reticulum (Palade and Siekevitz, 1956)) were initially labelled more intensively (Hultin, 1950; 1955; Khesin, 1954; Keller et al., 1954).

Several workers later succeeded in separating microsomes into various fractions. By using the detergent, deoxycholate, the membrane portion can be solubilised leaving the granules containing 80% of the RNA and 20% of the protein of the

original microsomes (Palade and Siekevitz, 1956; Littlefield et al., 1955). Alternatively, the particles can be removed by treatment with pyrophosphate (Sachs, 1958) or versene (Palade and Siekevitz, 1956), leaving the membrane which contains much of the original microsomal protein, all of the phospholipid and some 30-40% of the RNA. Such work made it possible to pin point the ribonucleoprotein particles (now come to be known as ribosomes) as the major site of protein synthesis. Much work has therefore been done on the structure of these particles in an attempt to elucidate this mechanism.

The first problem encountered in animal cells was the fact that some ribosomes appeared to be present free in the cytoplasm whereas others were attached to the endoplasmic reticulum. Palade and Siekevitz (1956) have shown that these two types of ribosomes are not identical in function. Those ribosomes which occur free may be involved in protein synthesis for intracellular purposes, while those attached to the endoplasmic reticulum may be involved in making secretory proteins (Porter, 1961). Thus, although the protein formed by the ribosome may be used for various purposes, this need not reflect a basic difference in the mechanism whereby these proteins are made in the ribosome.

Once extracted from the cell, the stability of the

ribosomes is a function of the ionic strength and concentration of divalent cations in the suspending medium. The variation of particle size with Mg^{++} concentration can be summarised as follows:-

$$(100\ S) \rightleftharpoons 2(70\ S) \rightleftharpoons 2(50\ S) + 2(30\ S)$$

Mg^{++} concentration decreasing →

(Tissieres et al., 1959; Roberts et al., 1959; Petermann and Hamilton, 1961).

Early electron micrographs showed that mammalian 70 S particles were spherical, with a diameter of 150 to 200 Å (Palade, 1958). More recently, Huxley and Zubay (1960) have produced electron micrographs of E.coli ribosomes. Particles of 30 S appear irregularly shaped with average dimensions 95 by 170 Å; particles of 50 S are nearly spherical with a diameter of about 160 Å; particles of 70 S are units of unequal size joined to give irregularly shaped structures and 100 S particles appear as dimers of these 70 S particles.

The RNA content is about 40 to 50% for mammalian ribosomes (Roberts, 1958) and 60 to 65% for E.coli ribosomes (Tissieres et al., 1959). The RNA has been extracted by various methods and shown to have sedimentation coefficients ranging from 16 to 18 S and 25 to 28 S, giving molecular weights between 5 and 6×10^5 and 1.1 and 1.3×10^6. Ribosomes

with a sedimentation coefficient of 30 contain only 16 S RNA but 50 and 70 S ribosomes yield a mixture of 16 and 25 S RNA. It is possible that the 25 S component is a dimer of the 16 S RNA (Kurland, 1960).

Nucleotide analysis of ribosomal RNA shows a high guanylic acid content; uridylic and cytidylic acids are present in small but about equal amounts; the quantity of unusual nucleotides is negligible (Monier et al., 1960; Ofengand et al., 1961). The protein content of ribosomes has not been studied in great detail but it would appear that there is no difference in the proteins associated with the various particles (Spitnik- Elson, 1962).

Although the structure of the ribosome has been widely studied, the relationship of this structure to the ability of the ribosome to synthesise predetermined protein molecules is not understood. At one time, the ribosomal RNA was thought to act as a template for the synthesis of proteins (Crick, 1958) but the present theory is that the template RNA is manufactured in the nucleus and becomes attached to the ribosome only transiently by an unknown linkage. Warner et al., (1962) have recently suggested from electron microscope studies with rabbit reticulocyte ribosomes that ribosomes act in groups of five, the template or messenger RNA forming links between them. This suggestion is supported by the work of Wettstein et al. (1963).

There remains the soluble fraction of the cell. This fraction contains a vast variety of activities but we will restrict ourselves to those fractions which are involved in protein synthesis. By adjusting the pH of this soluble fraction to 5 it is possible to concentrate a fraction which contains enzymes which can activate amino acids and also a soluble RNA which plays a part in protein synthesis (Hoagland et al., 1956). Also present in the soluble fraction of the cell but not precipitable at pH 5 is an enzyme which is involved in the transfer of amino acids to the ribosomes for the synthesis of protein (Nathans and Lipmann, 1960; Grossi and Moldave, 1960).

This concludes the discussion of the intracellular location of the various reactions involved in the synthesis of protein. The salient points can be summarised as follows:-

In the soluble fraction of the cell are found the enzymes and soluble RNA which are responsible for the initial stages of protein synthesis. Energy is also required in these stages and is supplied in the form of ATP by the mitochondria. The ribosomes (small ribonucleoprotein particles) which may be present free in the cytoplasm or attached to the endoplasmic reticulum then arrange these amino acids into a specific polypeptide with the help of an RNA template supplied by the nucleus. On release from the ribosome, the polypeptide assumes the appropriate three dimensional structure and thus a specific protein is

formed. This protein may be used intracellularly or secreted by means of the endoplasmic reticulum to another part of the organism.

We must now consider in more detail the mechanisms involved at each of these stages in the formation of a protein.

Initial Stages of Protein Synthesis.

1. Amino Acid Activation.

The formation of a peptide bond is an endergonic process and from work by Snoke and Bloch (1955) on glutathione synthesis and Maas (1955) on pantothenic acid synthesis it was obvious that one energy-rich phosphate bond was required for each peptide bond formed. Maas suggested that the activation was a two step reaction involving first, the formation of an adenylate. This hypothesis was given weight when Berg (1955) showed that a similar mechanism was involved in the activation of acetate.

Using the procedures of ATP-pyrophosphate exchange and hydroxamate formation employed by these workers, Hoagland (1955) found a similar reaction involving amino acids to take place in the soluble fraction of rat liver. This initial break-through in the study of the mechanism of amino acid activation stimulated many workers to further investigation in this field.

The methods used in the study of 'activating enzyme'

activity have not changed markedly from that of ATP-^{32}PP exchange and the formation of hydroxamates as used by Hoagland (1955; 1956). The difficulty involved in the use of these methods is that most fractions under survey are contaminated with endogenous amino acids which tend to mask the activating enzyme activity brought about by the addition of amino acids. To overcome this difficulty, Bucovaz and Davis (1961) have used 'Norite' and 'Sephadex' treatment to lower the non-amino acid dependent ^{32}PP exchange rate of tissues. Also, two methods have been devised whereby the activity of a single amino acid activating enzyme can be estimated in the presence of a mixture of amino acids and activating enzymes. These methods are based on the formation of an hydroxamate from a ^{14}C-labelled amino acid and its subsequent separation on, in the first case, an Amberlite IRC-50 column (Coleman and Elliot, 1962) or, in the second method, on a strip of Amberlite IR-120 ion exchange paper (Loftfield and Eigner, 1961). Von der Decken (1961) has also devised a method involving a rapid scanning procedure for amino acid activating enzymes which is based on the paper chromatographic separation of labelled ATP formed by exchange with ^{32}PP.

By these methods, activating enzymes have been shown to be present in a large variety of mammalian tissues (Hoagland, 1961; Berg and Ofengand, 1958; Lipmann, 1958; Schweet et al., 1958; Novelli and De Moss, 1957), in plants

(Webster, 1959; Clark, 1958) and in microorganisms (Roberts et al., 1959; Wieland and Pfleiderer, 1957). Many of these enzymes have now been purified to a fairly high degree using techniques such as isoelectric precipitation, ammonium sulphate precipitation, DEAE cellulose columns, calcium phosphate gel and ethanol fractionation. Table 1 shows some of the enzymes which have been purified.

An interesting point about the occurrence of activating enzymes is that in mammalian tissues, the enzymes have been found almost entirely in the cell supernatant while in bacteria, although the bulk of the activating enzymes have been found in the supernatant fraction, some activating enzymes have been found associated with membrane fractions. However, the enzymes can be removed fairly easily from the membranes, although not by simple washing, and thus are unlikely to be an integral part of the membrane (Spiegelman 1959; Hunter et al., 1959; McCorquodale and Zillig, 1959).

Making use of the partially purified enzymes, the reaction mechanism for the activation of amino acids has been largely elucidated.

$$\underset{\text{Enzyme}}{\text{Activating}} + \text{Amino Acid} + \text{ATP} \rightleftharpoons \underset{\text{Acid}}{\text{Amino-AMP}}\text{-}\underset{\text{Enzyme}}{\text{Activating}} + \text{PP}$$

The available experimental evidence in support of the mechanism can be summarised as follows:-

Table 1.

Activating Enzymes which have been Purified.

Amino Acid Activated	Enzyme Source	Reference
L-alanine	Pig liver nuclei	Webster, (1960)
	Rat liver	Holley and Goldstein (1959)
L-arginine	E.coli	Boman et al. (1961)
L-iso-leucine	Guinea pig liver	Ogata et al. (1960)
	E.coli	Bergmann et al. (1961)
L-leucine	Guinea pig liver	Ogata et al. (1960)
L-methionine	Yeast	Berg (1956)
L-serine	Beef pancreas	Webster and Davie (1961)
L-threonine	Calf liver	Lipmann et al. (1959)
L-tryptophan	Beef pancreas	Davie et al. (1956)
	Pig pancreas	Cole et al. (1957)
L-tyrosine	Pig pancreas	Schweet et al. (1957)
		Schweet and Allen (1958)
	Beef pancreas	Bernlohr and Webster (1958)
	Yeast	Van de Ven et al. (1958)
	Guinea pig liver	Allen et al. (1960)
	Rat liver	Holley et al. (1961)
L-valine	E.coli	Bergmann et al. (1961)

The enzymes activating amino acids catalyse an amino acid dependent ATP-PP exchange, and an ATP dependent formation of hydroxamates (Davie et al., 1956; Hoagland et al., 1956). These facts can be fitted into the above equation :-

ATP-^{32}PP Exchange.

Activating enzymes + Amino acid + A-P-^{32}PP

⇌

Amino acid-AMP-Activating enzyme + ^{32}PP

Hydroxamate Formation.

Activating enzyme + Amino acid + ATP

⇌

Amino acid-AMP-Activating enzyme + PP

+

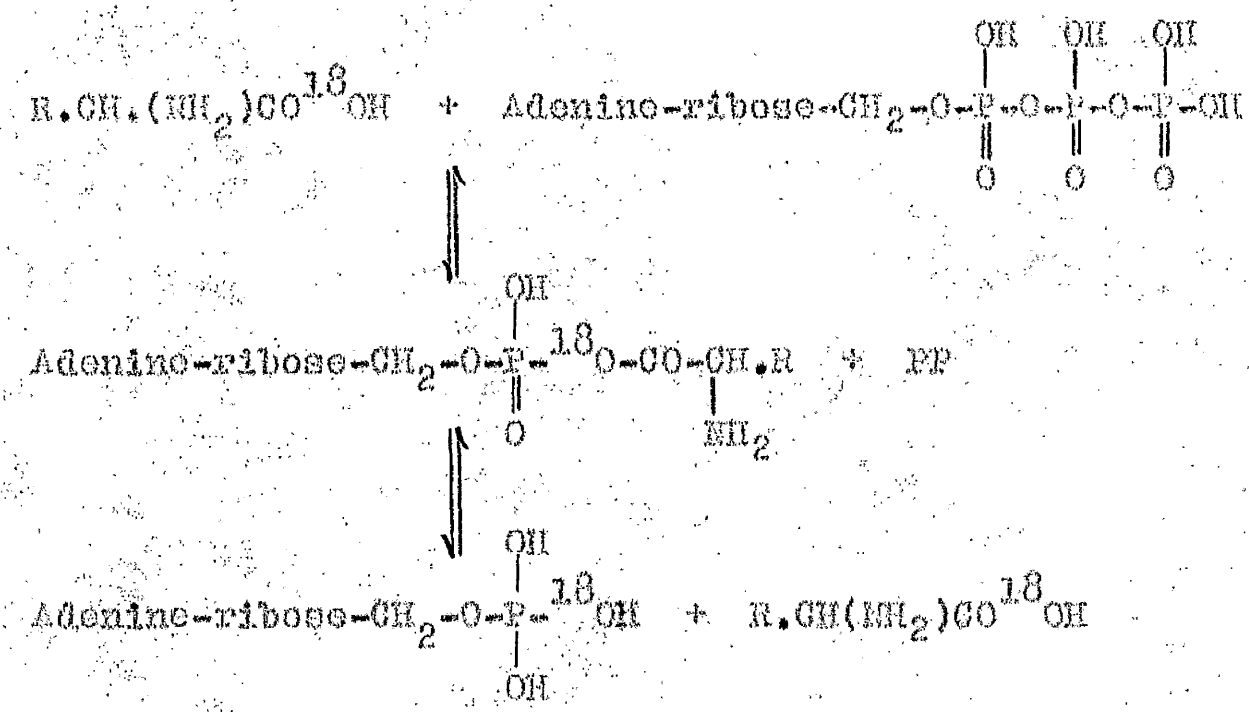
Hydroxylamine (NH_2OH)

⇌

Amino acid.NHOH + AMP + Activating enzyme
(hydroxamate)

Using tyrosine activating enzyme from beef pancreas, Bernlohr and Webster (1958) showed that ^{18}O from the carboxyl group of tyrosine was detectable in the released AMP. No ^{18}O could be found in the pyrophosphate liberated. This finding has been confirmed by other groups of workers (Hoagland et al., 1957), and is consistent with the above mechanism involving the formation of pyrophosphate but not with a reaction involving the formation of inorganic phosphate which is another possible method of activation.

Again, we can fit this finding into the above equation:-

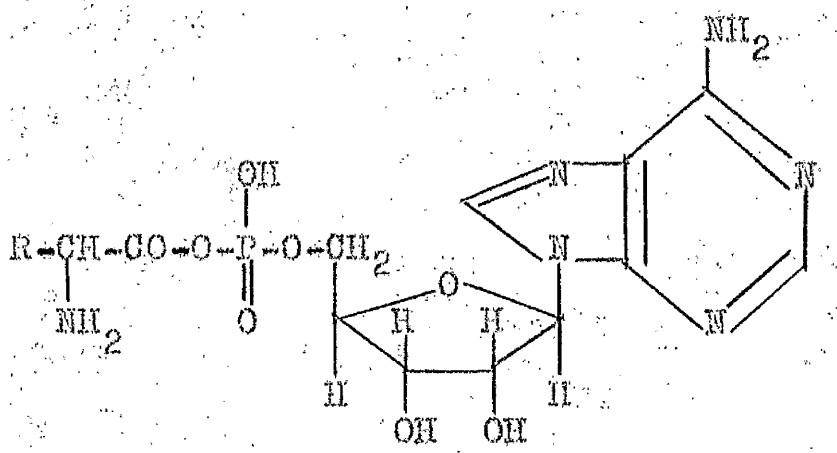


In 1955, the formation of a leucyl hydroxamate was demonstrated by Hoagland (1955) in a rat liver soluble system and by De Moss and Novelli (1955) in preparations from microorganisms, and in 1958 Kingdon et al., (1958) and Karasee et al. (1958) were able to isolate tryptophan-AMP from purified tryptophan activating enzyme. Another finding supporting the aforementioned mechanism is that chemically synthesised amino acyl adenylates give rise to ATP on incubation with pyrophosphate demonstrating the reversibility of the reaction (Krishnaswamy and Meister, 1960; De Moss et al., 1956; Novelli, 1958; Borg, 1957).

The remaining point for which evidence must be presented is the binding of the amino acid adenylate to the enzyme. Free amino acid adenylates are highly reactive and rapidly

form random polypeptides by a non-enzymic mechanism (Askonas et al., 1957; Moldave et al., 1959; Castelfranco et al., 1958; Wong et al., 1959). However, in the cell no such random condensations occur. Similarly, free amino acyl adenylates react with 10 mM hydroxylamine but Molar hydroxylamine is required to form hydroxamates in a cell free system which will activate amino acids. Obviously, during protein synthesis, the amino acyl adenylate is protected from non-enzymic reactions by some method, the most likely one being by binding to the activating enzyme. This theory is supported by the finding of no detectable amount of free amino acyl adenylates in the reaction mixture by Hoagland et al. (1956) and also by the fact that Kingdon et al. (1958) and Karasec et al. (1958) were able to obtain free amino acyl adenylates only when the activating enzyme was completely denatured. The bound amino acid adenylate is so unreactive, in fact, that Askonas et al., (1957) suggest that the nitrogen of the amino acid may well be involved in a temporary covalent link giving a phosphoramide link.

Thus, the mechanism of amino acid activation has been fairly extensively explored and shown to involve the formation of an amino acyl adenylate of the form shown on page 17. The nature of the linkage of the adenylate to the enzyme has not as yet, been determined.



There are, however, one or two outstanding problems still to be clarified in the field of activation of amino acids. In many of the early experiments with crude preparations amino acid activating enzymes could not be detected for all amino acids by ^{32}PP exchange or by the formation of hydroxamates. Some workers were, however, able to show activity for all the naturally occurring amino acids (Nisman et al., 1957; Cole et al., 1957; Lipmann, 1958). However, Webster (1959), although initially finding activity for only seven amino acids, showed that by adjusting the pH and amino acid concentration of the incubation medium activity for all 18 naturally occurring amino acids could be obtained. Other differences have been found in the optimal conditions for activity of various activating enzymes. Webster (1961) has found that highly purified alanine activating enzyme from pig liver is inhibited by

sulphydryl groups and is unaffected by p-chloromercuri-benzoate, in contrast, for example, to the tryptophan enzyme of Davie et al. (1956), the tyrosine activating enzyme of Schweet and Allen (1958) or to the serine enzyme of Webster and Davie (1961) all of which require free sulphydryl groups and are inactivated by p-chloromercuri-benzoate. Similarly, tyrosine activating enzyme from rat liver (Holley et al., 1961), guinea pig liver (Allen et al., 1960) and hog pancreas (Schweet and Allen, 1958) is stimulated by potassium ions which inhibit most other activating enzymes. Such variation in optimal conditions for activity can probably explain the difficulty in showing the presence of activating enzymes for some amino acids in some tissues. Also, variability in stability of the various enzymes under the conditions of preparation seems highly probable.

A second problem is that ^{32}PP-ATP exchange and hydroxamate formation have been shown not to give the same picture of activating enzyme activities (Aes et al., 1959; Webster and Davie, 1959; Nohara and Ogata, 1959). The probable explanation of this discrepancy is that the ^{32}PP-ATP exchange estimates the formation of the amino acyl adenylate-enzyme complex and the hydroxamate formation estimates the second stage of the reaction i.e. it substitutes for the soluble RNA (Nohara and Ogata, 1959).

Another problem is that some amino acids can be

incorporated into protein without any ^{32}PP-ATP exchange being demonstrable (Beljanski and Ochoa, 1958; Rendi and Hultin, 1959; Nisman and Fukuhara, 1959). Is this due to a different mechanism of activation? Cormier et al. (1959) have demonstrated a mechanism of activation of glycine by Photobacterium fischeri which involves the formation of inorganic phosphate rather than pyrophosphate. Similarly, Beljanski (1961) has shown a method of peptide formation in Alcaligenes faecalis involving the formation of inorganic phosphate and not pyrophosphate in the initial activation stage. However, in mammalian systems no such mechanisms have been demonstrated. A study of the rates of the various reactions involved in the incorporation of amino acids into protein has shown that this first step is not rate limiting and that concentrations of enzyme-AMP-amino acid too small to be detectable by ^{32}PP-ATP exchange could be enough to give the observed rate of incorporation of amino acids into protein (Gutfreund, 1959). Thus, failure to demonstrate the presence of activating enzymes does not exclude their participation.

There remains the question of the specificity of the activating enzymes for their substrates. In the early experiments of Hoagland et al. (1956) ^{32}PP-ATP exchange was shown to vary directly with the number of amino acids present as well as with their concentration. This finding, which has been amply supported by the findings of other workers

indicates that there is no competition between amino acids for activating enzymes and there is, in fact, an activating enzyme present for each amino acid. However, although the specificity of the activating enzymes is high, it is not absolute. Using enzymes purified from E.coli (Bergmann et al., (1961) have shown that iso-leucine activating enzymes will catalyse the formation of L-valine adenylate and that valine activating enzyme also activates L-threonine. Similarly, Nisman and Hirsch (1958) have shown competition for activation between valine and leucine. This lack of specificity can, however, probably be explained by a variation in the affinity or rate of the reaction for one particular substrate. An additional, interesting, though unexplained finding, is that of Tuboi (1960) who showed the activation of a dipeptide by activating enzymes.

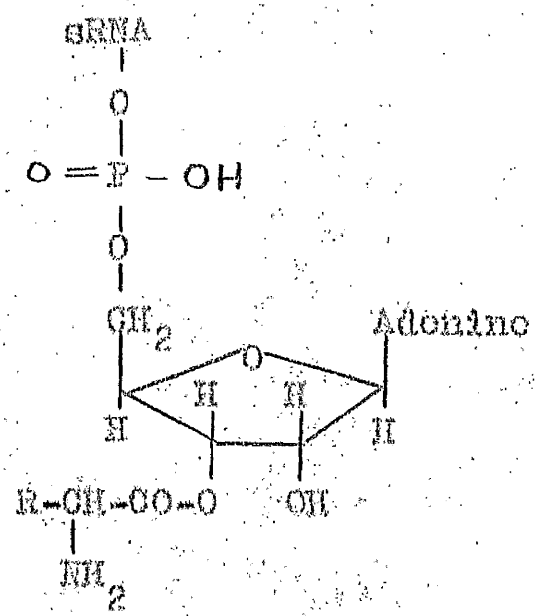
Several studies with amino acid analogues have also been made. In studies with analogues of tryptophan and purified tryptophan activating enzyme Sharon and Lipmann (1957) found some analogues which were activated and some which were not. It is of interest that those analogues which can be activated are found in proteins when in vivo incorporation is studied, and those not incorporated only inhibit the incorporation of tryptophan, presumably by competitive inhibition.

This short account of the specificity of activating enzymes completes the picture of the activation of amino

acids. What is the next step in the pathway of their incorporation into protein?

2. Transfer to Soluble RNA.

In 1957, Holley (1957) showed the presence of a ribonuclease-sensitive, alanine dependent, ^{32}PP-ATP exchange catalysed by alanine activating enzyme. From this initial indication, Hoagland et al. (1957a) and Ogata and Nohara (1957) showed that the amino acid was transferred from the enzyme-bound adenylate to a soluble RNA (sRNA) with the formation of an unstable linkage. Weiss et al. (1959) suggested that this might be an ester linkage to the 2' or 3' hydroxyl of the terminal adenosine of the sRNA molecule and chemical studies by Zachau et al. (1958), Berg and Ofengand (1958) and Preiss et al. (1959) have shown that this is indeed the type of linkage.



So far workers have not been able to distinguish between the

2' and 3' hydroxyl groups however (Zamecnik, 1962).

The specificity of this transfer from activating enzymes to sRNA is obviously important. In the very first experiments (Hoagland et al., 1957a) it was apparent that rat liver sRNA could bind several amino acids and that there was no competition between these. Since then, similar observations have been made in E.coli (Berg and Ofengand, 1958), in reticulocytes (Schweet et al., 1958) and in plants (Webster, 1959). Evidence has now been accumulated to suggest that this second step is even more specific than the initial activation step. With iso-leucine activating enzyme from E.coli Bergmann et al., (1961) have shown that although this enzyme will activate valine as well as iso-leucine, it will only transfer iso-leucine to sRNA. Similar results have been obtained with a tryptophan activating enzyme (Sharon and Lipmann, 1957; Krishnaswamy and Meister, 1960; Wong and Moldave, 1960).

Is there only one species of RNA with specific sites for the various amino acids or is there a species of sRNA for each amino acid? Recent work on the fractionation of sRNA has pointed to the probability that there is a specific sRNA molecule for each amino acid. Due to the great similarity of these molecules, separation work is difficult and so far, only partial separations have been achieved. Most of the published methods of separation involve the use of ion exchange or partition chromatography

(Everett et al., 1960; Ofengand et al., 1961; Smith et al., 1960), electrophoresis (Lipmann et al., 1959), and counter current distribution (Holley et al., 1960; 1962; Apgar et al., 1961). Zamecnik et al. (1960) have developed a chemical method which, at least in principle, is applicable to the isolation of all the individual soluble (or acceptor) RNAs.

At present, the physical and chemical nature of the sRNA molecule is being studied by many groups of workers. The chain length of the polynucleotide has been determined for mammalian, yeast and bacterial sRNA and although there is a wide variation in the results, most of the values obtained lie between 75 and 100 nucleotide residues. (Allen et al., 1960; Klee and Cantoni, 1960; Monier et al., 1960; Goldthwait and Starr, 1960; Ofengand et al., 1961; Brown and Zubay, 1960). Such a chain length is consistent with a molecular weight of 25,000 to 35,000. Sedimentation coefficients obtained have varied between 2.5 and 4.5 (Klee and Cantoni, 1960; Ofengand et al., 1961; Cox and Littauer, 1960), although previously the value was thought to be 1.85 (Hoagland et al., 1958). Recently Klee and Staehelin (1962) have isolated sRNA from yeast using sodium dodecylsulphate. The preparation obtained was heterogeneous containing 4.01 S and 1.04 S particles. The 1.04 S particles had much greater ability to accept activated amino acids than the 4.01 S particles. Ingram and Sullivan

(1962) have reported the presence of amino acids bound to sRNA which are not enzymically attached. Similar observations have been made by Berg (1961) which show a change in S value from 2.7 to 4 on removal of the amino acids. Such effects possibly explain the variations in S values so far obtained.

Forty to sixty per cent of the bases in sRNA are estimated to be involved in hydrogen bonding (Cox and Littauer, 1960; Luborsky and Cantoni, 1962). Fresco et al. (1960) have proposed a model involving looping out of non-complementary base pairs, thereby allowing a maximum number of adjacent hydrogen bonded nucleotide pairs. Recent evidence using X-ray diffraction on crystalline sRNA suggests a uniform double helical structure for the sRNA molecule with the 'unusual' nucleotides located at the 'hairpin bend' where hydrogen bonding does not exist (Spencer et al., 1962). The amino acid specificity does not, however, seem to depend on the secondary structure of the molecule (Brown and Zubay, 1960).

The most striking features about the chemical composition of sRNA can be summarised as follows:-
All active acceptor RNAs so far studied have the same terminal trinucleotide:- Adenyl 5'-3' Cytidyl 5'-3' Cytidyl 5'-3' RNA (Hecht et al., 1958, Preiss et al., 1961). This has been shown to be the acceptor end of the molecule by many workers (Hecht et al., 1958; Herbert and Wilson, 1962; Kalousek et al., 1962). The non-acceptor end of the

molecules have been shown to be guanosine-5-monophosphate (Zillig et al., 1960; Singer and Cantoni, 1960). Current studies have shown that the nucleotide next to the terminal trinucleotide varies from molecule to molecule. About 60% of molecules have AMP, 20% GMP and 15% UMP in E.coli sRNA (Lagerkvist et al., 1961) and in liver and yeast sRNA (Herbert and Wilson, 1962).

sRNA from all sources has a relatively high proportion of "unusual" nucleotides including 5-ribosyluridylate, ribothymidylate, 2-methyladenylate and 6-methylaminopurine nucleotide (Dunn et al., 1960). In rabbit liver sRNA, Cantoni et al. (1962) have shown an average of three 5-ribosyluridylate residues per sRNA chain. These unusual nucleotides have been shown to be located in a group in the molecule (Nihei and Cantoni, 1962) but no specific function has been assigned to them as yet.

Recent work on the "coding of amino acids" which will be discussed later, suggested that there might be more than one sRNA molecule for each amino acid. Some evidence for this has been obtained although the technical difficulties of separating sRNA species has severely handicapped investigations. To date, most studies have shown differences in specific sRNAs from different species rather than from the one soluble fraction (Berg et al., 1961; Allen et al., 1960; Webster, 1960). Physical evidence of heterogeneity amongst sRNA chains specific for a single amino acid has

also been obtained (Everett et al., 1960).

This concludes the description of the activation of amino acids and their subsequent transfer to sRNA. A brief summary is presented below.

The amino acid in the presence of ATP is converted to an amino acid adenylate by a specific activating enzyme. Such enzymes are present in the soluble fraction of all tissues so far studied, but a certain degree of species specificity exists (Benzer and Weisblum, 1961). The amino acid adenylate is tightly bound to the activating enzyme and is transferred directly to an sRNA molecule, an ester linkage of high energy content being formed. Each amino acid has a specific sRNA molecule. A physical and chemical investigation of these molecules has, however, shown very little gross difference between them. Each molecule has a chain length of about 70 to 100 nucleotides with a guanosine-5-monophosphate at one end and the trinucleotide -cytidyl-cytidyl-adenosine at the acceptor end.

However, in conclusion, the absolute specificity of the activation and transfer to sRNA of an amino acid appears to depend on the ability of the activating enzymes to discriminate between closely related amino acids and to recognise an RNA chain which has a characteristic sequence of nucleotides at some point on the chain.

Transfer of Amino Acyl-sRNA to the Ribosome.

It is only recently that much headway has been made in this study and thus, little is known of the detail of the mechanism.

Nathans et al. (1962) have studied the reaction and shown that with E.coli ribosomes only GTP, Mg ions, an energy generating system (such as phosphoenolpyruvate and pyruvate kinase) and an enzyme present in the cell supernatant are required to promote the transfer. Similar studies with mammalian ribosomes have shown a need for all those factors and also in many cases for a sulphydryl compound such as glutathione (Bishop and Schweet, 1961; Hulsmann and Lipmann, 1960).

The mechanism of this transfer is not at all clear. It would appear that initially, the amino acyl-RNA becomes attached to the ribosome (Bosch et al., 1962; Elson, 1962; Takanami, 1962; Hoagland and Comly, 1960) but whether the whole of the sRNA molecule or only part of it is involved is not known. In fact, it would appear from the work of Bloemendal et al. (1961) that the presence of neither an amino acid attached to the sRNA or of activating enzymes is required for the incorporation of the polynucleotide. However, the terminal trinucleotide of the sRNA molecule must be complete and ATP, GTP and an energy generating system have been shown to be necessary for the incorporation of sRNA. It has been suggested that the GTP is required in

in the interaction between the sRNA and ribosomal RNA, whereas the ATP is required only to restore the terminal trinucleotide in the absence of an amino acid (Bloemendal et al., 1962). Webster and Whitman (1962) have shown that one molecule of GTP is degraded for each molecule of amino acid that is transferred from sRNA to protein using a pea seedling preparation. However, Nathans et al. (1962) working with a rabbit reticulocyte system have shown that a pyrophosphate split of GTP does not occur stoichiometric with the transfer of an amino acid to the ribosome. No more definite function of GTP in the transfer system has as yet been offered.

There is some difference of opinion concerning the nature of the bond between the amino acyl-sRNA and the ribosomal RNA. Bloemendal et al. (1960; 1961) have suggested some form of covalent linkage through the terminal adenosine of the sRNA molecule. Takanami (1962) on the other hand from his studies on the effect of variation in the Mg ion concentration on the linkage, reckons that the bond cannot be covalent.

Attempts to purify the enzymes catalysing the transfer of amino acids to the ribosomes have met with some success. Grossi and Moldave (1959) and Hoagland et al. (1958) have shown the presence of such an enzyme activity in the pH 5 fraction of the cell supernatant. However, Takanami (1961; Takanami and Okamoto, 1960) have shown that the fraction

of the supernatant not precipitable at pH 5 is a much more potent source of transfer enzyme and he has purified the enzyme from this source several fold. His results indicate the presence of one enzyme only which is capable of transferring all amino acids from their specific sRNA molecules to ribosomes. This finding is supported by the work of Nathans and Lipmann (1961) but von der Decken and Hultin (1960) have suggested the existence of separate enzymes for each amino acyl-sRNA complex.

A system synthesising a specific determinable protein is essential for the study of the condensation of amino acids into polypeptides on the surface of the ribosomes. A rabbit reticulocyte system synthesising haemoglobin has been found suitable for such experiments. In such a system, Bishop et al. (1960) reckon that, in vitro, haemoglobin chains can be completed which are already partly formed but that new chains cannot be initiated. These studies and those of Dintzis (1961) suggest that the polypeptide is built up by the sequential addition of amino acids from the N-terminal end of the chain. Results obtained with the antibiotic puromycin may be interpreted to agree with these findings, if it is assumed that the puromycin molecule takes the place of an sRNA molecule at some position in the chain thus preventing further growth of that chain (Yamolinsky and de la Haba, 1959; Allen and Zamecnik, 1962).

The mechanism of release of the finished protein and the taking up of its three dimensional structure are not well documented. Webster (1961) has obtained some evidence of the presence of an enzyme catalysing the removal of the protein from the ribosome in a pea seedling system and Lamfrom (1961) has obtained similar evidence in a reticulocyte system. Lingrel and Webster (1961) have shown that an increased K^+ ion concentration will also bring about release of soluble protein. However, contradictory evidence has been obtained by von Ehrenstein and Lipmann (1961) when they found no requirement for a soluble fraction in the release of protein from reticulocyte ribosomes. As far as the formation of the three dimensional structure of the protein is concerned, it is assumed at present that once the amino acid sequence is arranged, the polypeptide automatically assumes the correct three dimensional structure (Perutz et al., 1960).

Determination of Amino Acid Sequences.

There remains the question of the specification of the amino acid sequence of proteins. As has previously been discussed, the initial stages in protein formation, that is the activation of amino acids and their transfer to sRNA leads to the specific attachment of each amino acid to its own characteristic sRNA molecule.

As early as 1958, Crick had put forward the 'Adaptor

Hypothesis' on purely theoretical grounds. In this, he suggested that each amino acid became attached to a small characteristic polynucleotide (a trinucleotide, in fact) which could recognise its complementary trinucleotide in the ribosomal RNA and thus the sequence of amino acids could be determined. Since this theory was put forward, new knowledge has made it necessary to modify it to some extent but, in essence, this is still the accepted theory of amino acid coding. Crick's 'Adaptor trinucleotide' has now been shown to be a somewhat larger polynucleotide (sRNA) of approximately 100 nucleotide residues but it seems highly probable that only three of these are involved in the coding of the amino acid. Similarly the role of the ribosomal template RNA has been somewhat modified.

From experiments with sRNA-amino acids from one species and ribosomes from a different one, it has been concluded that there is some factor present in the ribosome determining the sequence of amino acids in the protein formed (Campbell et al., 1959; 1960; Bishop et al., 1961; von Ehrenstein and Lipmann, 1961; but see Lamfrom, 1962). The homogeneity of ribosomes, both as regards size (Kurland, 1960) and RNA (Belozersky, 1957) makes it seem highly improbable that ribosomal RNA can act as a template in the formation of specific protein molecules which have vastly different molecular weights and amino acid composition.

Also, some very elegant work on the amino acid sequence of the various haemoglobin molecules as compared with mutations of the gene (Benzer et al., 1958; Hunt and Ingram, 1959; 1960) has left no doubt as to the involvement of DNA in the determination of the amino acid sequence of proteins.

Such findings led Jacob and Monod (1961) to predict the existence of an RNA synthesised on a DNA template in the nucleus and subsequently secreted into the cytoplasm whereupon it becomes attached to a ribosome and directs the synthesis of a protein. Using synthetic DNA polymers and a crude enzyme system from E.coli Hurwitz and Furth (1962) were able to synthesise RNA molecules complementary to the DNA polymers. Obviously, the E.coli cell contains a mechanism for synthesising RNA with a sequence of bases determined by DNA. What is the function of the RNA so formed?

A good system for studying this RNA is a culture of E.coli infected with a bacteriophage. In such a system, there is a sudden change in the protein to be manufactured by the cells and thus there ought to be a concomitant rapid synthesis of RNA if the theory of Jacob and Monod is correct. Such a synthesis could not be demonstrated by several groups of workers using the E.coli system, however (Volkin and Astrachan, 1956; Cohen, 1948) but Hershey (1953), Volkin et al. (1958) and Gros et al. (1961)

have shown that there is a rapid synthesis of RNA followed by just as rapid a breakdown.

The base ratios of the newly formed RNA have been studied and shown to be quite different from those of any of the bacterial RNAs but similar to that of the infecting viral DNA (Volkin and Astrachan, 1956; Astrachan and Fisher, 1961; Kitazume et al., 1962). On separation of phenol prepared ribosomal RNA and sedimentation analysis an 8 S peak was obtained as well as the normal 18 S and 25 S ribosomal RNA peaks. (Hayashi and Spiegelman, 1961; Munro and Korner, 1962). It is clear that phage infection of E.coli evokes the formation of a new kind of RNA but whether this RNA is really a special kind of phage specific RNA has not been completely proved. The similarity of the base ratios to those of DNA are significant only if they reflect a similar sequence of nucleotides. The previously mentioned results of Hurwitz and Furth (1962) on the formation of RNA from synthetic DNA polymers support this hypothesis, as does the formation of RNA-DNA hybrids as reported by Hall and Spiegelman (1961) and Otaka et al. (1962).

Some evidence has also been obtained for the presence of this type of RNA in normal uninfected bacterial cells (Hayashi and Spiegelman, 1961; Astrachan and Fisher, 1961;) in plants (Loening, 1962) and in mammalian cells (Hiatt, 1962; Arnstein et al., 1962; Wood and Berg, 1962; Munro

and Korner, 1962; Kruh et al., 1962).

Using an ingenious combination of radioactive and nonradioactive isotopes of carbon and nitrogen Brenner et al. (1962) were able to distinguish between ribosomes existing in E.coli cells before infection with a virus and any that might have been formed after infection. They also could demonstrate the formation of virus specific RNA after infection and whether it became attached to old or new ribosomes. Using this technique, they showed that a messenger RNA was formed on infection and was associated with the old ribosomes. They could not, in fact, detect the formation of any new ribosomes on infection. By using radioactive sulphur, they were able to show also that the newly formed virus protein was associated with the ribosome before it was released into the soluble fraction. This experiment thus shows very elegantly the involvement of a template RNA in the synthesis of a specific protein.

To test the probability of such a mechanism, Nirenberg and Matthaei (1961) investigated the use of a simple synthetic polynucleotide as a template in the E.coli system. They found that when they used polyuridylic acid they got a 1000 fold increase in the incorporation of phenylalanine. From this initial observation, assuming that polyuridylic acid is acting as a template in the synthesis of polyphenylalanine, it would appear that a sequence of unknown length of uridylic acid residues is the code for phenyl-

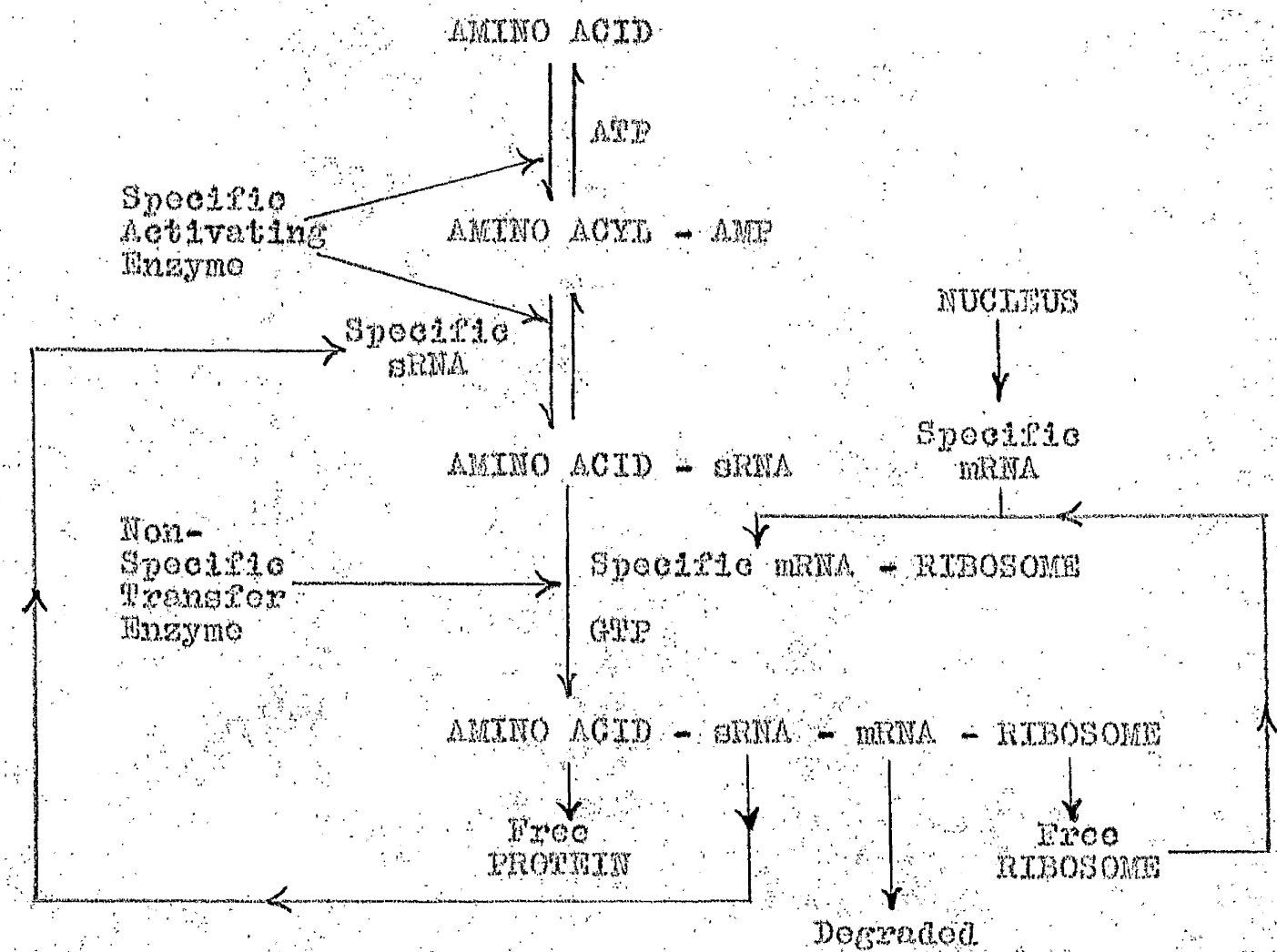
alanine. By using various synthetic copolymers and studying the incorporation of different amino acids in their presence, the laboratories of Ochoa (Lengyel et al., 1962; 1962a; Speyer et al., 1962) and Nirenberg (Martin et al., 1962; Matthaei et al., 1962) have worked out the nucleotide composition, although not the sequence, of the codes for all the amino acids which occur in proteins. Crick et al. (1961) have used a system involving amino acid replacements due to genetic changes brought about by phage infection, to suggest a non-overlapping triplicate code. Such a code is "read" by starting at a fixed point and working along the nucleotide taking the bases three at a time.

So far all the synthetic nucleotide polymers used have contained uridylic acid, as the presence of phenylalanine prevents the formation of a soluble polypeptide which would not be detected by the procedures used (Nirenberg et al., 1962). All the "code" words so far determined contain uridylic acid but whether this is an indication of the degeneracy of the code and that the use of polynucleotides not containing uridylic acid will provide different code words for the amino acids has yet to be determined.

This brings us to the end of our discussion of the mechanisms involved in the biosynthesis of proteins as presently envisaged. Fig. 2 summarises the present picture.

Fig. 2.

Summary of the Reactions Involved in Protein Synthesis.



Alternative Mechanisms of Biosynthesis of Proteins.

Although this scheme is currently held to be the main mechanism of synthesis of proteins, many workers have reported conflicting evidence at many of these stages. Thus, Beljanski (1961) has reported the presence of a fraction in Alcaligenes faecalis which can form peptides though it has no detectable activating enzymes or sRNA. There is some doubt about the presence of such a system in mammalian preparations however (Beljanski and Ochoa, 1958a; Campbell, 1960). Nisman and Fukuhara (1959) have demonstrated a similar system in E.coli and B.megaterium and Zalta (1960) has demonstrated a nonconventional method of amino acid uptake in a rat microsome preparation. Some work has been done on a soluble fraction of rat liver which contains neither activating enzymes nor sRNA (Sachs, 1957; Rendi and Campbell, 1959; Rendi and Hultin, 1959; 1960; von der Decken and Hultin, 1960).

We have obtained a fraction from rat liver which will incorporate amino acids into protein but again this fraction does not conform to the presently accepted mechanism of synthesis of proteins. This fraction has been partially explored by McLean (1962). This work is mostly unpublished and therefore an account of the main observations will now be presented. This is necessary, as the present thesis is a continuation and extension of the previous studies.

Post-microsomal Pellet.

Post-microsomal pellet (Goldthwait, 1959) is prepared from the supernatant remaining after the isolation of microsomes from a rat liver homogenate by centrifugation at 105,000g for 3 hours. Hereafter, we shall refer to the sediment so obtained as "post-microsomal pellet" and the supernatant as 3 hour cell sap to distinguish it from the microsomal supernatant which we shall call 1 hour cell sap. The initial interest in post-microsomal pellet arose from dietary studies on rat liver preparations. When a rat is given a diet free of protein, or is fasted, there is a rapid fall in the RNA content of the microsome fraction of the liver cell (Wikramanayake et al., 1953). This loss of RNA has been shown not to be due to a decline in the rate of synthesis (Campbell and Kosterlitz, 1948; Munro et al., 1953) and, in fact, from studies of ^{32}P incorporation it appears that the diminished amount of RNA has a more rapid turnover than normal liver cell microsomal RNA. However, this readjustment to a more rapid rate of turnover is a delayed response, only taking place after the initial rapid breakdown phase is over and the quantity of RNA has become stabilised at a lower level (Clark et al., 1957). During the initial period of breakdown which lasts for the first day or two after removal of protein from the diet, the rate of turnover of RNA is greatly decreased, probably due to dilution of the precursor pools by the breakdown

products.

It was first suggested that the main product of degradation during this phase of RNA loss was sRNA (Clark and Munro, 1959) and for this reason McLean (1962) studied the changes in the RNA content of the cell sap (supernatant after centrifugation at 105,000g for 1 hour) brought about by varying the dietary conditions. In agreement with the above theory, it was first shown that, in animals fasting overnight after receiving a diet adequate in protein, there is a significant rise in the amount of RNA present in the cell sap as compared with animals fed a meal of protein shortly before death. If the theory of Munro and Clark (1960), that this represents sRNA released from microsomes on fasting, is correct, this larger amount of RNA would be expected to have a greater biological activity. However, this was not the case and, in fact, the cell sap RNA from fasted animals was found to be much less active than that from the non-fasted animals, thus invalidating the above hypothesis.

Can the sRNA of the cell supernatant be separated from the inactive RNA produced by breakdown of the microsomal RNA? Hoagland et al. (1958) have demonstrated that further centrifugation of the cell supernatant obtained after centrifugation for 1 hour at 105,000g caused sedimentation of 50% of the supernatant RNA without any loss of the total soluble RNA activity of the pH 5 fraction. Can the

previously mentioned active and inactive RNAs be separated by such a method? By centrifugation at 105,000g for 3 hours under the conditions described by Goldthwait (1959) we separated 1 hour cell sap into a post-microsomal pellet and 3 hour supernatant. By this means, the changes in the level of RNA present and its biological activity due to the dietary regimen of the animal before death were shown to be confined entirely to the post-microsomal pellet, the 3 hour cell sap being unaffected by the dietary conditions both as regards content and acceptor ability of the RNA.

During the course of these studies on post-microsomal pellet RNA, it was observed that the protein of this fraction has an appreciable capacity to incorporate labelled amino acids and that the conditions for this uptake differed from those described in Fig. 2 - the "classical" system. A beginning was made by McLean (1962) to study this system and the present thesis represents a further study of the properties of the system. It is, therefore, appropriate to summarise here the points established by McLean.

The evidence suggests that the post-microsomal pellet protein can incorporate amino acids by an energy-dependent system. Post-microsomal pellet will take up labelled amino acids into a form stable to hot perchloric acid (PCA), presumably protein bound, in the presence of ATP, $MgCl_2$, $KHCO_3$, KCl and potassium phosphate buffered at pH 7.8; incorporation proceeds vigorously for a period of at least

2 hours of incubation. The presence of GTP, PEP and pyruvate kinase inhibit this incorporation whereas in the "classical" cell sap- ribosome system (Fig. 2) these adjuivants are essential. The concentration of ATP and Mg^{++} ions in the incubation medium is critical for incorporation to occur into post-microsomal pellet (Table 2). There is an absolute requirement for ATP, the activity falling to about 10% of the maximum in its absence. A concentration of 1 mM ATP is optimal and a high concentration of Mg^{++} ions is beneficial to the system.

Further properties of this <u>in vitro</u> system were noted by McLean (1962). The optimum pH of the incorporation of leucine by the fraction is fairly precise, lying between pH 7.6 and 7.9 (Fig. 3). The addition of RNAase has no inhibitory effect on the system and possibly even a stimulatory one (Table 3). In the classical system, on the other hand, RNAase completely inhibits the incorporation due to the sensitivity of sRNA to treatment with RNAase. Another interesting property of the post-microsomal pellet system is the marked stimulation of activity caused by preincubation (Table 3). No satisfactory explanation was found for this phenomenon; it may be due to a similar mechanism to that of RNAase.

The incorporation outlined above appears to be characteristic of the post-microsomal pellet. Fig. 4 shows the incorporation of ^{14}C-DL-leucine into a hot-PCA-soluble

Table 2.

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

Post-microsomal pellet (0.99 mg. protein; 0.11 mg. RNA) was incubated for 2 hours at 37°C in a total volume of 1 ml. Campbell buffer. ATP and Mg^{++} were added in the concentrations shown below; 1 uC ^{14}C-DL-leucine was also present.

ATP umoles/ml.	0.005M $MgCl_2.6H_2O$		0.01M $MgCl_2.6H_2O$	
	ug. RNA recovered	PCA stable cpm./min./mg. protein	ug. RNA recovered	PCA stable cpm./min./mg. protein
10	65	172	73	265
5	57	161	64	290
1	74	225	93	355
0	80	24	98	17

Fig. 3.

The Effect of pH on Leucine Uptake by Post-microsomal Pellet.

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

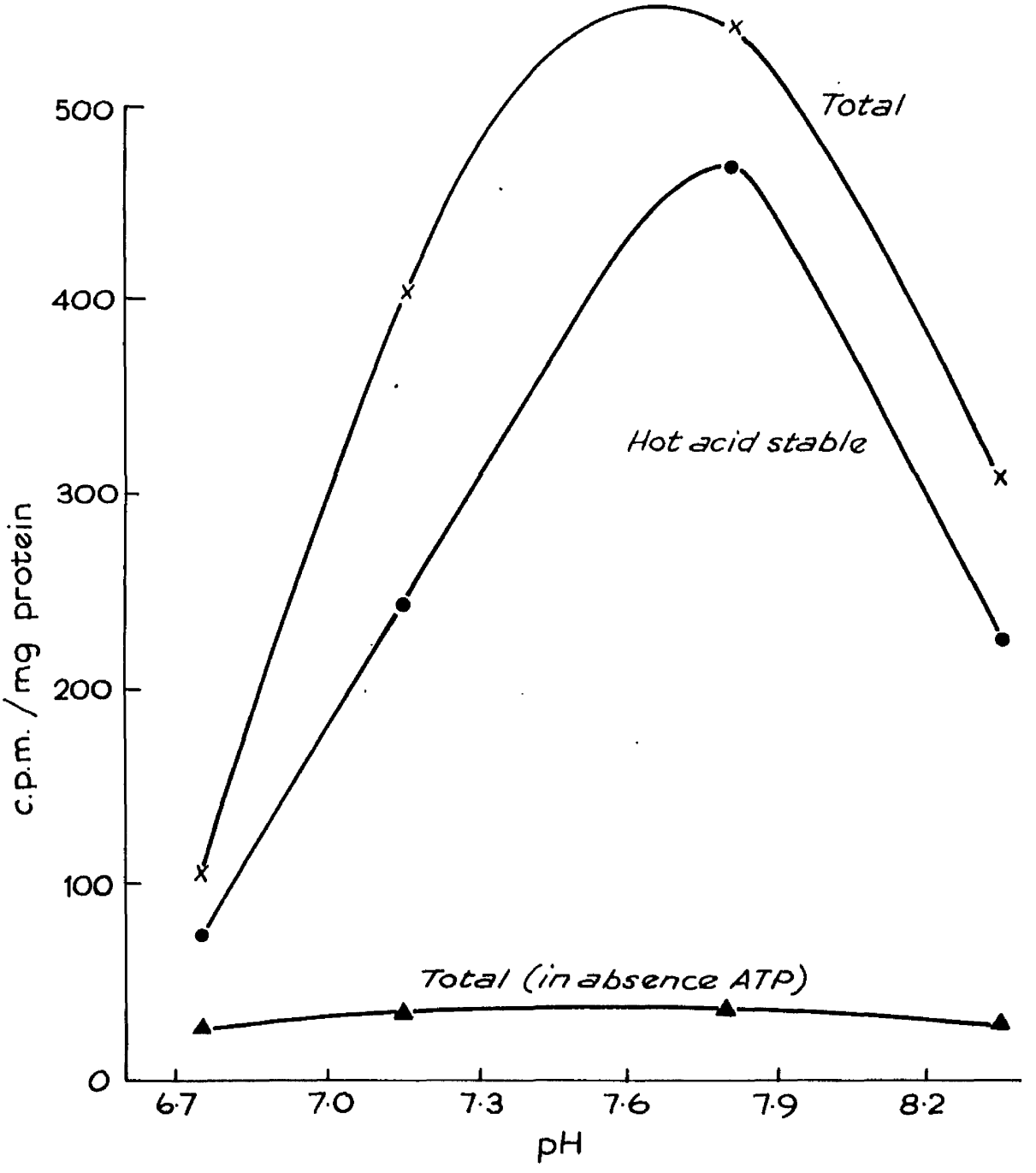


Table 3.

Influence of Preincubation and the Addition of Ribonuclease on the Amino Acid Incorporating Ability of Post-microsomal Pellet.

Aliquots of post-microsomal pellet (1 mg. protein) were incubated for 1 hour in 0.5 ml. Campbell buffer with continuous shaking in the presence or absence of 300ug. RNAase. The tubes were then chilled and 10 umoles ATP and 1 uC ^{14}C-DL-leucine added. These samples and a control sample which had not been preincubated were incubated at 37^{o}C for a further 1 hour in a total volume of 1 ml. Campbell buffer.

Addition before pre-incubation	Pre-incubation	ug. RNA/ tube in relation to Final incubation		Hot PCA soluble cts./min. /mg. protein	Hot PCA stable cts./min. /mg. protein
		Before	After		
None	-	146	79	70	130
None	+	79	62	225	338
RNAase	+	16	7	230	102

Fig. 4.

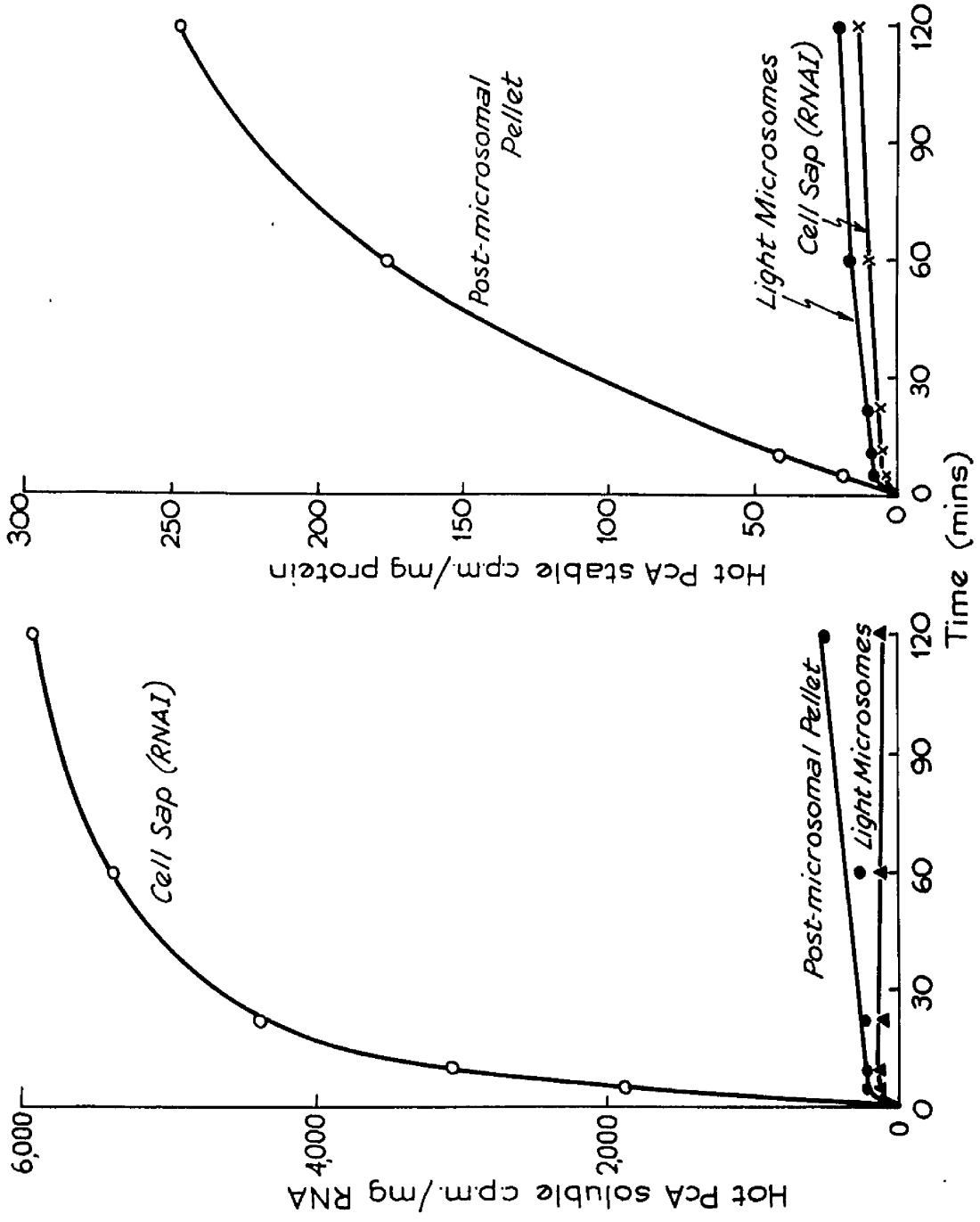
In Vitro Incorporation of ^{14}C-leucine into Subcellular Fractions of Rat liver.

Portions of 3 hour cell sap, post-microsomal pellet and microsomes of equal protein content were incubated in 1 ml Campbell buffer with 10 umoles ATP and 1 uC ^{14}C-DL-leucine at 37^{o}C for the times noted.

Each point is a mean of 2 identical experiments.

Fig. 4.

In Vitro Incorporation of ^{14}C-leucine into Sub-cellular Fractions of Rat Liver.



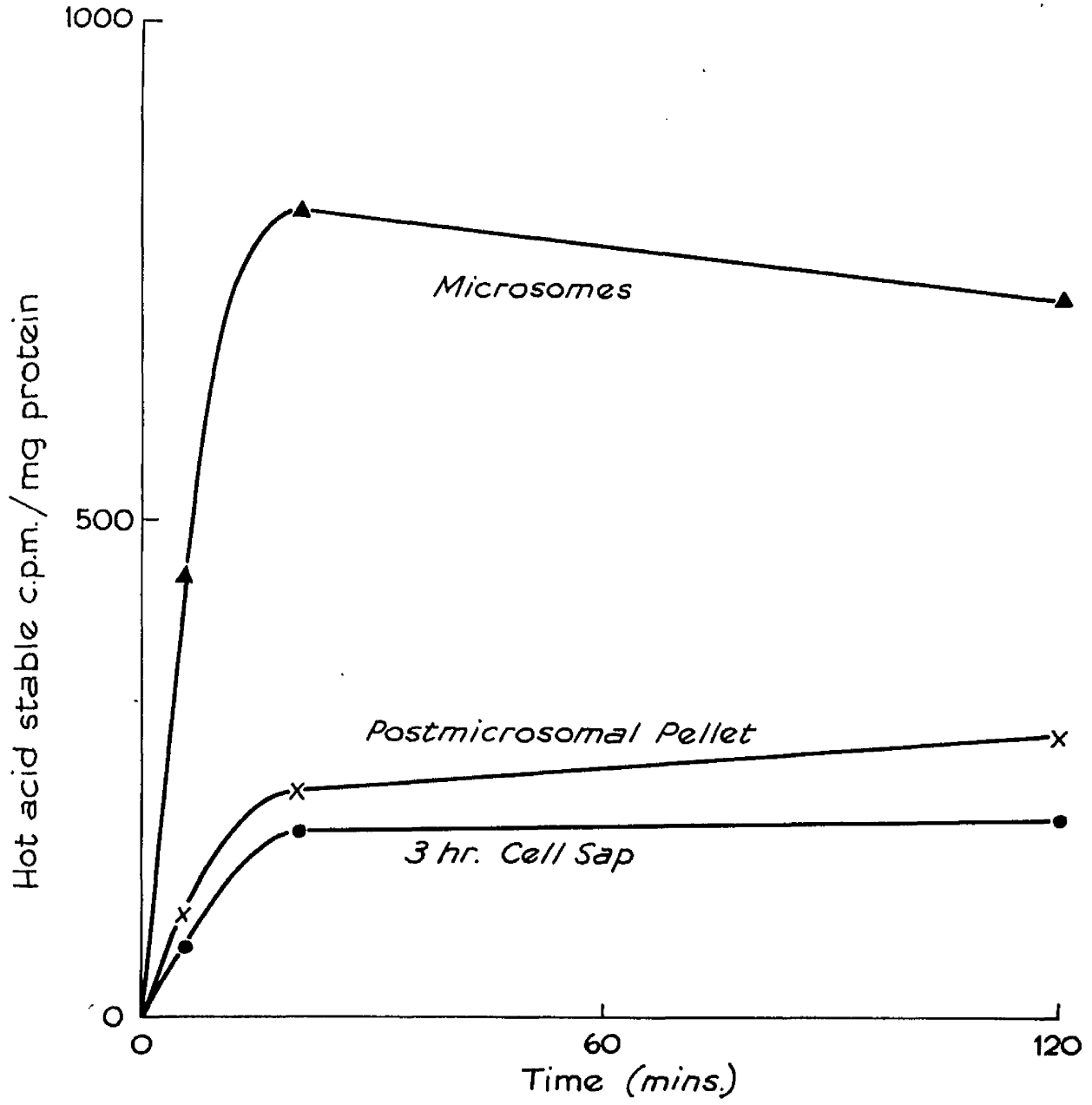
form, presumably RNA, and into a hot-TCA-insoluble form, probably protein, by various cell fractions under the conditions previously described. Only cell sap has any ability to incorporate leucine into RNA. This incorporation presumably representing uptake by mRNA, is very rapid over the first 30 minutes but the rate then decreases reaching a constant level after 2 hours. Both post-microsomal pellet and microsomes have very little capacity to incorporate leucine into RNA, although post-microsomal pellet is at all times slightly more active. The picture with regard to the activity in the protein fraction is quite different, post-microsomal pellet being the most active; the incorporation continues at a fairly steady rate for at least 120 minutes. Under these conditions i.e. without the addition of cell sap, GTP, PEP and PK, microsomes show no ability to incorporate amino acids into protein. Likewise, cell sap is inactive in this system.

All the results reported above were obtained in an *in vitro* labelling system. The picture obtained in an *in vivo* experiment is presented in Fig. 5. The microsome fraction, the most important one in protein biosynthesis, has the highest specific activity, rising to a peak 10 minutes after the injection of the amino acid, thereafter declining slowly. The post-microsomal pellet has only about 30% of the specific activity of the microsome fraction but has at all time intervals a greater specific activity than cell sap.

Fig. 5.

In Vivo Incorporation of ^{14}C-leucine into Sub-cellular Fractions of Rat Liver.

12.5 uC ^{14}C-leucine in 0.9% saline were injected into the tail vein of rats which had previously received a protein free diet. The animals were killed at the times noted and the sub-cellular fractions separated. Aliquots of each fraction were used to determine the specific activity of each fraction.



The specific activity of the post-microsomal pellet protein rises rapidly over the first 10 minutes and continues at a much reduced rate for at least two hours. This suggests that the post-microsomal pellet protein, under natural conditions, does not accumulate labelled amino acids to the same extent as it does over a long period *in vitro*.

The properties of the post-microsomal pellet studied by McLean (1962) can be summarised:-

1. Post-microsomal pellet can incorporate amino acids into protein in the presence of ATP in an ionic medium. The system is completely dependent on the presence of ATP.
2. There is little or no incorporation into the RNA of the post-microsomal pellet.
3. The system has an optimum pH of 7.6 to 7.9.
4. The incorporation is insensitive to RNAase although post-microsomal pellet RNA is sensitive to the enzyme.
5. Preincubation increases the capacity of post-microsomal pellet to incorporate amino acids into a protein form.
6. Incorporation continues for at least 2 hours.
7. The post-microsomal pellet protein does become labelled *in vivo* but to a much smaller extent than the microsome fraction.

Plan of the Present Experiments.

From this point we have continued to explore the properties of post-microsomal pellet in an attempt to elucidate its biological significance.

In Section I, the physical and chemical characteristics of the fraction are compared with those of other sub-cellular fractions.

In Section II, the relationship of the post-microsomal incorporating system to the "classical" system is presented. This investigation includes:-

1. An estimation of the activating enzymes present.
2. A study of the ability of the activating enzymes present in post-microsomal pellet to transfer amino acids to sRNA.
3. A study of the capacity of post-microsomal pellet to replace cell sap in the "classical" system of protein biosynthesis.

In Section III, the nature of the incorporation of amino acids into the protein of post-microsomal pellet is considered. This study can be subdivided as follows:-

1. The position of the incorporated amino acid in the protein chain.
2. An investigation of the conditions under which incorporation can occur in the absence of ATP.
3. Some attempts to separate the various activities of post-microsomal pellet.

In Section IV, some attempts to obtain an active post-

microsomal pellet by breakdown of various sub-cellular fractions are presented.

SECTION I.

SECTION I.

Introduction.

Before any investigations of the function of post-microsomal pellet were carried out, it was obviously of interest to ascertain some facts about the physical and chemical nature of post-microsomal pellet. (a) Does post-microsomal pellet have a constant morphology and chemical composition? (b) Is it a homogeneous fraction? (c) Is it only a non-specific aggregation of some of the components of cell sap?

These questions are considered in this section of the thesis. Electron microscopy, sedimentation coefficient determinations and electrophoresis have all been used in the investigations of the physical properties of the fraction. The chemical composition has also been examined and a nucleotide analysis of post-microsomal RNA is presented. In all these studies, other cell fractions were examined concurrently so that comparisons could be made.

Experimental Methods.

Animals. Male or female albino rats in the weight range 150 to 200g were used. However, the sex and size of the animals was not critical.

Materials. Ponceau S was obtained from Gurr. Otherwise, only common laboratory reagents were used in this section of the work.

Media used for Homogenisation.

1. Campbell Buffer. (Rendi and Campbell, 1959).

   0.01M $MgCl_2.6H_2O$

   0.03M $KHCO_3$

   0.025M KCl

   0.02M Potassium phosphate buffer, pH 7.8

   (KH_2PO_4 : K_2HPO_4 = 1 : 9)

   0.35M Sucrose.

2. Sucrose/Phosphate, pH 7.8 (S/P, pH 7.8.)

   0.02M Potassium phosphate, as above.

   0.35M Sucrose.

3. Sucrose/phosphate/EDTA, pH 3.4. (S/P/E/ pH 3.4.)

   0.02M Potassium phosphate, as above.

   0.35M Sucrose

   0.001M Ethylenediaminotetraacetic acid (EDTA).

Preparation of Sub-cellular Fractions by Differential Centrifugation.

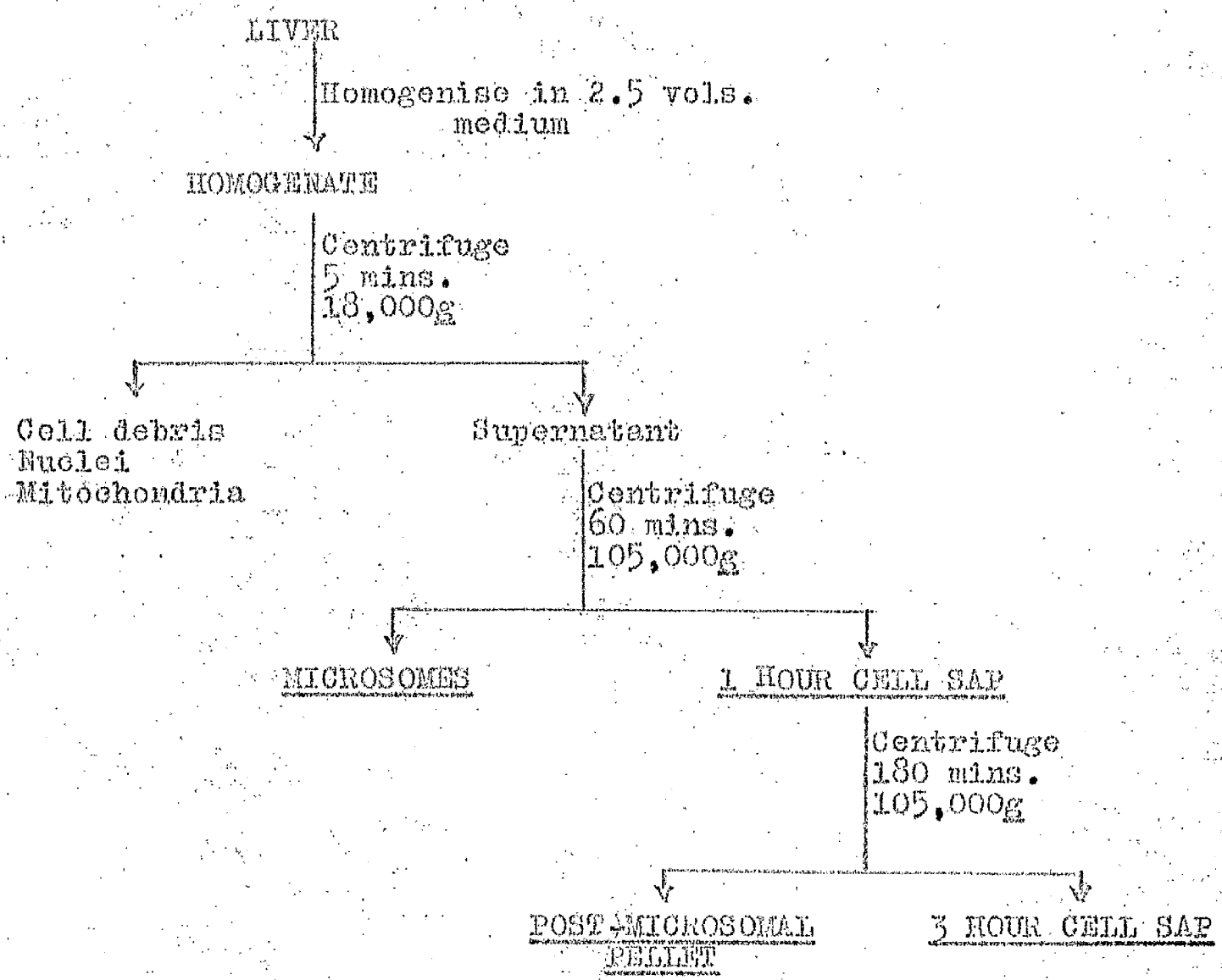
All operations were carried out at 0°C.

The animals were killed by a blow on the head, the livers rapidly excised and plunged into chilled distilled water. They were blotted dry, weighed and homogenised in 2.5 vols. of the appropriate buffer - Campbell buffer, Sucrose/phosphate, pH 7.8 or Sucrose/phosphate/EDTA, pH 3.4 - using a Potter-Elvejhem (1936) homogenizer. After homogenisation, the large cell debris, nuclei, and mitochondria were removed by centrifugation at 18,000g for 5 minutes in the Rotor 30 of the Spinco ultracentrifuge, Model L. The supernatant was decanted and recentrifuged at 105,000g for 1 hour in the Rotor 40 of the Spinco. The precipitate from this centrifugation will hereafter be called "Microsomes" and the supernatant "1 Hour Cell Sap". To obtain post-microsomal pellet this 1 hour cell sap was removed carefully from above the microsome pellet using a Pasteur pipette. To ensure that no contamination with the microsome fraction was obtained, only two thirds of the supernatant was removed. "Post-microsomal Pellet" was sedimented by centrifugation of this supernatant for 3 hours at 105,000g. The remaining supernatant will be termed "3 Hour Cell Sap" Fig. 6 gives a flow sheet for this preparative sequence.

To minimise the contamination of sedimented fractions by supernatant fractions, after centrifugation, the super-

Fig. 6.

The Preparation of Sub-cellular Fractions by Differential Centrifugation.



natant fraction was removed and the surface of the pellet and the sides of the tube washed several times with the appropriate medium. The sides of the tube were dried out with filter paper and the pellet suspended in medium by gentle homogenisation or careful manual stirring. The latter method was always used with post-microsomal pellet as a homogeneous suspension was not obtained by homogenisation.

Acid Precipitation of Fractions.

The pH of the resuspended sedimented fractions and of the soluble fractions was adjusted to 5.1 to 5.2 by the dropwise addition of N acetic acid at 0°C. The pH was determined by the use of a glass electrode. The precipitate obtained was separated centrifugally at 2,000 r.p.m. for 10 minutes in an M.S.E. refrigerated centrifuge. The supernatant was decanted, the sides of the tube dried out with filter paper and the pellet suspended in the appropriate medium.

Estimation of the Chemical Constituents of Sub-cellular Fractions.

1. Ribonucleic acid.

The procedure used was that of Fleck and Munro (1962) in a slightly modified form.

The RNA and protein of the sample were precipitated by the addition of 0.5 vols. of 0.6N PCA. After standing for

10 minutes the precipitate was sedimented by centrifugation and the supernatant discarded. The precipitate was resuspended in 0.3N PCA and resedimented. This washing was repeated, the supernatant being discarded in each case. Three ml. of 0.3N KOH were added to the sample and the RNA hydrolysed by incubation at 37°C for 1 hour. After incubation the samples were chilled and the protein reprecipitated by the addition of sufficient PCA to neutralise the KOH present and bring the final PCA concentration to 0.3N PCA. The protein precipitate was allowed to form for 10 minutes and was then separated centrifugally. The supernatant, containing the hydrolysed RNA, was retained and the precipitated protein washed twice with 0.3N PCA to complete the extraction of the RNA. The combined supernatant and washings were made up to a known volume and the absorption at 260mu was measured. The RNA content was calculated using the equation:-

$$\text{O.D. of } 1.0 \equiv 32.87 \text{ ug. RNA/ml.}$$

2. Protein.

The method of Lowry et al. (1951) was used.

Reagents.

A. 2% Na_2CO_3 in 0.1N NaOH.

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

(This reagent precipitates on storage. To circumvent this difficulty equal volumes of 1% $CuSO_4$ and 2% Na or K tartrate were mixed just before use.)

C. Alkaline Copper Solution.

(Mix 50 ml. reagent A with 1 ml reagent B.)

D. Diluted Folin-Ciocalteu Reagent.

(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 give a N solution.)

Procedure.

The specimen was adjusted to a protein concentration of between 40 and 400ug per ml. One ml. of such a solution was added to 5 ml of reagent C and, after 10 minutes, 0.5 ml. of reagent D was added. Instantaneous mixing was essential and a Vortex mixer was used to achieve this. The blue colour was found to be completely developed after 20 minutes standing and remained constant for at least a further 30 mins.. The optical density was read at 750mu and the protein content determined from a calibration curve prepared in this laboratory using bovine serum albumin as standard.

3. Phosphorus.

The method used was that developed by Allen (1940) but modified to some extent.

The total phosphorus is converted by digestion with PCA to inorganic phosphate from which a phosphomolybdate complex is formed by reaction with ammonium molybdate. Reduction with amidol (2,4 diaminophenol hydrochloride) gives a blue pigment. From the intensity of this colour the phosphorus

content of the sample could be calculated.

Reagents

73% PCA

10N H_2SO_4

8.3% Ammonium molybdate

1% Amidol in 20% Na metabisulphite

(1g amidol was dissolved in 100ml. 20% metabisulphite; the reagent was then filtered into a brown bottle and kept at 0°C. Fresh reagent was made up every 2 or 3 days.)

Procedure.

As the estimations fell in the range 5 to 50 ug of phosphorus, the one fifth Allen method was used (Allen 1940).

A suitable sample of the solution to be assayed was evaporated to dryness in a sand bath at about 200°C. 0.4 ml. 73% PCA was added and the sample digested until the solution was clear (about 2 hours). After cooling, the sides of the tube were washed down with 4 ml. distilled water. The blue colour was developed by the addition of 0.4 ml amidol reagent followed by 0.2 ml. ammonium molybdate. The colour was allowed to develop for 10 mins. and the O.D. was read at the absorption maximum, 725mu, in a Unicam SP 600 against a reagent blank. A standard phosphorus solution was estimated at the same time.

For the estimation of inorganic phosphate no digestion

was necessary. In this case, to a suitable aliquot of the sample, 0.24 ml. 10N H_2SO_4 and distilled water to a total volume of 4.4 ml. were added. 0.4 ml. amidol reagent and 0.2 ml. ammonium molybdate were then added and the blue colour allowed to develop as before.

4. Phospholipid Phosphorus.

The method used was that of Folch et al. (1957).

Reagents.

A. 2 : 1 (w/v) chloroform : methanol

B. 8 : 4 : 3 chloroform : methanol : 0.58% NaCl

(This mixture was shaken together, allowed to stand and the bottom layer discarded. "Pure solvents - Upper phase")

Proceedure

A suitable volume of a solution of the cell fraction was shaken with 20 vols. of reagent A for 15 minutes. The mixture was then centrifuged at 1500 r.p.m. for 15 mins. and the chloroform : methanol quantitatively transferred into a 10 ml. stoppered graduated centrifuge tube. This crude extract was then mixed thoroughly with 0.2 vols. of 0.73% NaCl. The aqueous layer was separated by centrifugation as before and discarded. To completely remove the aqueous layer the interface was rinsed three times with reagent B leaving the lower layer undisturbed. The lower layer and remnants of the rinsing fluid were made into one phase and made up to 10 ml. by the addition of methanol.

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

Preparation of Sub-cellular Fractions for Determination of S Values and for Electrophoresis on Cellulose Acetate Strips.

Post-microsomal pellet and 1 hour and 3 hour cell sap were separated as given in Fig. 6 using sucrose/phosphate, pH 7.8 as the homogenisation medium.

After sedimentation, post-microsomal pellet was suspended in 0.06M barbitol buffer and used in this form; also, some was precipitated at pH 5 from this buffer and resuspended in it before use. Cell sap preparations both in the "native" state and precipitated at pH 5 were also used. The cell sap preparations which were not precipitated at pH 5 were passed through a Sephadex 25 column (about 1 ml. of cell sap through a column about 1 cm by 15 cms.) to remove the sucrose present which would interfere with S value determinations and electrophoresis. The material was eluted from the column in 0.06M barbitol buffer. The samples of cell sap precipitated at pH 5 were precipitated from sucrose/phosphate, pH 7.8 as previously described using 0.06M barbitol buffer to resuspend the precipitate.

Each fraction was adjusted to approximately equal protein content before use for sedimentation coefficient determinations or electrophoresis.

Determination of S Values.

Each fraction prepared as above, was centrifuged at 44,770 r.p.m. in the Spinco, Model E, analytical ultracentrifuge at 22°C. The Schlieren optical system was used, and photographs were taken at 4 min. or 8 min. intervals. The S values were calculated using the equation:-

$$w^2 s = \frac{d \log_e r}{dt}$$

where

w = Angular velocity (radians/sec.)

r = Distance of the boundary from the centre of rotation (cms.)

t = Time (secs.)

s = Sedimentation coefficient.

The results were expressed in Svedberg units (S). A sedimentation coefficient of 10^{-13} sec. is termed one Svedberg unit.

Electrophoresis on Cellulose Acetate.

The method described by Kohn (1960) was used.

20 ul. of a solution of the fraction to be investigated, prepared as previously described, was applied to a cellulose acetate strip 12 cm. by 2.5 cm.. The strip was immersed in 0.06M barbitol buffer, pH 8.6 and electrophoresis carried out by applying a voltage of 200 for 1¼ hours. The strips were then carefully removed and stained using Ponceau S. The excess stain was washed out by successive immersions in 5% acetic acid

and the strips were then dried at room temperature. To ensure that the strips remained flat, the final period of drying was carried out with the strips between filter paper underneath a heavy weight.

Preparation of RNA from Sub-cellular Fractions by Phenol Extraction.

The methods used by Kirby (1956) and Hoagland et al. (1958) were used in a modified form.

The subcellular fraction, prepared by differential centrifugation, was precipitated at pH 5 and the precipitate obtained suspended in 0.2M potassium phosphate buffer, pH 6.85. An equal volume of 90% (w/v) phenol was slowly added and the mixture shaken for 1 hour at 0°C. The aqueous layer was isolated by centrifugation at 2,000 r.p.m. for 1 hour. The phenol layer was washed twice with 0.2M potassium phosphate buffer and the aqueous layer and these washings combined. Excess phenol was removed by three extractions with ether and the resulting solution was made 2% with respect to acetate by the addition of the appropriate volume of a 20% potassium acetate solution at pH 5. The RNA was precipitated from this solution by the addition of 2.5 vols. of absolute ethanol and the precipitate was allowed to stand overnight at -10°C. The precipitate was separated by centrifugation and redissolved in 0.2M phosphate buffer. Precipitation was carried out by the addition of ethanol as before and the reisolated RNA was dissolved in distilled water adjusted to pH 8. This

RNA solution was dialysed for 48 hours with constant stirring against frequent changes of distilled water at pH 8.

Protein estimations on the resulting RNA solutions showed the presence of a maximum of 2.5% protein.

Fig. 7 shows the sedimentation pattern obtained for an RNA preparation from 3 hour cell sap. It can be seen that under these conditions, only one peak is evident. The S value calculated for this peak is 3.8 which agrees well with published values for sRNA. (See page 23.)

Determination of the Base Composition of RNA from Various Sub-cellular Fractions.

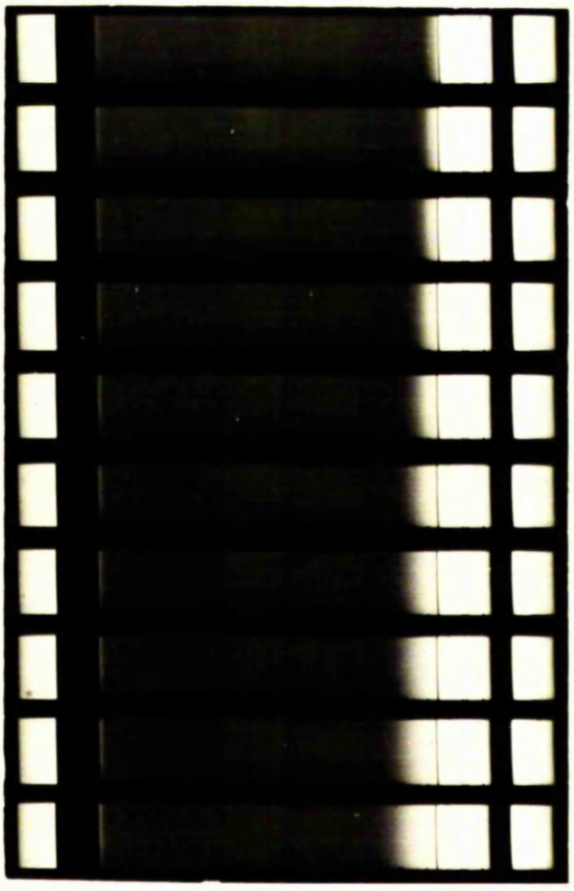
Specimens of RNA prepared by phenol extraction were hydrolysed in 0.3N KOH for 18 hours at 37°C. and the nucleotides separated by the paper chromatographic procedure of Lipshitz and Chargaff (1960). The amount of each nucleotide present was measured by the ultraviolet absorption of the material eluted from the paper. Goswami, Barr and Munro (1962) have slightly modified this method and are able to measure quantitatively the amount of nucleotide present, even when the paper is contaminated with UV absorbing material. A two wavelength procedure is involved.

By these methods, the four major nucleotides can be separated quantitatively, and the minor components can also be estimated.

Fig. 7.

The Ultracentrifugation Pattern of RNA prepared by Phenol Extraction from 3 Hour Cell Sap pH 5 Enzyme.

Phenol RNA was prepared by the normal procedure from 3 hour cell sap pH 5 enzyme. A sample of this solution (containing about 200ug. RNA per ml.) was examined in the Analytical Ultracentrifuge, using UV optics.



Results.

Electron Microscopy of Post-microsomal Pellet.

In Fig. 8 is given an electron microscope picture of post-microsomal pellet. Fig. 9 gives the equivalent picture for a microsome preparation for comparison. The most striking difference between post-microsomal pellet and microsomes is the lack of membrane structure visible in the post-microsomal pellet electron micrograph. It appears as a fairly uniform granular material but further pictures at a higher magnification would be required to determine the fine details of its structure. It has not been possible to obtain a further, more detailed examination of the preparation.

The Chemical Composition of Post-microsomal Pellet.

In Tables 4 and 5 is presented the detailed composition of post-microsomal pellet compared with cell sap and microsomes. The data are presented for the fractions prepared in three different media, as post-microsomal pellet prepared in each of these media is used in ensuing parts of this thesis. An explanation for the use of these rather unusual media will be given in the appropriate section of the thesis.

The major points to be noted about the differences between post-microsomal pellet and the other fractions, irrespective of the medium used for preparation, are :-

1. Post-microsomal pellet is a very small fraction representing only about 6 mg. of solids (RNA + protein + phospholipids) per

Fig. 8.

Electron Micrograph of Post-microsomal Pellet.

Immediately after preparation in Campbell buffer, a small portion of the sedimented post-microsomal pellet was removed and placed in osmic acid buffered at pH 7.4. After dehydration, the material was embedded in n-butyl methacrylate and sections cut.

Magnification: 13,300.

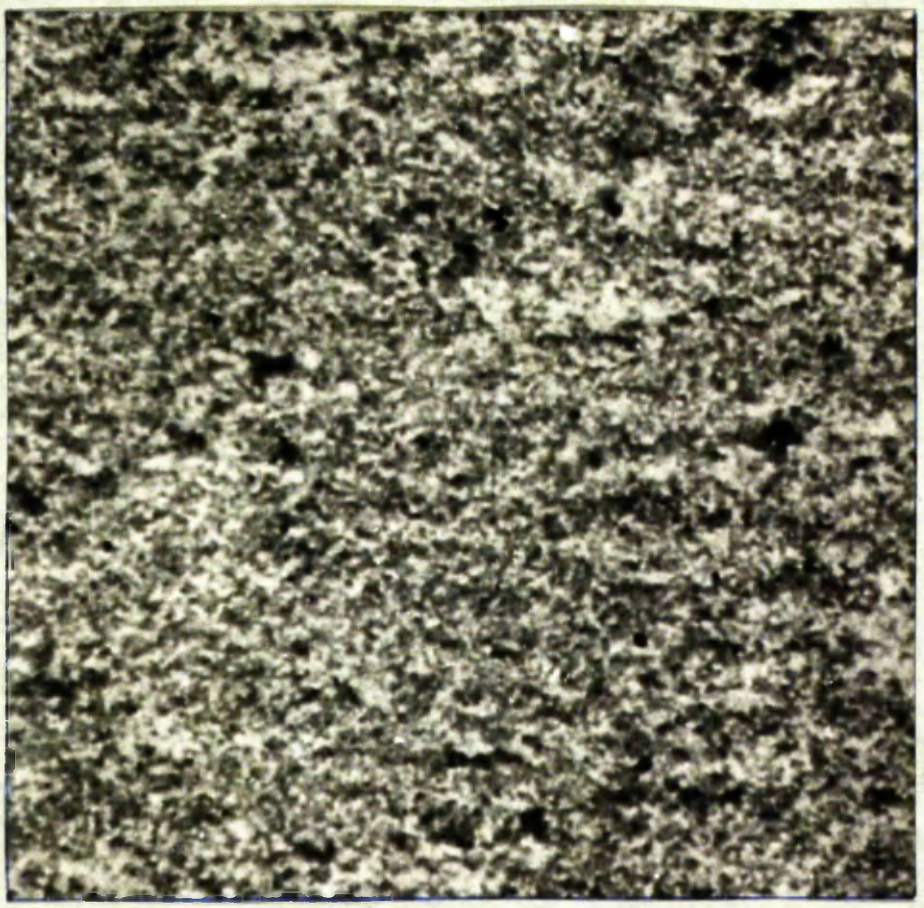


Fig. 9.

Electron Micrograph of the Microsome Fraction.

The procedure used was identical to that given for Fig. 8.

Magnification: 13,300

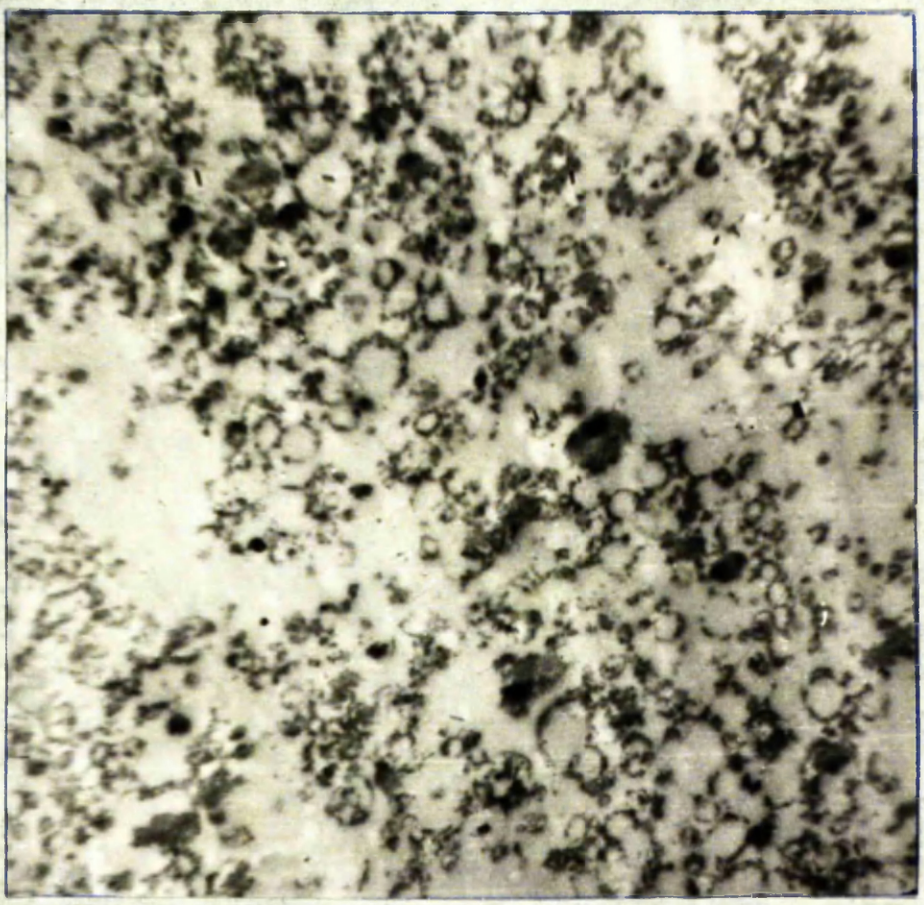


Table 4.

The Chemical Composition of Various Fractions prepared in Different Media.

Each cell fraction was prepared as shown in Fig. 6, in the medium shown in the Table below. C represents Campbell Buffer; S/P/E, sucrose/phosphate/EDTA, pH 3.4; and S/P, sucrose/phosphate, pH 7.8. RNA was estimated by the method of Fleck and Munro (1962); protein by the method of Lowry (1951) and phosopholipid by the method of Folch et al. (1957).

Fraction	pH 5 ppt.	mg. RNA/5 g. liver			mg. protein/5 g Liver			mg. phospholipid per 5g liver		
		Preparation medium			Preparation medium			Preparation medium		
		C	S/P/E	S/P	C	S/P/E	S/P	C	S/P/E	S/P
Post-microsomal Pellet	+	0.17	0.15	0.51	0.63	1.14	3.45	0.07	0.07	0.18
	-	0.21	0.20	1.00	2.88	5.58	10.7	-	-	-
3 Hour Cell Sap	+	1.06	-	1.13	23.7	-	51	-	-	0.35
	-	1.32	1.43	3.52	154	151	201	1.05	0.08	-
Microsomes	-	9.0	7.7	9.2	52.1	42	80	14	19	40

Table 5.

The RNA/Protein and Phospholipid/Protein Ratios of Various Cell Fraction.

Fraction	pH 5	RNA/Protein			Phospholipid/Protein		
		C	S/P/E	S/P	C	S/P/E	S/P
Post-Microsomal Pellet	+	0.26	0.10	0.19	0.12	0.06	0.05
	-	0.07	0.04	0.09	-	-	-
3 Hour Cell Sap.	+	0.04	-	0.03	-	-	0.02
	-	0.01	0.01	0.02	0.02	0.01	-
Microsomes	-	0.17	0.17	0.11	0.28	0.46	0.44
* Ribosomes	-	0.25 to 0.4			0.03		

* From Chauveau (1962).

5 g. of liver, compared with about 160 mg. for 3 hour cell sap and 70 mg. for microsomes (Table 4.).

2. Post-microsomal pellet, after pH 5 precipitation, has a much higher RNA/protein ratio than cell sap and a similar one to that of microsomes (Table 5). The figures of Chauveau (1962) for ribosomes occurring free in the cell are given for comparison. The RNA/protein ratio for this ribosome fraction is much higher than that of post-microsomal pellet.

3. The ratio of phospholipid/protein in post-microsomal pellet is very small compared with that of microsomes but similar to that of ribosomes as determined by Chauveau (Table 5). In comparison, cell sap has negligible amounts of phospholipid.

4. On pH 5 precipitation of post-microsomal pellet and 3 hour cell sap there is a 50% recovery of RNA and a 20% recovery of protein.(Table 5.)

From these observations we can conclude that post-microsomal pellet is intermediate between microsomes and ribosomes in compositionand quite different from cell sap. It has an RNA/protein ratio similar to that of microsomes but resembles ribosomes in having a very low amount of phospholipid. This latter finding presumably indicates the presence of very little membranous material in post-microsomal pellet, in agreement with the picture obtained by electron microscopy.

Considering now the changes in the composition of each fraction due to preparation from homogenates made in various

buffers, there are, once more, several well marked effects:-

1. As compared with Campbell buffer, preparation in sucrose/phosphate, pH 7.8 seems to increase the yield of RNA, protein and phospholipid in all the cell fractions studied.

2. Preparation in sucrose/phosphate/EDTA, pH 3.4 appears to increase the yield of post-microsomal protein compared with that in Campbell buffer, but has little effect on the yield of RNA. Consequently, post-microsomal pellet prepared in sucrose/phosphate/EDTA, pH 3.4 has a lower RNA/protein ratio. Preparation in sucrose/phosphate, pH 7.8 gives a post-microsomal pellet with an RNA/protein ratio between these two preparations.

3. Microsomes prepared in Campbell buffer have a lower phospholipid/protein ratio than those prepared in sucrose/phosphate, pH 7.8 or sucrose/phosphate/EDTA, pH 3.4 (Table 5). It may be significant that post-microsomal pellet prepared in Campbell buffer has a higher ratio of phospholipid/protein than that prepared in either of the other buffers. However, it must be noted that this increase in the phospholipid/protein ratio is due rather to a decrease in the protein content of post-microsomal pellet than an increase in the phospholipid content, as seen from the absolute yields (Table 4).

4. Apart from point (1) above, cell sap shows very little variation in composition due to preparation in the various buffers.

We can conclude that the homogenisation medium has an

effect on the composition of some of the sub-cellular fractions studied, including post-microsomal pellet. However, the magnitude of these effects is small and, regardless of the preparation medium, post-microsomal pellet can be considered as a small fraction with an RNA/protein ratio of 0.2 and a phospholipid/protein ratio of about 0.07. The homogenisation medium has more effect on the yield of post-microsomal pellet than on its chemical composition. About 4 mg. solids i.e. RNA + protein + phospholipid is obtained from 5 g. liver when post-microsomal pellet is prepared in sucrose/phosphate, pH 7.8 and precipitated at pH 5, about 1.5 mg. when preparation is carried out in sucrose/phosphate/EDTA, pH 3.4 and about 1 mg. when Campbell buffer is used.

The main difference in the homogenisation media used is that Campbell buffer contains various inorganic ions, notably Mg^{++} whereas neither sucrose/phosphate, pH 7.8 nor sucrose/phosphate/EDTA, pH 3.4 contain Mg^{++}. Indeed, the latter contains a strong Mg^{++} binding agent. It might be expected that in these two latter media the absence of Mg^{++} would lead to a breakdown of the ribosomes giving an accumulation of breakdown products in the post-microsomal pellet. The increase in yield of post-microsomal pellet prepared in the buffers which do not contain Mg^{++} may be taken as support for this hypothesis, but, on the other hand, a complementary increase in the RNA/protein ratio would also be expected, and is not apparent - indeed, the RNA/protein

is decreased rather than increased. Consequently, we can conclude that the extra material obtained when post-microsomal pellet is prepared in a Mg^{++} free buffer does not seem to arise from ribosomal disintegration.

The Heterogeneity of Post-microsomal Pellet.

1. A Determination of the S Values of the Particles Present.

Although the chemical composition of post-microsomal pellet is constant when prepared under standard conditions a homogeneous particle must not be envisaged. Samples of post-microsomal pellet prepared in sucrose/phosphate, pH 7.8 show the presence of 4 peaks when examined in the analytical Spinco. Figs. 10, 11, 12, 13,14 and 15 give the sedimentation patterns as seen using the Schlieren optical system with the ultracentrifuge. (The patterns obtained for 1 hour and 3 hour cell sap are also given for comparison.) In Table 6 the sedimentation coefficients calculated for each peak are summarised.

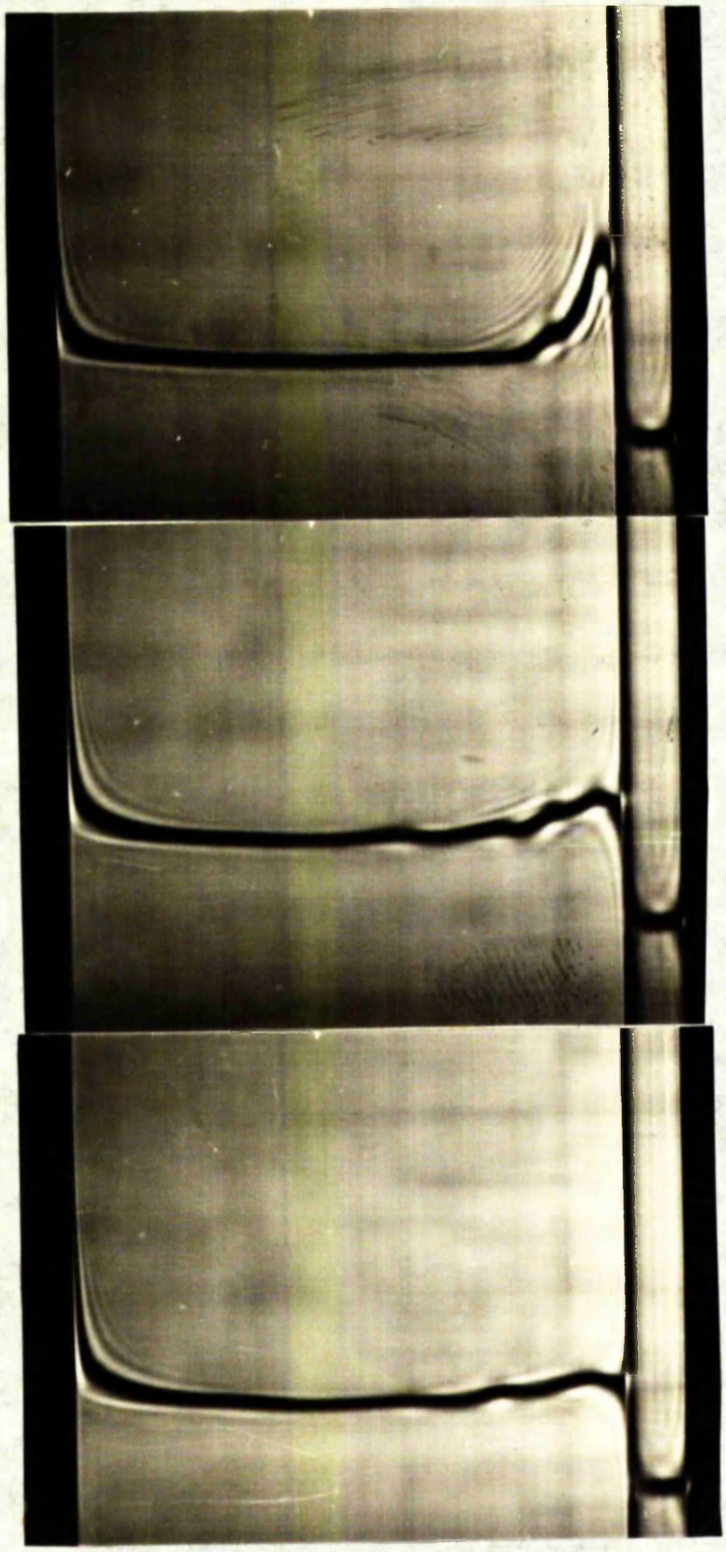
All three fractions, whether precipitated at pH 5 or not, contain a major peak varying between 5 and 7 S (Table 6). Many workers have suggested that the sRNA molecule is of about these dimensions, and it is noteworthy that sRNA as prepared by us from 3 hour cell sap by the phenol extraction process, was found to consist of a 4 S particle (Fig. 7). This peak may indicate therefore, the presence of sRNA, although why it should be sedimentable by centrifugation at 105,000g for 3 hours is difficult to explain. In cell sap

Fig. 10.

Post-microsomal pellet was prepared in sucrose/phosphate, pH 7.8 and suspended in 0.06M barbitol buffer. It was precipitated at pH 5 by the normal procedure and re-suspended in this buffer. A sample of this solution was centrifuged in the Analytical Ultracentrifuge at 22^{o}C at 44,770 r.p.m.. The Schlieren optical system was used and photographs were taken at 4 minute intervals.

Fig. 10.

The Ultracentrifugation Pattern of Post-microsomal Pellet Precipitated at pH 5 before Examination.



4 minutes

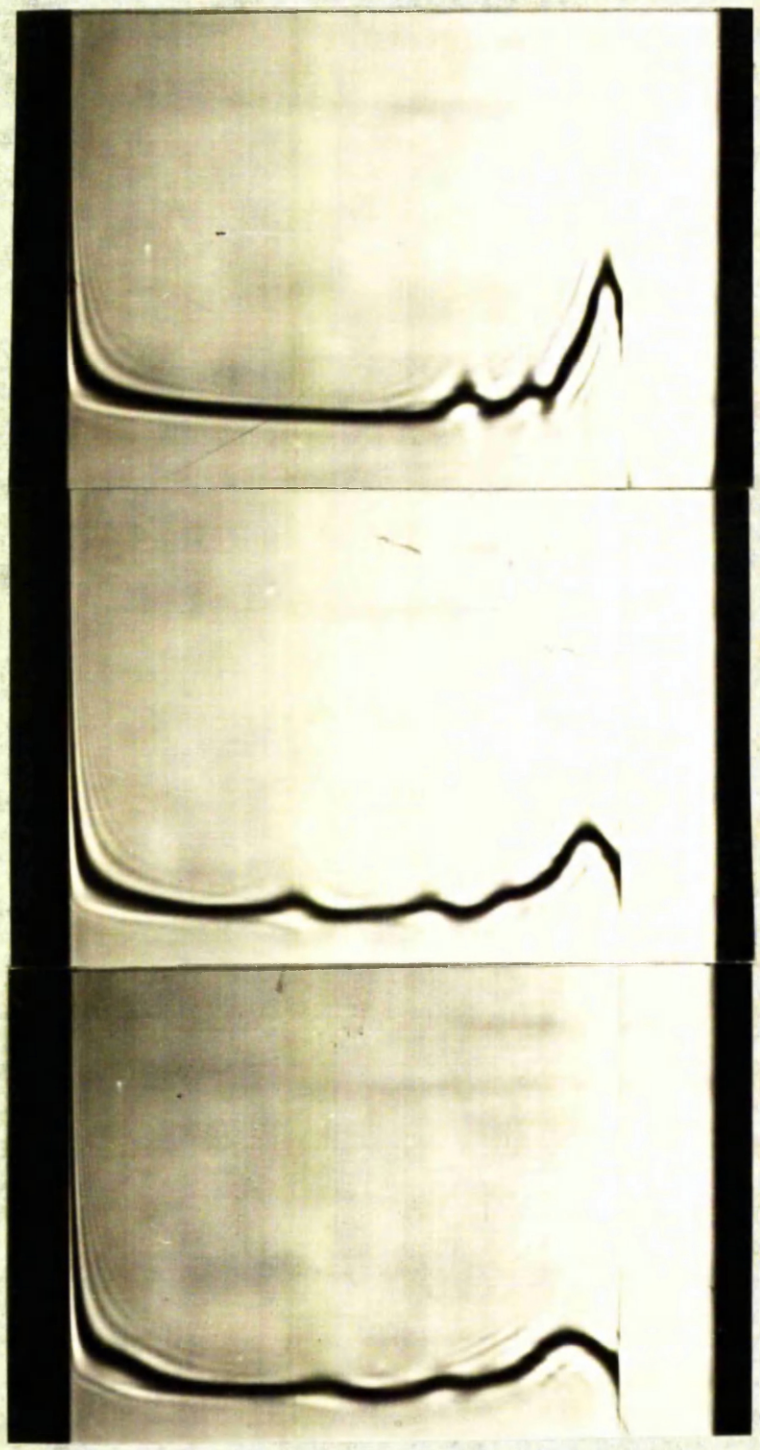
8 minutes

12 minutes.

Fig. 11.

The Ultracentrifugation Pattern of Post-microsomal Pellet not Precipitated at pH 5 before Examination.

The conditions of the analysis were exactly as described for Fig. 10 except that the post-microsomal pellet was not precipitated at pH 5 before analysis.



4 minutes

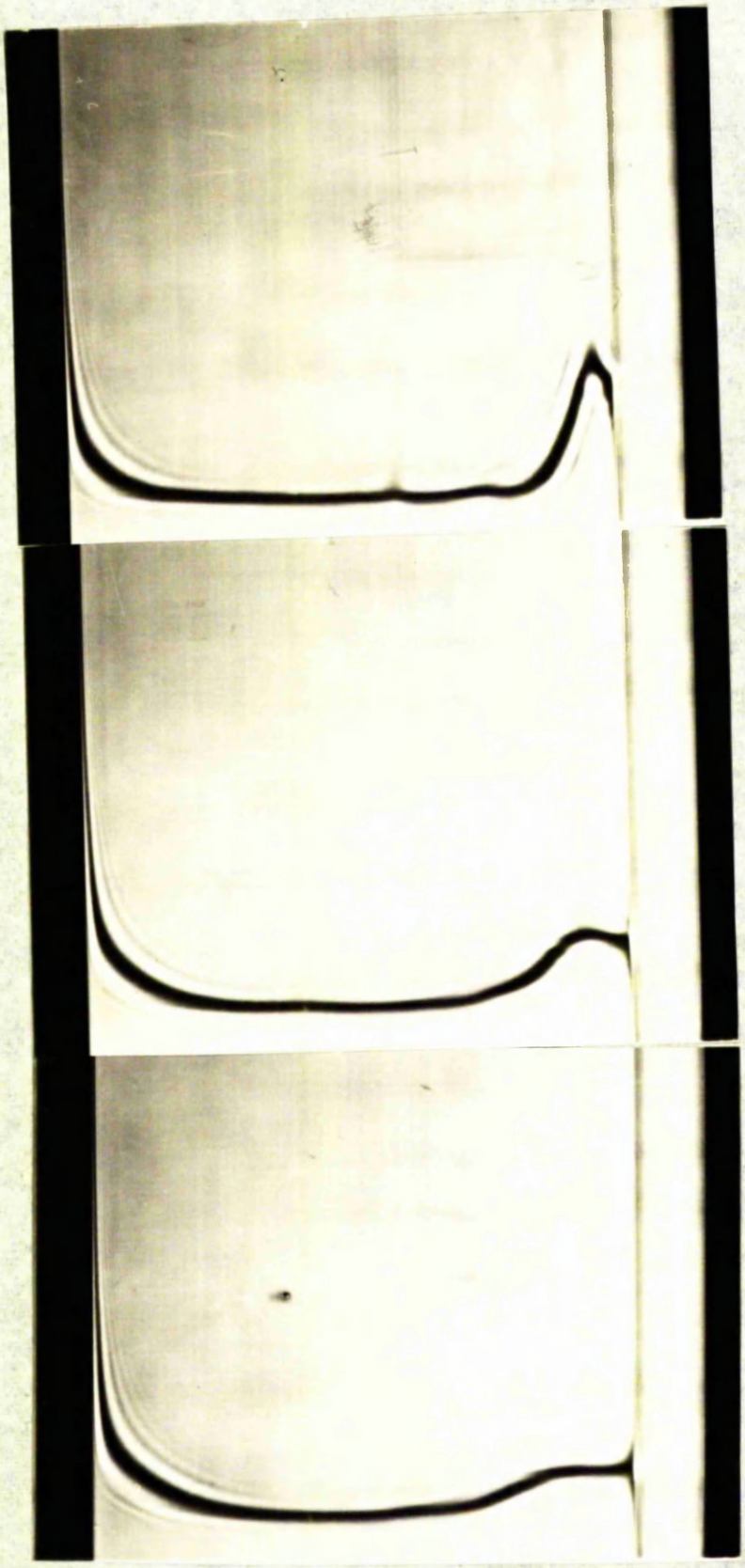
8 minutes

12 minutes

Fig. 12.

Ultracentrifugation Pattern of pH 5 Enzyme from 1 Hour Cell Sap.

Conditions were as described for Fig. 10.



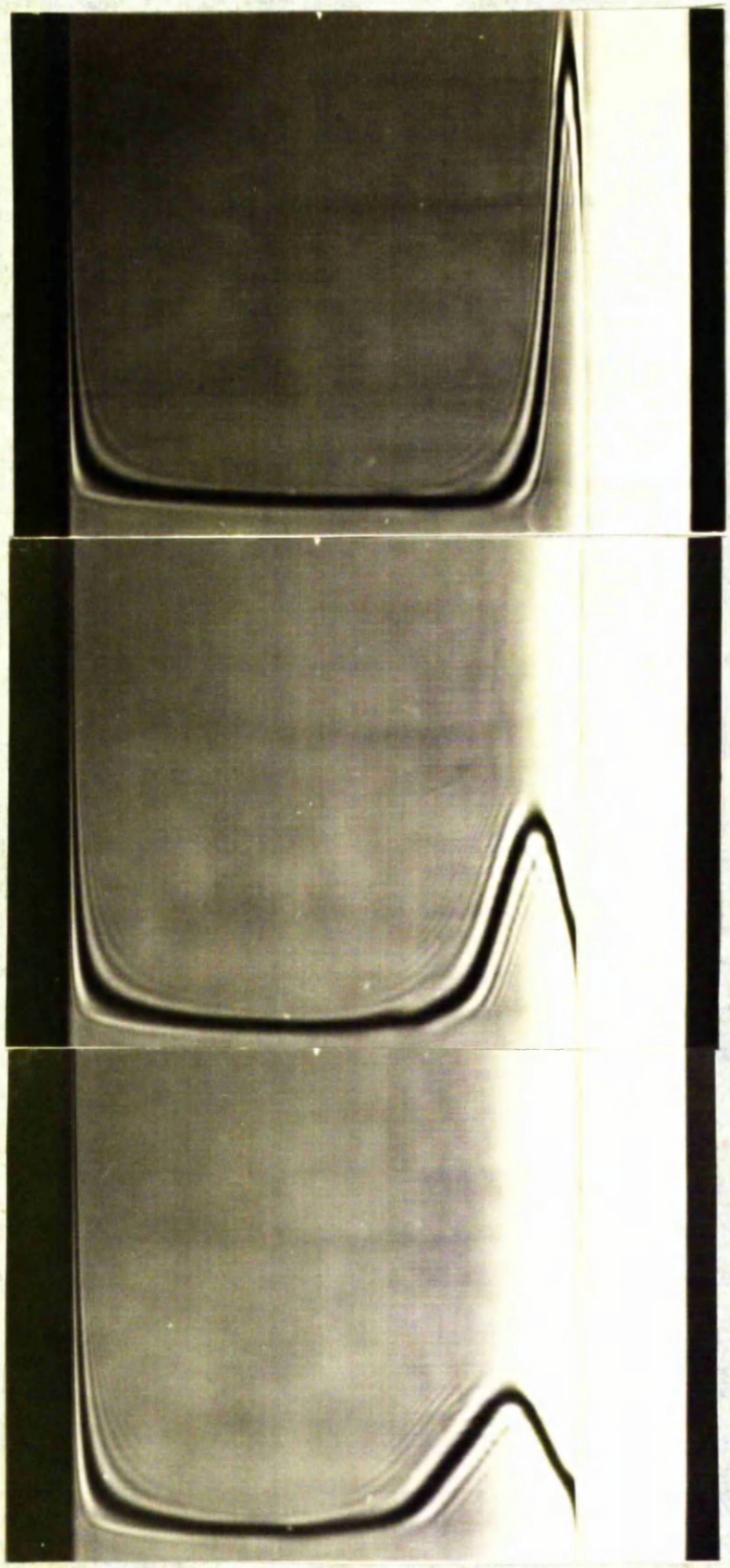
8 minutes

12 minutes

16 minutes

Fig. 13.

Ultracentrifugation Pattern of 1 Hour cell Sap not Precipitated at pH 5.



12 minutes

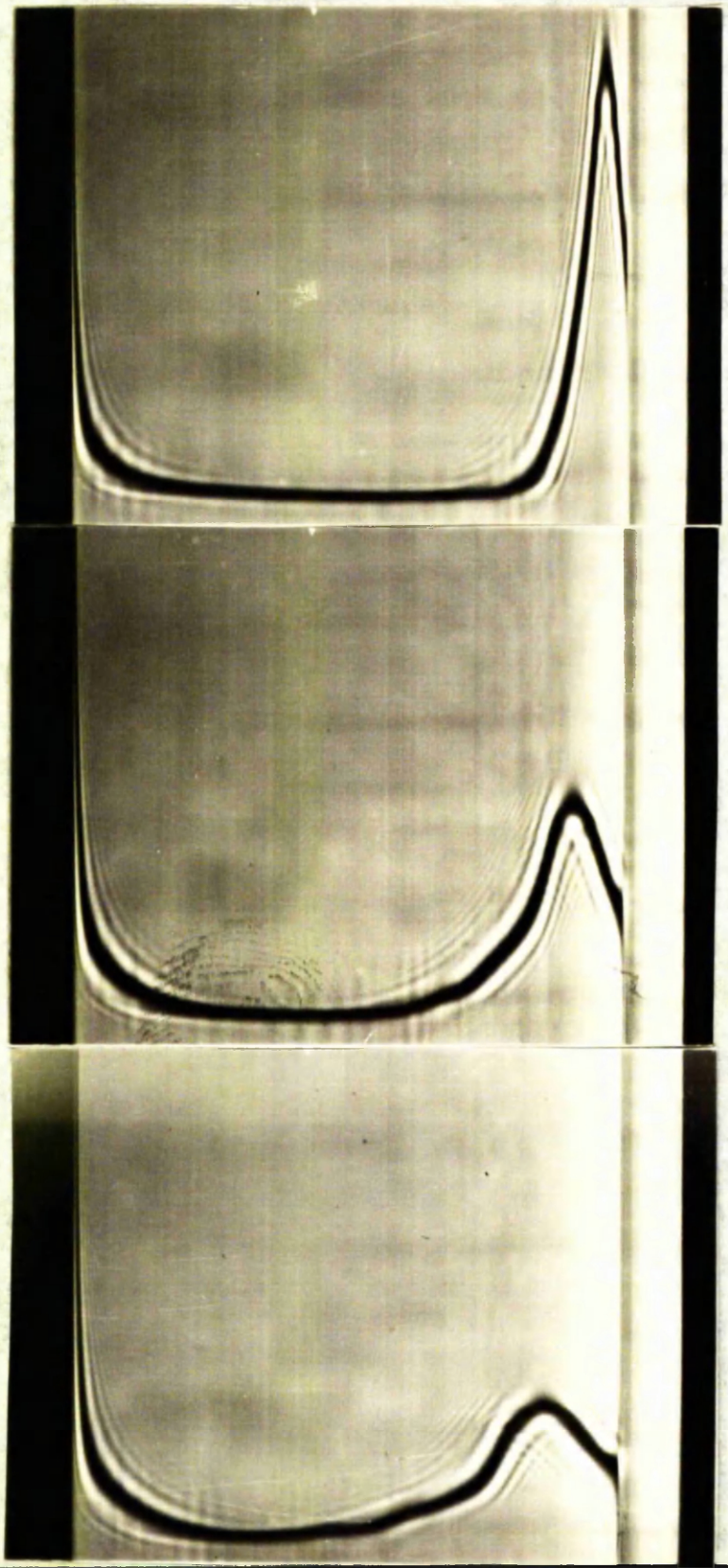
16 minutes

20 minutes

Fig. 14.

Ultracentrifugation Pattern of pH 5 Enzyme from 3 Hour Cell Sap.

Conditions were as described in Fig. 10.



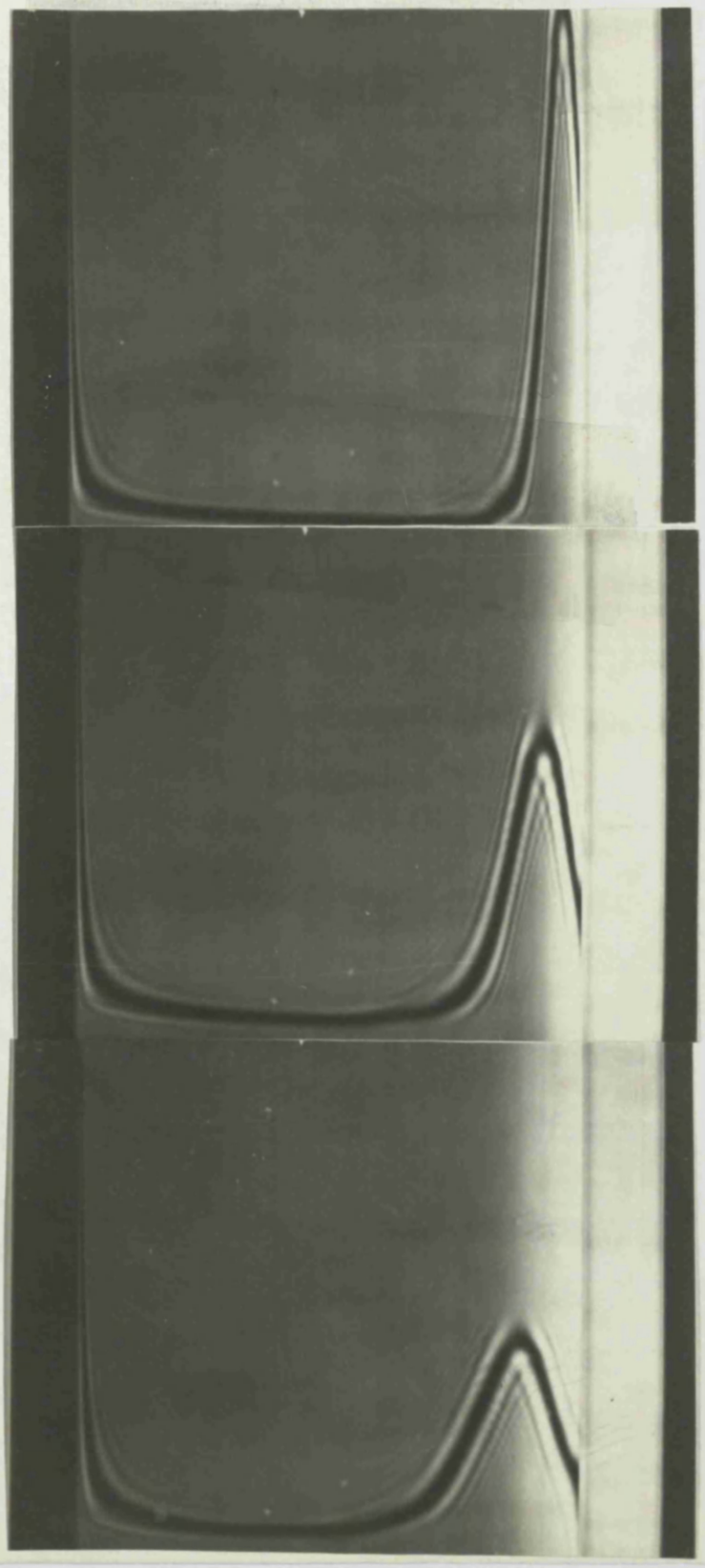
8 minutes

12 minutes

16 minutes

Fig. 15.

Ultracentrifugation Pattern of 3 Hour Cell Sap not Precipitated at pH 5.



8 minutes

12 minutes

16 minutes

Table 6.

S Values of the Particles Present in Post-microsomal Pellet and in 1 Hour and 3 Hour Cell Sap.

Each fraction was prepared in sucrose/phosphate, pH 7.8 and resuspended in 0.06M barbitol buffer for the determination of the sedimentation coefficients of the particles present. All of the samples were centrifuged at 44,770 r.p.m. in the Spinco, Model E ultracentrifuge and photographs taken at 4 min. or 8 min. intervals using the Schlieren optical system. Each fraction had approximately equal protein concentrations.

pH 5 Precipitation	Post-Microsomal Pellet S	1 Hour Cell Sap S	3 Hour Cell Sap S
+	5	5	5
	21		13
	32		18
	57	34	
		47	
-	7	5	5
	21		(19)
	33		
	53		

preparations not precipitated at pH 5, this is the only peak present although a very small peak of a 19 S particle can be detected in 3 hour cell sap. Post-microsomal pellet, on the other hand, contains three small peaks as well as a major 7 S peak. The S values of these three small peaks have been calculated and are 21, 33 and 53. (At this point, it may be relevant to mention that ribosomes initially present as 70 S particles break down to a 30 S and a 50 S component on removal of Mg^{++} ions from the suspending medium (See page 8). Possibly, the 33 S and 53 S particles present in post-microsomal pellet represent ribosomal breakdown products.)

The ultracentrifugation pattern of post-microsomal pellet is unchanged by precipitation at pH 5. On the other hand, two peaks, with S values of 34 and 47, appear in 1 hour cell sap on precipitation. In 3 hour cell sap, a 13 S peak appears and the 18 S peak present to a small extent in the non-precipitated sample becomes much more pronounced. The 34 S and 47 S particles which appear in 1 hour cell sap are probably equivalent to the 32 S and 57 S particles present in post-microsomal pellet. The fact that these two larger particles are not present in 3 hour cell sap pH 5 fraction indicates that these particles are removed by centrifugation at 105,000g for 3 hours.

An explanation of the finding that these peaks are not evident in total 1 hour cell sap but only appear on precipitation at pH 5, might be a concentration effect. Thus

when the pH of 1 hour cell sap is reduced to 5, only 25% of the total substance is precipitated. Presumably, this 25% is a specific fraction of the cell sap material and not just a random precipitation of molecules. Consequently, the 34 S and 47 S components may be concentrated in the 25% of the cell sap substance which is precipitated at pH 5. Alternatively, an aggregation effect may explain the appearance of these peaks. Thus, after precipitation at pH 5 and resuspension, some of the components of cell sap might aggregate, giving rise to particles which are not present in the total cell sap preparation. However, the fact that the particles observed in the 1 hour cell sap preparation have sedimentation coefficients similar to those of post-microsomal pellet, yet the 3 hour cell sap does not produce these particles, indicates that these peaks are not formed by a purely random condensation of molecules from cell sap. Consequently, these particles appear to be formed from some specific components of the substance of 1 hour cell sap, which is absent from 3 hour cell sap. If post-microsomal pellet does arise from aggregation of components in the cell sap, these components must be wholly removed by centrifugation at 105,000g for 3 hours.

2. Electrophoresis on Cellulose Acetate.

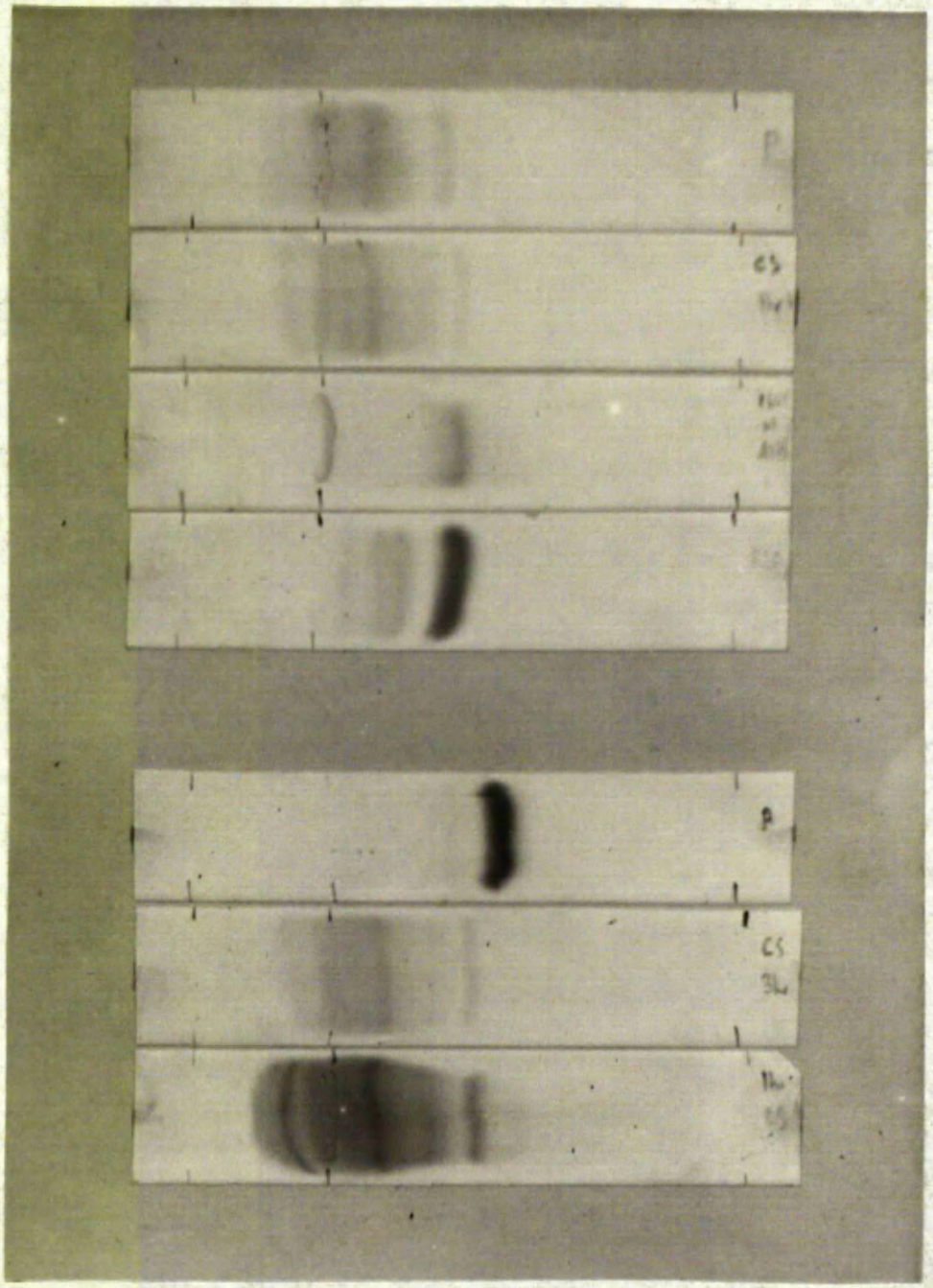
Electrophoresis on cellulose acetate also reveals the presence of several components in post-microsomal pellet.

In Fig. 16, strips obtained in two separate experiments are shown. In the upper part of the diagram, the patterns obtained for post-microsomal pellet and 1 hour cell sap are shown. A sample of rat serum albumin and one of albumin prepared from rat liver microsomes are also included as controls. Post-microsomal pellet and 1 hour cell sap exhibit very similar bands. However, the patterns are not identical. The main difference is thatpost-microsomal pellet contains very much less basic protein than cell sap (i.e. the band moving to the left of the strip from the origin). One hour cell sap has a band in an identical position to that of rat serum albumin; it is doubtful from these strips whether such a band is present in post-microsomal pellet. There appears to be two bands in this fraction, one very faint one which has moved further than rat serum albumin and one which appears rather less mobile than the band of rat serum albumin.
In the lower part of Fig. 16, samples of 1 hour and 3 hour cell sap are compared. In this experiment bovine serum albumin was usedas control instead of rat serum albumin; in this solvent system, it is more mobile than rat serum albumin. Twice as much 1 hour cell sap as 3 hour cell sap was applied but the picture is essentially similar. The band of basic protein is very well marked in the 1 hour preparation; no differences can be detected between the 1 hour and 3 hour cell sap preparations. This suggests that the separation of post-microsomal pellet does not remove one specific protein but

Fig. 16.

Electrophoresis of Post-microsomal Pellet and 1 hour and 3 Hour Cell Sap on Cellulose Acetate.

20 ul. of a solution of the fraction noted below (not precipitated at pH 5) in 0.06M barbitol buffer was applied to a cellulose acetate strip and electrophoresis carried out in 0.06M barbitol buffer for 1½ hours at 200 volts. The strips were stained with Ponceau S and dried in air.



Post-microsomal Pellet

1 Hour Cell Sap

Albumin isolated from rat liver microsomes

Rat Serum Albumin

Bovine Serum Albumin

3 Hour Cell Sap

1 Hour Cell Sap

rather some of each band. However, since post-microsomal pellet protein is quantitatively very small compared with the proteins of cell sap, the possibility of detecting the deletion of a protein from cell sap on the removal of post-microsomal pellet is remote.

Studies on the RNA Present in Post-microsomal Pellet and in 1 Hour Cell Sap.

The sequence of nucleotide residues in post-microsomal RNA and in cell sap RNA has been determined and is given in Table 7. In this table, the figures are also given for nuclear RNA and microsomal RNA for comparison. We are indebted to Mr. G.C. Barr and Dr. P. Goswami for carrying out this part of the work. From Table 7, it can be seen that post-microsomal RNA resembles nuclear RNA to a large extent, the presence of a significant amount of pseudouridine being the major point of difference.

Although post-microsomal pellet and cell sap occur together in the soluble fraction of the cell, the RNAs of these two fractions bear very little resemblance, only the adenine and guanine content being similar in concentration. However, the presence of a significant amount of pseudouridine in post-microsomal RNA is an indication of the presence of sRNA, as pseudouridine occurs mainly in sRNA. Post-microsomal RNA has half the pseudouridine content of cell sap RNA, which suggests that 50% of post-microsomal RNA may be sRNA. The fact that the base ratios of the major bases in post-microsomal RNA do

Table 7.

The Nucleotide Composition of RNA prepared from Various Liver cell Fractions.

RNA prepared from the fractions noted below by phenol extraction was hydrolysed with KOH and the bases separated by paper chromatography. The amount of each base present was estimated by UV absorption. The data presented are the percentage composition of the RNA.

Fraction	Percentage					
	Adenylic	Guanylic	Cytidylic	Uridylic	Pseudo Uridylic	Others
Nuclear	18.7	31.8	31.3	18.4	0	0
Microsomal						
Membrane	17.0	34.1	32.3	16.8	0.3	0
RNP Particles	20.9	27.0	27.1	23.0	2.2	0
Post-Microsomal Pellet	19.1	31.6	29.7	17.9	1.8	0
1 Hour Cell Sap.	19.9	31.1	26.9	15.6	3.5	3.0

not resemble those of sRNA does not rule out the possibility of the presence of sRNA. The presence of a second RNA with no pseudouridine and a very different base composition could account for the variations in the base ratios of the major nucleotides. This hypothesis, of the presence of sRNA in post-microsomal pellet, is consistent with the finding of a large peak of material with an S value of about 5 in post-microsomal pellet. However, investigations of the biological activity of post-microsomal RNA have produced no evidence for the presence of an RNA with sRNA-like activity in post-microsomal pellet. (See Section II.) Also, studies by McLean (1962) using Ecteola columns failed to reveal the presence of an RNA with an elution pattern similar to sRNA.

An hypothesis consistent with the above, apparently conflicting, evidence is that there is sRNA present in post-microsomal pellet but it is in a degraded form and is, therefore, incapable of accepting activated amino acids. Degradation of the RNA could also explain the inability of McLean (1962) to demonstrate the presence of sRNA in post-microsomal pellet using Ecteola columns. (See also Goldthwait, 1959.)

Summary of Section I.

In summary, we may say that post-microsomal pellet consists of a fairly uniform mass of very small granules when examined by electron microscopy but both ultracentrifugation and

electrophoresis reveal the presence of several components. Of the 4 peaks shown by ultracentrifugation the major one has a sedimentation coefficient of about 6 S, the 3 minor peaks being about 21 S, 32 S and 55 S. None of these peaks is detectable in 1 hour cell sap (the soluble fraction from which post-microsomal pellet is prepared) but precipitation at pH 5 reveals the presence of a 34 S and a 47 S peak in 1 hour cell sap. Precipitation of 3 hour cell sap at pH 5 does not produce these particles; in this case, a 13 S and an 18 S peak appear.

Electrophoresis shows the presence of 4 major proteins in post-microsomal pellet. The electrophoretic pattern is only slightly different from that of either 1 hour or 3 hour cell sap which appear to be identical.

In chemical composition, post-microsomal pellet is quite different from the parent cell sap. It has an RNA/protein ratio similar to that of microsomes and a phospholipid/protein ratio of the same order as that of ribosomes. Preparation in a Mg^{++} free buffer leads to the production of an increased yield of protein and a smaller increase in the yield of RNA, thus ruling out the possibility that the increased yield is due to an accumulation of ribosome breakdown products.

Post-microsomal RNA resembles nuclear RNA in base composition except that it contains demonstrable amounts of pseudouridine. The amount of pseudouridine present suggests that 50% of post-microsomal RNA may be sRNA; this sRNA is in an

inactive form, however, as shown by later tests in Section II.

Conclusions.

In the introduction to this section we asked three questions. The first one was "Does post-microsomal pellet have a constant morphology and chemical composition?" All the results presented in this section indicate that this question may be answered in the affirmative. The chemical composition of post-microsomal pellet is constant when preparation is carried out in a specific medium; indeed, for the three different media used the variations in chemical composition of post-microsomal pellet were very small. Also, although the results of only one typical experiment have been given in the sections dealing with ultracentrifugation and electrophoresis several experiments were conducted, all of which gave similar results.

The answer to the second question, "Is post-microsomal pellet a homogeneous fraction?" must definitely be negative. Both electrophoresis and ultracentrifugation reveal the presence of at least four components.

The third question "Is post-microsomal pellet only a non-specific aggregation of some of the components of cell sap?" is more difficult to answer. Again, however, it is probably to be answered in the negative. The following evidence may be cited in support of this conclusion:

1. The chemical composition of post-microsomal pellet is

constant from experiment to experiment and quite different from that of the parent 1 hour cell sap. It would therefore have to be a very specific aggregate of cell sap material.

2. The ultracentrifugation pattern and the bands obtained on electrophoresis do not change from preparation to preparation.

3. The 32 S and 57 S peaks present in post-microsomal pellet are present in 1 hour cell sap but not in 3 hour cell sap after pH 5 precipitation, presumably indicating the complete removal of some specific material on sedimentation of post-microsomal pellet.

However, contrary to these findings, it may be said that:-

1. No difference can be demonstrated in the electrophoretic patterns of 1 hour and 3 hour cell sap. This, however, is not strong evidence, because of the abundance of cell sap protein compared with post-microsomal protein.

2. Electrophoresis shows the presence of several bands in post-microsomal protein similarly positioned to those of cell sap.

3. The presence of sRNA in post-microsomal pellet is suggested by the presence of pseudouridine in post-microsomal RNA and also by the large peak of material with an S value of about 6 demonstrable by ultracentrifugation. Again, this suggests that some of the components of cell sap are present also in post-microsomal pellet.

SECTION II.

SECTION II.

Introduction.

Having established something of the chemical composition and morphology of post-microsomal pellet in Section I, we will now investigate some of its properties. In Section I indications were obtained that post-microsomal pellet may contain some of the components of cell sap. Consequently, we first investigated the presence, in post-microsomal pellet, of any of the activities normally associated with cell sap enzymes. The work has been subdivided into three parts:-

1. An investigation of the activating enzymes present in post-microsomal pellet.
2. A study of the ability of post-microsomal pellet to transfer amino acids to sRNA.
3. A study of the capacity of post-microsomal pellet to replace cell sap pH 5 enzyme in the classical system of protein biosynthesis.

Experimental Methods.

Materials. The materials used in this section are:- ATP obtained as the sodium salt from B.D.H.; Guanosine triphosphate (GTP) as the disodium salt from Sigma; Pyruvate kinase from Boehringer; Phosphoenolpyruvate as the disodium salt from Sigma; ^{14}C-1-DL-alanine (2.25 umoles/uC), ^{14}C-1-DL-glutamic acid (3.20 umoles/uC), ^{14}C-2-glycine (2.61 umoles/uC), ^{14}C-1-L-lysine (1.66 umoles/uC), ^{14}C-1-L-methionine (1.85 umoles/uC) and ^{14}C-1-DL-phenylalanine (2.36 umoles/uC) from the Radiochemical Centre Amersham; ^{32}P sodium pyrophosphate from the same source; the L forms of the 18 common amino acids from B.D.H.; Sodium deoxycholate from Merck.

Preparation of Ribosomes.

Media used.

1. Medium A.

0.004M $MgCl_2.6H_2O$

0.025M KCl

0.35 M Sucrose

0.05M Trihydroxyamino methane (Tris), pH 7.6

2. Medium C.

0.01M $MgCl_2.6H_2O$

0.05M KCl

0.5M Sucrose

0.05M Tris, pH 7.6.

The method used was that of Kirsch (1962).

The rats used were starved for 24 hours prior to killing to deplete the glycogen store of the liver. The animals were killed by a blow on the head, the livers rapidly excised and plunged into ice cold 0.35M sucrose to remove any excess blood. They were blotted dry, weighed by displacement and homogenised in 2.4 volumes of Medium A in a Potter-Elvejhem homogeniser at the slowest speed compatible with the formation of a homogeneous suspension. The homogenate was centrifuged at 18,000g for 5 mins. in the Rotor 30 of the Spinco ultra-centrifuge, Model L, to remove the cell debris, nuclei and mitochondria. The supernatant was centrifuged at 105,000g for 1 hour in the Rotor 40 of the Spinco giving a pellet which will subsequently be called microsomes and a supernatant which was discarded. The microsome pellet obtained from each tube was resuspended in 1 ml. Medium C. Five ml. of this medium containing 1% sodium deoxycholate (DOC) was added to each tube and the mixture stirred briefly over a period of about 15 mins. until a clear solution was obtained. (To make up Medium C containing DOC equal volumes of twice as concentrated solutions of Medium C and DOC were mixed together just before use. If this precaution was not taken, Mg DOC tended to precipitate.)The deoxycholate treated microsome solutions were pooled and centrifuged at 105,000g for 2 hours. The surface of the pellet so obtained "Ribosomes" was washed several times with Medium A, the sides of the tube dried out

with filter paper and the pellet suspended in a small volume of Medium A for use in subsequent incubation experiments. If the ribosomes were to be stored overnight before use in an experiment, they were stored in the form of the pellet and not resuspended in Medium A until the next day.

Standard Incubation Conditions for Uptake of ^{14}C-leucine by Post-microsomal pellet.

One mg. of post-microsomal protein prepared as in Fig. 6 was incubated for 2 hours at 37^{o}C with constant shaking, in the presence of 1 umole ATP and 1 uc ^{14}C-DL-leucine. 10 umoles $MgCl_2.6H_2O$, 30 umoles $KHCO_3$, 25 umoles KCl, 20 umoles potassium phosphate buffer, pH 7.8 and 350 umoles sucrose were also added in a total volume of 1 ml..

Termination of Incubation and Preliminary Treatment of Samples for Counting.

After the period of incubation the samples were chilled and the protein and RNA precipitated by the addition of 5 vols. 0.3M PCA. After 10 minutes the precipitate was centrifuged off and washed three times with 0.3M PCA at 0^{o}C to remove any adherent acid soluble material. The lipids were then removed by washing twice with ethanol:ether:chloroform = 2:2:1 and once with ether at room temperature.(Rendi and Campbell, 1959).

The recovery of RNA and protein at each of the stages in this process was investigated. The results are given in Table 8.

Table 8.

An Investigation of the Procedure used to Obtain Radioactive Proteins after Incubation.

1.31 mg. post-microsomal protein (containing 110 ug. RNA) was incubated with 1 umole ATP and 1 mC ^{14}C-DL-leucine for 2 hours at 37°C in a total volume of 1 ml. Campbell buffer. The RNA and protein were precipitated by the addition of 4 vols. 0.36N PCA. RNA and protein estimations were carried out on this precipitate and again, after each sequence of washes, as indicated.

Washing Fluid		Conditions during Incubation	ug. RNA recovered	% Loss	ug. Protein recovered	% Loss	Total Cts./min./mg. Protein Incubated
0.3N PCA	Normal Lipid Solvents						
-	+	+ ATP	132	0	970	26	89,200
		- ATP	114	0	896	32	79,730
+	-	+ ATP	117	0	902	31	679
+	+	+ ATP	94	14	825	37	494

Immediately after precipitation and before washing, there is considerable contamination of the RNA and protein with non-incorporated ^{14}C-DL-leucine and also some ATP, as would be expected. Thus, there is a greater than 100% recovery of RNA and the radioactivity of the samples is extremely high. There is, however, a loss of 26% of the protein due either to incomplete precipitation, or to breakdown of the protein during incubation. On washing the precipitate with 0.3N PCA there is a loss of a further 5% of the protein but 100% of the RNA is recovered. Additional washing with lipid solvents reduces the protein recovered by a further 5% making the overall recovery 63%. There is a loss of about 14% of the RNA at this stage giving an overall recovery of 86%. This wash with lipid solvents appears to remove also about 30% of the radioactivity presumably attached to the RNA and protein which is also lost at this stage.

Obviously, this method is not ideal giving only a 60% recovery of protein and an 80% recovery of RNA but an investigation of the use of other normalities of PCA showed no other method to be better than this one (Table 9). Therefore, the method outlined above was adopted.

By plating the lipid-free dry powder obtained, the total amount of ^{14}C -leucine incorporated by the post-microsomal pellet could be measured. In some cases, however, we were interested in the amount of leucine incorporated into the protein portion of the fraction only. To obtain this, the RNA

Table 9.

The Effect of Various Normalities of PCA on the Precipitation of Post-microsomal RNA.

Post-microsomal pellet (unincubated) was precipitated with 5 vols. of PCA of the normality given in column 1 below. After standing for 10 mins. the precipitate was centrifuged off and washed with 5 ml. of the appropriate PCA (column 2). Washing with PCA was repeated twice. The pellet so obtained was washed twice with ethanol:ether:chloroform = 2:2:1 and once with ether. RNA was estimated at each stage of the washing procedure.

Normality PCA used for Precipitation	Normality PCA used for Washes	Lipid Solvents Wash	ug. RNA recovered	% Loss
0.4	-	-	272	-
	0.2	-	234	14
		+	222	18
0.2	-	-	246	10
	0.2	-	232	15
		+	216	21
0.7	-	-	258	5
	0.7	-	237	13
		+	215	21

was extracted using 0.4N PCA at 70°C. Two twenty minute extractions were carried out leaving a precipitate of protein free from RNA. The extracted RNA could be measured from the ultraviolet absorption of the extract at 260 and 290 mu. This estimate agreed very well with that obtained by the alkaline hydrolysis method of Fleck and Munro (1962).

Determination of the Radioactivity of Samples.

The lens-paper method of Garrow and Piper (1955) was used. The dry powder obtained as above, was dissolved in three drops of N NaOH by heating in a boiling water bath for a few minutes. The solution obtained was transferred to lens paper discs on steinless steel planchettes, 5.15 cm.^{2} in area using a Pasteur pipette. The tube was washed out with a further three drops of N NaOH at 100°C, and the washings added to the planchette. The planchettes were dried under infra red lamps and counted with a Nuclear-Chicago windowless-gas-flow-automatic counter.

To ensure that a linear relationship existed between the amount of protein plated and the radioactivity obtained, varying amounts of a radioactive protein were plated, and the planchettes counted. Up to 3mg. of protein, the activity obtained varied directly with the amount of protein plated. Above this level, the counting efficiency fell off and above 20mg. the activity was completely independent of the amount of protein plated. As normally we are dealing with 1 mg. of protein,

and in every case with less than 3mg., this method proved to be entirely satisfactory.

Assay of Activating Enzyme Activity.

The ATP-^{32}P pyrophosphate exchange method of Hultin and von der Decken(1958) was used.

One mg. of protein of the fraction under investigation was incubated at 37°C for ten minutes with constant shaking. The incubation medium contained also 4 umoles ^{32}PP, 6 umoles ATP, 10 umoles $MgCl_2.6H_2O$, 30 umoles $KHCO_3$, 25 umoles KCl, 60 umoles KF, 20 umoles potassium phosphate buffer, pH 7.8 and 350 umoles sucrose in a total volume of 1 ml.. In some cases, an amino acid mixture containing three umoles of glycine and 3 umoles of the L form of each of the following amino acids was added:- alanine, valine, leucine, isoleucine, serine, threonine, methionine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylalanine, tryptophan and proline. In other experiments only one amino acid was added, 3 umoles again being used.

After incubation the samples were chilled and the protein precipitated by the addition of 6 ml. 0.36N PCA. After standing for 10 minutes, the precipitate was sedimented by centrifugation and 5 ml. of the supernatant was removed and added to 100 mg. of Norit A. The charcoal adsorbed the ATP, leaving the pyrophosphate in solution (Orano and Lipmann, 1953). To ensure complete adsorption of the ATP, the mixture was allowed to stand for 30 minutes at 0°C with frequent stirring (McLean, 1962).

Thereafter, the charcoal was separated by centrifugation and washed five times with chilled distilled water. The supernatant and washings were combined and made up to 25 ml. with distilled water. This fraction contained the pyrophosphate of the incubation mixture. The radioactivity of the pyrophosphate was measured using a liquid counter (Veall). The amount of pyrophosphate present was estimated using the method of Allen (1940). One ml. aliquots were used to determine the orthophosphate present before and after digestion with 2 ml. 2N H_2SO_4. (When acid hydrolysis of samples was carried out the amount of 10N H_2SO_4 added to the final colour reaction was adjusted to give the same acid concentration as in the non-hydrolysed samples.) From the difference in these two estimations the amount of pyrophosphate present could be calculated. McLean (1962) has shown that this method is valid. Thus, non-hydrolysed pyrophosphate does not interfere with the estimation of inorganic phosphate, and hydrolysis at 100°C for 15 mins. with 2N H_2SO_4 does convert pyrophosphate quantitatively to a form which is estimable by the Allen method. The specific activity of the recovered pyrophosphate could therefore be calculated.

The specific activity of the ATP adsorbed on the charcoal was determined as follows:- The β and γ phosphate groups of the ATP can be hydrolysed from the charcoal by heating at 100°C with 2 ml. 2N H_2SO_4. As this is the part of the ATP molecule

which becomes labelled by exchange with pyrophosphate, the remainder of the molecule need not be isolated to determine the specific activity. After hydrolysis for 30 mins. at 100°C with constant shaking, 4 ml. of distilled water was added to each tube and the charcoal sedimented by centrifugation. Five ml. of the supernatant was removed and diluted to 10 ml.. Aliquots of this solution were used to determine the radioactivity of the ATP (liquid counting) and the amount recovered (Allen method). From these results the specific activity of the ATP could be calculated.

The percent exchange was calculated using the equation of Hoagland (1956):-

$$\% \text{ Exchange} = \frac{\text{Specific activity of ATP}}{\text{Specific activity of ATP + PP}} \times 100$$

Standard Incubation Conditions for the Transfer of Amino Acids to sRNA by Post-microsomal Pellet.

One mg. of post-microsomal protein was incubated for 10 minutes at 37°C in 1 ml. Campbell buffer containing 10 umoles $MgCl_2.6H_2O$, 30 umoles $KHCO_3$, 12.5 umoles KCl, 20 umoles potassium phosphate buffer, pH 7.8 and 350 umoles sucrose. Also present were 1 umole ATP, 1 uC ^{14}C-DL-leucine and 100 ug. sRNA prepared by phenol extraction from 3 hour cell sap. After incubation, the method used to stop the reaction and to prepare the samples for plating was identical to that used previously (page 73.).

Standard Incubation Conditions for the Incorporation of Amino Acids into Ribosomes.

About 2 mg. ribosomal protein and 0.5 mg. cell sap pH 5 enzyme protein were incubated at 37°C for 20 minutes in 1 ml. Medium A containing 4 umoles $MgCl_2.6H_2O$, 25 umoles KCl, 350 umoles sucrose and 50 umoles tris buffer, pH 7.6 (Kirsch, 1962). Also present in the incubation mixture were 1 umole ATP, 0.25 umoles GTP, 10 umoles PEP, 0.02 mg. pyruvate kinase, 0.72 uC ^{14}C-1-DL-leucine and an additional 10 umoles Mg^{++}. As PEP is unstable in solution, it was dissolved in Medium A and added immediately to each incubation tube. In some experiments 5 umoles ATP were added instead of only 1 umole; under these conditions PEP and PK are not essential for the activity of the system. In these experiments an additional 5 umoles of Mg^{++} were also added (Korner, 1960).

Termination of the Incorporation into Ribosomes and Preparation of the Samples for Counting.

The reaction was stopped by chilling the samples and immediately adding 1 volume of 0.4N PCA containing 4 mg./ml. of ^{12}C-leucine at 0°C. The samples were frequently stored overnight at - 10°C at this stage. After standing for 10 minutes, The precipitate was separated and washed twice with 5 ml. of 0.2N PCA at 0°C. The RNA was removed by the addition of 3 ml. 0.3N KOH and hydrolysed at 37°C for 1 hour. The protein was reprecipitated at 0°C by the addition of 2.5 ml. of 0.6N PCA

containing ^{12}C-leucine. The precipitate obtained was separated and washed twice with 0.2N PCA draining the tubes carefully and drying out the sideswith filter paper after the second wash. The protein was dissolved in 1 ml. 0.1N KOH by standing overnight at room temperature. The potassium perchlorate present was centrifuged off and 0.5 ml. aliquots of the supernatant were plated on lens paper discs and counted as previously described.

Results.

Part 1.

The Amino Acid Activating Enzyme Content of Post-microsomal Pellet.

As post-microsomal pellet is prepared from the cell supernatant, the main site of the activating enzymes of the cell, it was of some interest to investigate the level of the activity of these enzymes in this fraction. This is particularly important to establish in a particle which is capable of incorporating amino acids by a reaction requiring ATP.

The method used in this estimation was that of ^{32}PP-ATP exchange dependent on the presence of amino acids. Certain precautions were necessary in carrying out this study. Crude fractions prepared from a homogenate by differential centrifugation have a high level of endogenous amino acids which conceals the effect of the addition of an amino acid. To minimise this effect, each sedimented fraction was dissolved in a very large volume of medium and the supernatant fractions were diluted to a large volume before precipitation at pH 5. Another requirement was the addition of potassium fluoride to the incubation mixture to suppress the action of any ATP-hydrolysing enzyme which may be present. Such an enzyme, by removing one of the products of the reaction, would completely alter the equilibrium conditions. With these precautions,

reliable and reproducible results were obtained.

Fig. 17 shows a concentration curve obtained for activating enzymes in post-microsomal pellet and in 1 hour and 3 hour cell sap in the presence and absence of a complete amino acid mixture. This was obtained by incubating between 0 and 4 mg. of protein of each fraction for 10 minutes at 37°C with the standard additions described in the methods section. The percent exchange achieved by each fraction at each protein level was calculated and plotted against the mg. of protein incubated. A control sample, containing no enzyme, was also processed.

The first point to be noted in Fig. 17 is that in the absence of added enzyme there is no exchange between ^{32}PP and ATP even in the presence of added amino acids. This presumably indicates that there is no non-enzymic exchange between ATP and PP in the system and also that the method of separation of the ATP and PP by charcoal adsorption (Crane and Lipmann, 1953) is quite satisfactory.

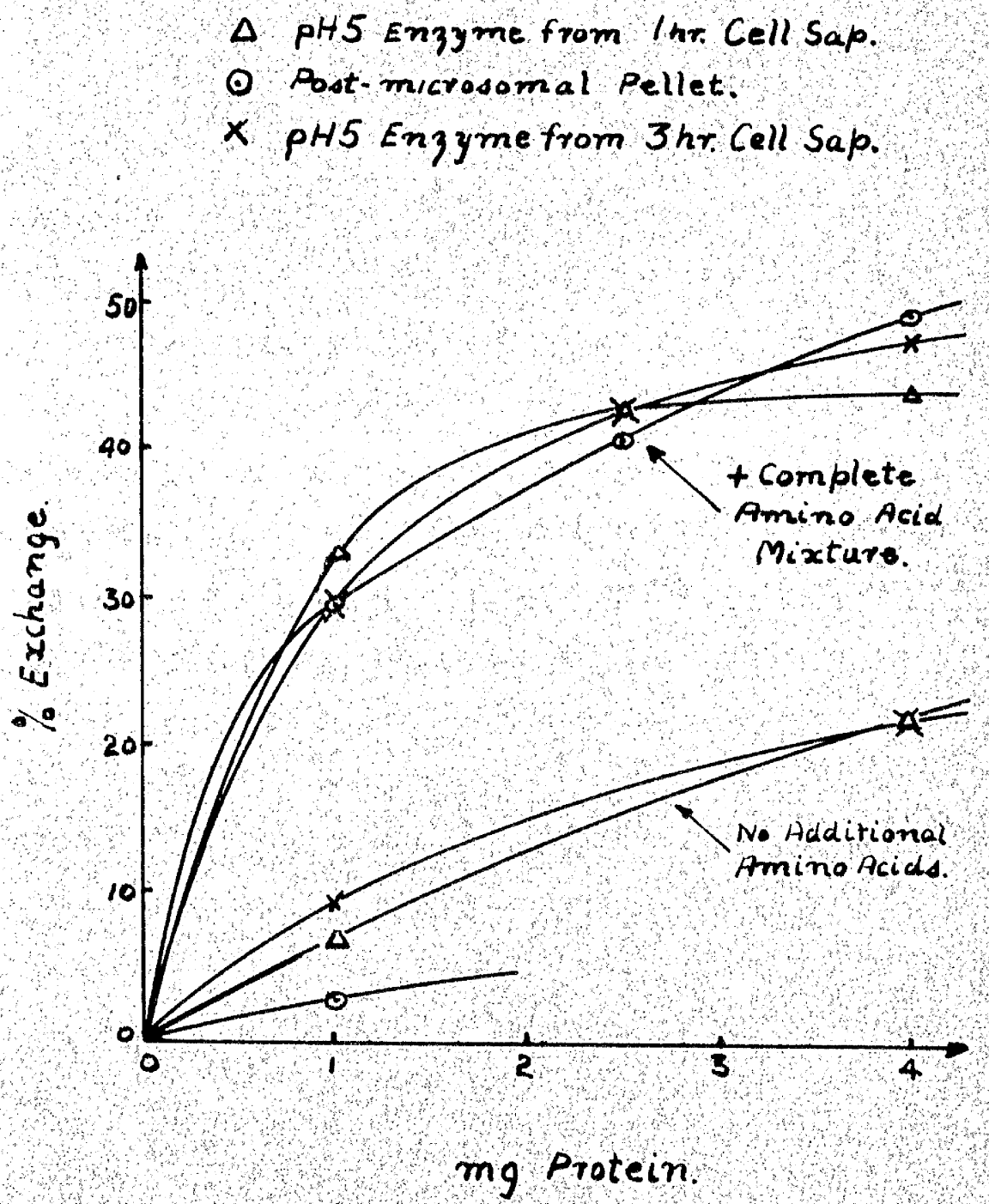
Secondly, on the addition of activating enzymes from any of the three sources mentioned, but in the absence of an amino acid mixture, there is a considerable exchange especially in the cell sap preparations. This shows that the precautions taken have not completely removed the endogenous amino acids. However, Hultin and von der Decken (1958) have obtained just as high a level of endogenous exchange after extensive dialysis of their fractions and, therefore, we did not deem it

Fig. 17.

Various amounts of protein (from 0 to 4 mg.) of the fractions shown were incubated for 10 minutes at 37°C with 6 umoles ATP, 4 umoles ^{32}PP and 60 umoles KF. A complete amino acid mixture containing 3 umoles of each amino acid was also added to some tubes. The total volume of the incubation mixture was 1 ml. in each case and the concentration of $MgCl_2$, $KHCO_3$ and KCl were adjusted to be the same as those of Campbell buffer. After incubation, the ATP was separated from the pyrophosphate by adsorption on charcoal. The specific activities of the ATP and the PP were calculated and hence the % Exchange.

Fig. 17.

Activating Enzyme Activity of Various amounts of Protein from Post-microsomal Pellet and pH 5 Enzyme from 1 Hour and 3 Hour Cell Sap.



Each point is a mean of two experiments.

profitable to treat our fractions further to lower this level of endogenous exchange. In post-microsomal pellet the endogenous exchange is only about 50% of that of the two cell sap preparations which are similar in activity.

On the addition of a complete amino acid mixture there is a large stimulation in the percent exchange. As shown in the enzyme concentration curve, all three fractions behave in exactly the same way, with a rapid rise in activity from 0 to 1 mg. protein and then a slower rise from 1 to 4 mg.. The catalytic activity of each fraction per mg. protein over the range of concentrations tested is the same, if the endogenous level is ignored, but if the endogenous value is subtracted before comparison, post-microsomal pellet shows the greatest activity. When 1 mg. enzyme protein is used, the difference between the activity due to endogenous and exogenous amino acids is greatest (about 4 times in the case of cell sap preparations and about 10 times for post-microsomal pellet). Therefore, in all the following experiments 1 mg. enzyme protein is used.

Having demonstrated the presence of a high level of activating enzymes in post-microsomal pellet, the following investigations were carried out.

First, a survey of the enzyme activity for various amino acids, added singly, was made, and the activities compared with those of the pH 5 precipitate from 3 hour cell sap and of the cell microsome fraction. For all the amino acids tested

the 3 hour cell sap preparation, the post-microsomal pellet and the microsomal fraction had different activities (Table 10). None of the fractions were able to activate alanine, glycine or arginine and the activity for phenylalanine was fairly low, although this was the only amino acid which was activated to any extent by the microsome preparation. Leucine and methionine were activated by cell sap and post-microsomal pellet but lysine was only significantly activated by post-microsomal pellet. Summarising, it may be said that the distribution of activating enzymes for various amino acids in post-microsomal pellet and 3 hour cell sap pH 5 enzyme is similar but not identical.

The finding that the activating enzymes for leucine, methionine and phenylalanine in post-microsomal pellet are all about three times more active and that lysine activating enzyme is about five times more active than the corresponding enzymes in cell sap makes it most unlikely that the activating enzyme content of post-microsomal pellet is due solely to the contamination of the fraction with cell sap. This observation is also applicable to the microsome fraction where there appears to be a concentration of the phenylalanine activating enzyme although no other activating enzymes appear to be present.

Another finding on the activation of various amino acids was that the percent exchange due to a mixture of amino acids was equal to the sum of the activities for the various amino acids added separately. (Table 11.) This observation is in

Table 10.

The Activating Enzyme Content of Post-microsomal Pellet, pH 5 Enzyme from 3 Hour Cell Sap and Microsomes for Various Amino Acids.

1 mg. of post-microsomal protein or 1 mg. of pH 5 enzyme protein or 1 mg. microsomal protein was incubated at 37°C for 10 mins. with 6 umoles ATP, 4 umoles ^{32}PP, 60 umoles KF and 3 umoles of the amino acid noted, in a total volume of 1 ml. Campbell buffer.

The results are expressed as % Exchange relative to the % Exchange due to the addition of leucine to post-microsomal pellet.

Amino Acid	Post-microsomal Pellet	pH 5 Enzyme	Microsomes
Leucine	100	44	9
Lysine	57	7	0
Methionine	66	23	0
Phenylalanine	25	10	27
Alanine	1	5	2
Glycine	0	7	1
Arginine	4	0	0

Table 11.

The Effect of the Addition of Individual Amino Acids Added Separately and Together on the Activating Enzyme Activity of Post-microsomal Pellet and pH 5 Enzyme from 3 Hour Cell Sap.

The conditions of incubation were the same as given for Table 10. The results are expressed as % Exchange per mg. of protein.

	% Exchange	
	Post-microsomal Pellet	pH 5 Enzyme
Leucine	16	6
Methionine	10	4
Phenylalanine	4	2
Leucine + Methionine + Phenylalanine (Sum of above)	30	12
Leucine + Methionine + Phenylalanine (By incubation together)	30	12

agreement with that of Hoagland et al. (1956) and is an indication of the presence of separate activating enzymes for each amino acid.

Thus, having shown that leucine, lysine and methionine are extensively activated and other amino acids are not activated to any extent by post-microsomal pellet the obvious question to ask is "Does the capacity of post-microsomal pellet to incorporate ^{14}C-amino acids into its protein exhibit a similar spectrum?" Therefore, activating enzyme assays and ^{14}C-incorporation experiments were run concurrently on the same post-microsomal preparation. The same spectrum of amino acid activating enzymes was obtained as before. In general, the energy dependent incorporation of amino acids into the protein fraction followed this pattern (Table 12). Thus, leucine and methionine are highly incorporated in an energy dependent reaction, while alanine glycine and glutamic acid are not incorporated to any extent by such a mechanism. In opposition to this, however, lysine, and to a lesser extent phenylalanine, are incorporated into post-microsomal protein by a reaction which is independent of the presence of ATP, although the presence of activating enzymes is indicated. Also, alanine, glycine and glutamic acid show a significant incorporation into post-microsomal protein by a reaction which is not dependent on ATP, although no detectable activating enzymes are present for these amino acids. These findings suggest that there may be two systems

Table 12.

The Relationship of Activating Enzyme Activity to Incorporation of Amino Acids by Post-microsomal Pellet.

The conditions of incubation for activating enzyme assay were as given in Table 10.

To determine the incorporating ability of post-microsomal pellet for the various amino acids 1 mg. aliquots of post-microsomal pellet protein were incubated for 2 hours at 37°C with 1 umole ATP, and 1 uC of each of the following amino acids: 1-^{14}C-DL-leucine, 1-^{14}C-DL-methionine, 1-^{14}C-L-lysine, 1-^{14}C-DL-phenylalanine, 1-^{14}C-DL-alanine, 1-^{14}C-DL-glutamic acid and ^{14}C-glycine in a total volume of 1 ml. Campbell buffer. The results are expressed as total counts incorporated (in mumoles) per mg. protein incubated.

The data is the average of 2 experiments.

	% Exchange	mumoles incorporated/ mg. protein		
		+ ATP	- ATP	Difference i.e. energy dependent incorporation
leucine	100	2.6	0.6	2.0
methionine	67	2.6	0.5	2.1
lysine	59	3.2	2.6	0.6
phenylalanine	28	1.1	0.7	0.4
alanine	4	1.6	1.2	0.4
glycine	4	2.2	2.1	0.1
glutamic acid	5	0.8	0.6	0.2

present in post-microsomal pellet one of which involves activating enzyme activity, and the other incorporation into the post-microsomal pellet protein. Further evidence for the presence of these two pathways will be given in the following paragraphs and also in the sections on the transfer of amino acids to sRNA and the investigation of the ATP-dependence of the amino acid incorporating system of post-microsomal pellet.

Although simple contamination with cell sap has been ruled out as the source of the activating enzymes of the post-microsomal pellet, due to the presence of very much more active enzymes in post-microsomal pellet than in cell sap an experiment was carried out in which the post-microsomal pellet was resuspended in buffer after sedimentation and then reseparated. Such an operation should remove most of the contaminating cell sap material. Activating enzyme assays and ^{14}C-incorporation ability were studied in a preparation of this "washed" post-microsomal pellet (Table 13). It can be seen that this treatment has no effect on the percent exchange due to the endogenous amino acids (Table 13a). The activating enzyme activity for a total amino acid mixture falls by about 64% and that for leucine by about 73%. That for lysine is unaffected per mg. post-microsomal protein. It may be significant that the activating enzyme activity for lysine in the cell supernatant is very low (Table 10), suggesting that this enzyme is rather insoluble. It is of

Table 13

The Effect of Washing on the Activating Enzyme Activity and ^{14}C-Amino acid Incorporating Ability of Post-microsomal Pellet.

The conditions of incubation were the same as given for Table 12.

A. The Activating Enzyme Activity.

Amino Acid Added	% Exchange	
	Unwashed Pellet	Washed Pellet
None	8	6
Complete Mixture	66	28
Leucine	44	12
Lysine	21	25

B. The Incorporating Ability.

	PCA stable cts./min./mg. protein			ug. RNA recovered	
	+ ATP	- ATP	Difference i.e. ATP Dependent Incorporation	+ ATP	- ATP
Unwashed Pellet	374	23	351	170	182
Washed Pellet	602	245	357	246	238

course possible that the finding that the activity for the various amino acids is affected differently by washing the post-microsomal pellet, may reflect a difference in stability of the enzymes rather than a removal of the enzymes; this possibility will be considered later.

On turning to the incorporating ability of the post-microsomal pellet after washing (Table 13b), the incorporation of leucine is increased in spite of the large fall in the activity of the activating enzyme previously mentioned. However, the energy-dependent incorporating ability of the fraction is not altered by washing. Presumably, washing removes some protein from the pellet which is a potent source of activating enzyme for leucine but is not involved in the ATP-dependent incorporation reaction. The removal of this protein may unmask some site in the particle where incorporation can occur which is not ATP-dependent. (It is also noteworthy that washing removes much more protein than RNA, the RNA/protein ratio rising from 0.15 to 0.24). Again, the evidence indicates that there are at least two systems, and perhaps three, operating in the post-microsomal pellet, one involving the activating enzyme activity, one the energy-dependent incorporation of amino acids and the third the energy-independent incorporating ability.

"Does washing of post-microsomal pellet render the leucine activating enzyme unstable, or does it merely solubilise this enzyme?" The stability of the enzyme was studied in a

cell sap preparation and in post-microsomal pellet. 1 mg. of protein of each fraction was preincubated for 110 mins. before the addition of the ^{32}P pyrophosphate (Table 14). The results show that even after this long period of pre-incubation, the percent exchange catalysed by leucine activating enzyme is the same as that of a control sample which was not preincubated. Obviously, the enzyme involved must be stable to this treatment. Therefore, the large fall in the activity of the leucine activating enzyme on washing of post-microsomal pellet does not seem to be attributable to the instability of the enzyme, but must represent rather a solubilisation of the enzyme.

This concludes the observations made on the activating enzyme content of post-microsomal pellet as compared with other fractions. The results obtained can be summarised as follows:-

1. Between 0 and 4 mg. protein, the exchange caused by the presence of a complete amino acid mixture is the same for post-microsomal pellet, 1 hour and 3 hour cell sap.
2. The exchange caused by the presence of endogenous amino acids is much less for post-microsomal pellet than for either a 1 hour or 3 hour cell sap preparation, suggesting that free amino acids are less abundant in post-microsomal pellet than in the pH 5 fraction of either 1 hour or 3 hour cell sap.
3. The exchange for individual amino acids is additive when a mixture is used, implying that discrete enzymes are present

Table 14.

The Activating Enzyme Activity of Post-microsomal Pellet and pH 5 Enzyme from 3 hour Cell Sap after a Period of Preincubation.

The conditions of incubation were as given for Table 10. In the case of the preincubated samples the ^{32}P was added after the period of preincubation all the other additions being made before preincubation.

Duration of Preincubation	Duration of Incubation mins.	Amino Acid Mixture	% Exchange	
			Pellet	pH 5 Enzyme
-	10	+	15	12
"	10	-	4	6
110	10	+	18	15
110	10	-	4	6

for each amino acid.

4. The distribution of activating enzymes for various amino acids in post-microsomal pellet and pH 5 enzyme from 3 hour cell sap is qualitatively similar but not identical. There are activating enzymes for methionine, leucine, lysine and phenylalanine in both fractions; those for lysine and phenylalanine are five times more active and those for leucine and methionine three times more active in post-microsomal pellet than in cell sap pH 5 enzyme.

5. Microsomes show little activating enzyme activity under the conditions used, although this fraction does have some capacity to activate phenylalanine.

6. The ability of post-microsomal pellet to incorporate individual amino acids by an energy-dependent reaction follows a similar pattern to the activating enzyme activity except in the case of lysine. Lysine, and also those amino acids for which activating enzymes could not be demonstrated are incorporated into protein by a non-ATP-dependent reaction.

7. Washing of post-microsomal pellet reduces the activating enzyme content by 60% for a complete amino acid mixture and by 70% for leucine, but has no effect on the lysine activating enzyme activity. The ability to incorporate ^{14}C-leucine is increased, but only by a non-energy dependent mechanism.

8. The activating enzyme activity of pH 5 enzyme and post-microsomal pellet is stable to preincubation for 110 mins. at $37^{o}C$, thus showing that the decline in the activity on

"washing" cannot be due to the instability of the enzymes.

9. Conclusions: The main conclusion to be drawn from these experiments is that, although post-microsomal pellet does possess activating enzymes for several amino acids, the incorporation of amino acids into post-microsomal pellet protein does not depend on the presence of these enzymes, since the two properties do not change in parallel during various treatments. Two mechanisms appear to be operating in this incorporation process, one involving the participation of ATP and the other not dependent on the presence of ATP.

Part 2.

The Ability of Post-microsomal Pellet to Transfer Amino Acids to sRNA.

In Part 1 of this section, activating enzymes were shown to be present in post-microsomal pellet. These enzymes are involved in the first stage of the generally accepted mechanism of protein biosynthesis. The second step in this pathway is a transfer of the activated amino acids to the soluble polynucleotide, sRNA. (See Fig. 2.) Can post-microsomal pellet carry out this second reaction as well as the initial activation step? This aspect of the problem is examined in this part of the thesis.

1. The Absence of Acceptor RNA in Post-microsomal Pellet.

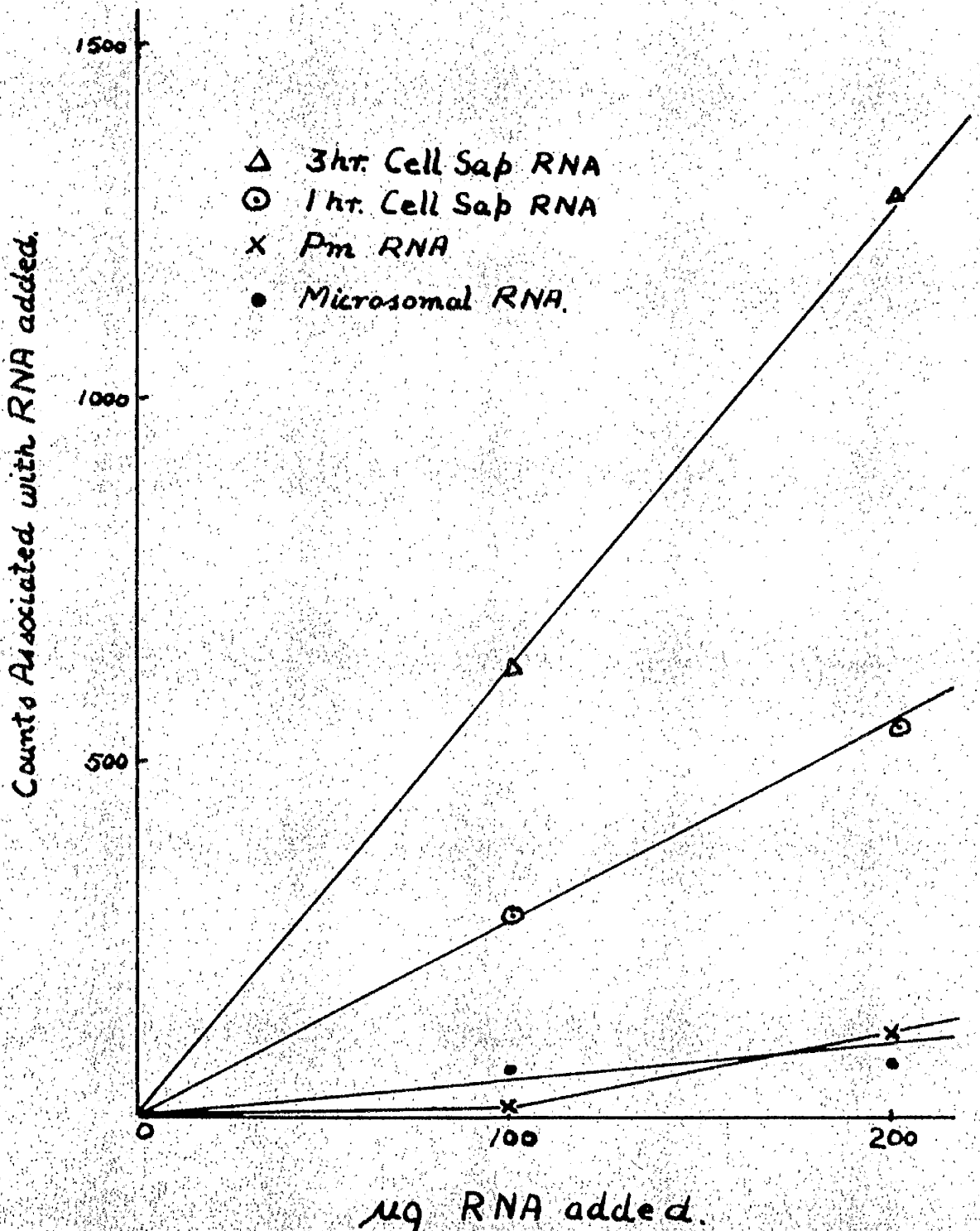
In Section 1 we concluded that post-microsomal RNA does not contain any active sRNA. (See page 66.) To confirm this finding, RNA was prepared from a variety of fractions by phenol extraction and its acceptor ability tested by incubation in a standard pH 5 enzyme system. The results (Fig. 18) show that in such a system, post-microsomal RNA does not act as an acceptor of amino acids since ^{14}C does not accumulate in this RNA. On the other hand, RNA prepared by phenol extraction from 1 hour and 3 hour cell sap does have this property which may be equated to sRNA activity. 3 hour cell sap RNA is more effective as an acceptor than 1 hour cell sap, consistent with a higher content of sRNA, the inactive post-microsomal RNA

Fig. 18.

1.7 mg. protein from pH 5 enzyme from 1 hour cell sap was incubated at 37°C for 10 minutes with 10 umoles ATP, 1 uC ^{14}C-DL-leucine and 100 or 200 ug. RNA as shown in the Fig., in a total volume of 3 ml. Campbell buffer. The results are expressed as total counts per sample less the counts obtained when pH 5 enzyme was incubated with no added RNA and thus are termed "counts associated with the added RNA".

Fig. 18.

The Addition of RNA prepared by Phenol Extraction from Various Cell Fractions to pH 5 Enzyme from 1 Hour Cell Sap.



having been removed. This is borne out by quantitative considerations. Post-microsomal RNA is approximately one third of 3 hour cell sap RNA; it is, consequently, noteworthy that 1 hour cell sap RNA has only two thirds of the activity of 3 hour cell sap RNA. These findings are consistent with the explanation that post-microsomal RNA is inactive as an acceptor of amino acids and dilutes the active RNA present in 1 hour cell sap. Microsomal RNA was included in this experiment as a control and as expected, showed no significant ability to accept activated amino acids (Fig. 18). These findings of no acceptor ability in post-microsomal RNA and microsomal RNA indicate that there is little contamination of these fractions by cell sap, but the possibility is not completely ruled out as there may be enzymes present in the post-microsomal pellet and microsome fraction which inactivate any sRNA which may be present due to contamination.

2. <u>The Capacity of Post-microsomal Pellet Enzymes to Transfer Amino Acids to sRNA.</u>

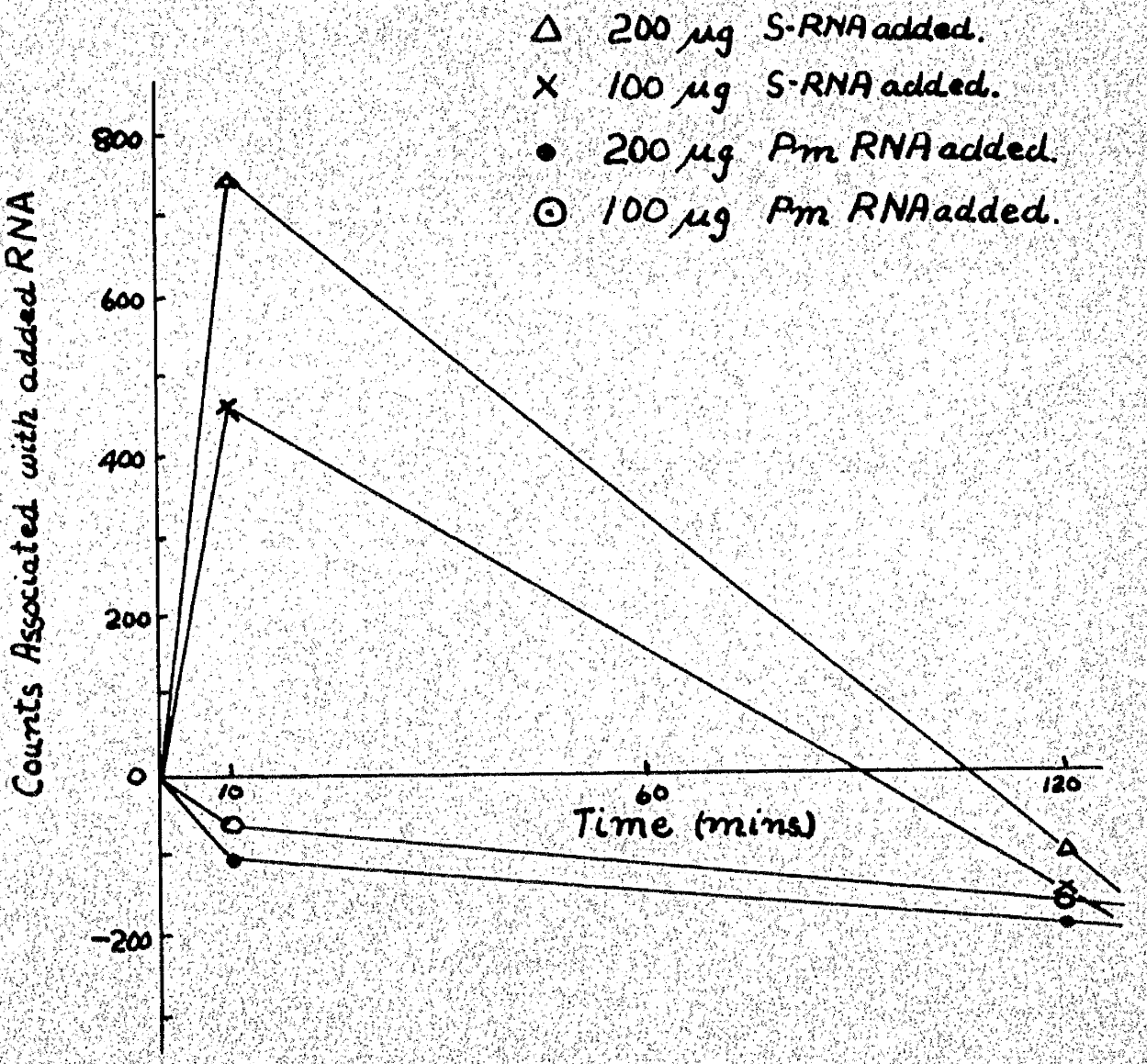
Although post-microsomal pellet does not contain any active sRNA it is possible that, if the activating enzymes present are supplied with active sRNA, they could transfer the activated amino acids to this RNA. Fig. 19 gives the result of such a test where post-microsomal pellet was incubated with the addition of phenol-prepared sRNA and post-microsomal RNA. The results show that after incubation

Fig. 19.

1 mg. post-microsomal protein was incubated for 10 minutes or 120 minutes at 37°C with 100 or 200 ug. RNA prepared from 3 hour cell sap or post-microsomal pellet by phenol extraction. 1 umole ATP and 1 uC ^{14}C-DL-leucine was also added in a total volume of 1 ml. Campbell buffer. The radioactivity incorporated into the added RNA is plotted against time.

Fig. 19.

The Addition of 3 Hour Cell Sap RNA and Post-microsomal RNA to a Post-microsomal Incorporating System.



for 10 minutes, the activating enzymes of post-microsomal pellet had transferred ^{14}C-leucine to sRNA but not to added post-microsomal RNA. When incubation was carried on for two hours, the label was lost from the sRNA, but there was still no labelling of the post-microsomal RNA. The most obvious explanation of this disappearance of label would be a falling off in the activity of the activating enzymes. However, in Part 1 of this section (Table 14) it was shown that the activating enzymes of this fraction are stable to at least 110 minutes of incubation. This explanation must, therefore, be ruled out.

Before any other explanation of this rapid loss of label can be considered, a more detailed examination of the time course of this incorporation of amino acids into sRNA and their subsequent removal is necessary. The results of such an experiment are shown in Fig. 20. The hot-PCA-soluble activity (presumably attached to RNA) rises very rapidly in the presence of added sRNA, and even after only 2 minutes of incubation there is a large incorporation of leucine into this fraction. After 10 minutes however, the activity falls off fairly rapidly, and by 120 minutes, it is not much greater than the activity obtained in the absence of added sRNA. When no ATP is provided, there is no incorporation of isotope into a hot-PCA-soluble form in the presence or absence of added sRNA. (Only the curve obtained in the presence of sRNA is shown for the sake of clarity; the curve obtained in its

Fig. 20.

The Time Course of the Incorporation of ^{14}C-leucine into sRNA by Post-microsomal Pellet.

1 mg. post-microsomal protein was incubated at 37°C for the times indicated with 1 umole ATP, 1 uC ^{14}C-DL-leucine and 200 ug. sRNA as shown in the Fig. The total counts obtained in a PCA soluble and PCA stable form are plotted against duration of incubation.

Each point is a mean of 2 experiments.

Fig. 20.

PCA Soluble.
Counts/min/mg/Protein.
1500
1000
500
0
0
10
30
60
90
120
Time (mins.)
PCA Stable.
Counts/min/mg Protein.
300
200
100
0
0
10
30
60
90
120
Time (mins.)
Δ + ATP + sRNA.
X + ATP
O - ATP

absence was, however, similar.) The picture obtained in the hot-PCA-stable (presumably protein) fraction of the post-microsomal pellet is quite different. In this case the presence of sRNA markedly inhibits the incorporation. The probable explanation of this finding would be that sRNA competes specifically with the protein for the amino acid. However, the independence of the two pathways is indicated by the fact that over the 120 minute period of incubation there is a rise and fall in the amount of leucine attached to the sRNA but the uptake into a hot-PCA-stable form rises steadily throughout the period. To establish completely this point of inhibition by RNA, an experiment was carried out in which the effect of microsomal RNA on the hot-PCA-soluble and stable activity was studied (Table 15). Microsomal RNA does not act as an acceptor of activated amino acids under the standard conditions as can be seen from the samples which were incubated for 10 minutes, but again, the microsomal RNA acts just as efficiently as sRNA in inhibiting the incorporation of leucine into the protein fraction of post-microsomal pellet on prolonging incubation for 2 hours. We can, therefore, conclude that the presence of RNA prepared by phenol extraction from various cell fractions inhibits the incorporation of leucine by post-microsomal pellet into a hot-PCA-stable form; this inhibition occurs whether the RNA is an amino acid acceptor type or not.

Returning to the investigation of the rapid decline in labelling of sRNA by post-microsomal pellet on prolonging

Table 15.

The Effect of the Addition of Microsomal RNA on the Incorporation of ^{14}C-leucine by Post-microsomal Pellet.

The conditions of incubation were as given for Fig. 19. 100 ug. microsomal RNA (mRNA) or sRNA was added where noted.

ATP	10 min. incubation			2 hour incubation		
	Total cts./min./mg. protein.			TCA stable cts./min./mg. protein		
	No added RNA	+ sRNA	+ mRNA	No added RNA	+ sRNA	+ mRNA
-	12	15	14	43	92	58
+	201	876	255	347	122	134

incubation, it seemed desirable to ensure that this effect was confined to the post-microsomal fraction and was not also a feature of the cell sap activation system. An identical time course experiment, using cell sap activating enzymes instead of post-microsomal pellet, was, therefore, carried out (Fig. 21). In this case, there is a similar initial rapid labelling of the sRNA but a plateau is reached after 10 minutes which is maintained throughout the 120 minute period of incubation. The hot-PCA-stable activity is much lower than in the post-microsomal pellet and the inhibition of this small incorporation into protein by the presence of sRNA is not marked.

Can the decline in the transfer ability of leucine to sRNA by post-microsomal pellet be due to the presence of a ribonuclease in the post-microsomal pellet? Fig. 22 gives the result of an experiment devised to test this possibility. Post-microsomal pellet was incubated in the presence and absence of sRNA under the standard conditions of incubation for the times indicated. The RNA recovered at each time interval, both in the presence and absence of additional RNA is shown on the graph. Apparently, there is little breakdown of either the endogenous post-microsomal RNA or of the added sRNA. In fact, the amount of sRNA recovered at the end of the 2 hour period of incubation (calculated from the difference in the amounts of RNA recovered in the presence and absence of added sRNA) is identical to that present at the beginning of

Fig. 21.

The Time Course of the Incorporation of ^{14}C-leucine into sRNA by 3 Hour Cell Sap pH 5 Enzyme.

1 mg. pH 5 enzyme protein was incubated under the same conditions as those described in Fig. 20.

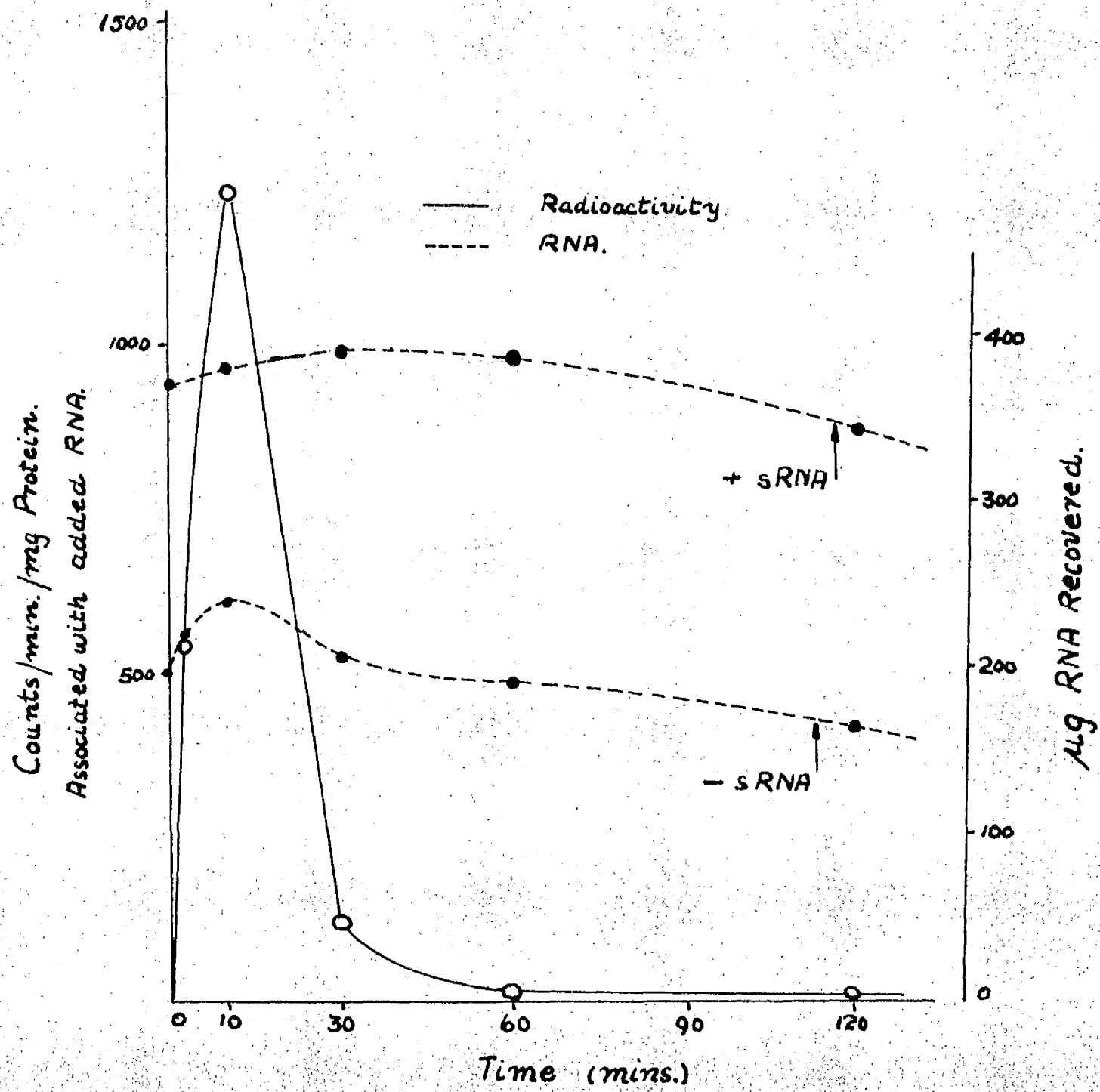
Fig. 21.

PCA Soluble
Counts / min / mg Protein
1500
1000
500
0
0
10
30
60
90
120
Time (mins)
PCA Stable
Counts / min / mg Protein
150
100
50
0
0
10
30
60
90
120
Time (mins)
Δ +ATP+sRNA
× +ATP
○ −ATP

Fig. 22.

The Relationship between the Rapid Decline in the Labelling of the sRNA and the Amount of RNA recovered.

The conditions of incubation were as given in Fig. 19. 200 ug. sRNA was used.



incubation. This finding presumably rules out the presence of a ribonuclease in post-microsomal pellet. Also, there is no correlation between the rapid fall in the activity associated with the RNA and the changes in the amount of RNA present at the various time intervals. Between 10 and 30 minutes there is a fall in the acceptor activity of the RNA of about 40% whereas there is no change in the amount of RNA present. However, although there are no gross changes in the amount of RNA present, removal of only the terminal trinucleotide would inactivate the RNA without necessarily being detectable by the amount of RNA present.

The degradative effect of post-microsomal pellet on sRNA was tested by preincubating post-microsomal pellet with sRNA for 110 minutes before the addition of ^{14}C-leucine. Control samples were incubated for 10 minutes and 120 minutes without any preincubation to ensure that (a) the sRNA preparation was initially active and that (b) the rapid decline in counts after 110 minutes incubation occurred, as usual. In such an experiment, if post-microsomal pellet is inactivating the added sRNA, at no time will there be any transfer of amino acid to sRNA after the initial period of preincubation. Table 16 gives the result of such an experiment. As expected, in the control samples incubated for only 10 minutes there is a high count in the hot-PCA-soluble fraction (RNA) in the presence of sRNA. Also, after 110 minutes incubation this label is lost, although the actual amount of RNA present has not

Table 16.

The Addition of ^{14}C-leucine to Post-microsomal pellet after Preincubation with sRNA for 110 minutes.

1 mg. post-microsomal protein or 3 hour cell sap pH 5 fraction protein was preincubated for 110 minutes with 1 umole ATP in the presence and absence of 200 ug. sRNA at 37°C in a total volume of 1 ml. Gempbell buffer. 1 uC ^{14}C-DL-leucine was then added and incubation contonued for a further 10 minutes.

Conditions during Preincubation	Conditions during Incubation	Post-microsomal Pellet			pH 5 Fraction		
		PCA sol cts./min./mg. protein	PCA stable cts./min./mg. protein	ug. RNA recovered	PCA sol cts./min./mg. protein	PCA stable cts./min./mg. protein	ug. RNA recovered
-	+ ATP	203	64	261	447	7	45
-	+ ATP + sRNA	1310	46	454	1959	16	232
+ ATP	+ ^{14}C-DL-leucine	18	10	172	388	16	42
+ ATP + sRNA		27	7	359	959	16	224

decreased to an appreciable extent. However, preincubation for 120 minutes before the addition of the ^{14}C-leucine led to the complete removal of the acceptor ability of the sRNA; again, the actual amount of RNA present was only slightly reduced. This effect is, presumably, due to the presence of an enzyme peculiar to post-microsomal pellet, since in a control experiment carried out in an identical manner but using cell sap pH 5 enzyme, only a slight loss in activity of the sRNA was noted (Table 16).

If post-microsomal pellet is inactivating sRNA by breaking down the terminal acceptor trinucleotide of the molecule, the addition of cytidine triphosphate (CTP) after preincubation should alter the equilibrium of the reaction in the direction of reformation of the terminal trinucleotide. An experiment was therefore carried out in which sRNA was preincubated with post-microsomal pellet for 30 minutes before the addition of ^{14}C-leucine and CTP. ATP was also added to ensure that an adequate supply was present. After these additions incubation was carried on for a further 10 minutes. Table 17 gives the result of this experiment. The experiment is not entirely satisfactory as the activity of the sRNA has not been completely inhibited by preincubation with post-microsomal pellet for 30 minutes. However, it has been decreased to some extent and the addition of CTP after pre-incubation does reconstitute the activity of the sRNA, the transfer obtained reaching the same level as in a sample which

Table 17.

The Effect of the Addition of CTP after Preincubation of sRNA with Post-microsomal Pellet.

1 mg. post-microsomal protein was incubated in the presence of 1 umole ATP and 100 ug. sRNA in a total volume of 1 ml. Campbell buffer at 37°C for 30 mins. The samples were chilled and 0.5 umoles CTP, 1 uC ^{14}C-leucine and a further 1 umole ATP were added where noted. The samples were then incubated for a further 10 mins. at 37°C.

The values obtained from duplicate samples are shown.

Duration of Preincub-ation mins.	Additions before Preincub-ation	Additions before Incubation	Duration of Incubation mins.	Total cts./ min./mg. Protein		
				Tube 1	Tube 2	Mean
-	-	ATP CTP ^{14}C-leucine	10	122	127	125
-	-	ATP sRNA ^{14}C-leucine	10	387	397	392
30	ATP sRNA	ATP ^{14}C-leucine	10	249	314	282
30	ATP sRNA	ATP CTP ^{14}C-leucine	10	370	370	370

was not preincubated. We may conclude, therefore, that post-microsomal pellet does contain an enzyme which is capable of inactivating sRNA by degrading the terminal acceptor tri-nucleotide. The reaction is reversible, however, the addition of CTP and ATP causing the reconstitution of the sRNA.

As a control for these preincubation experiments, an experiment was carried out in which sRNA was added after a period of preincubation. As would be expected, this sRNA can accept amino acids just as efficiently as a non-preincubated sample (Table 18). This finding validates the previous assumption that the effects of preincubation were due to the action of post-microsomal pellet on sRNA and not to any other effect on the system.

The above findings on the inactivation of sRNA by post-microsomal pellet can be summarised as follows:-

1. There is a rapid fall in the labelling of the sRNA after 10 minutes of incubation, although there is no commensurate fall in the amount of RNA present.
2. After a period of preincubation with post-microsomal pellet, sRNA loses its activity to accept amino acids.
3. The presence of CTP in the incubation medium after pre-incubation of sRNA with post-microsomal pellet reactivates the sRNA.

An explanation consonant with these findings is that post-microsomal pellet, although not able to degrade sRNA to a form which is not acid precipitable, does possess an

Table 18.

The Addition of sRNA to Post-microsomal Pellet after a Period of Preincubation.

1 mg. post-microsomal protein was incubated with 1 umole ATP, 1 uC ^{14}C-DL-leucine with or without 100 ug. sRNA for the times noted. In the preincubated samples, after incubation for 110 mins., 1 umole ATP and 100 ug sRNA were added where noted and incubation continued for a further 10 mins.

Duration of Preincub-ation mins.	Additions before Preincub-ation	Additions before Incubation	Duration of Incubation mins.	PCA soluble cts./ min./mg. Protein	PCA stable cts./ min./mg. Protein	ug. RNA
-	-	ATP	10	111	21	139
		ATP sRNA		407	23	238
		ATP	110	70	238	112
		ATP sRNA		81	83	178
110	ATP	H_2O	10	30	220	99
		sRNA	10	168	206	156
		sRNA 'cold' leucine	10	47	216	161

The results are a mean of two experiments.

enzyme or enzymes which can inactivate the molecule by removal of the terminal acceptor trinucleotide. The action of this enzyme would appear to be reversed by the addition of CTP.

3. Transfer of Post-microsomal-bound Amino Acids to sRNA.

As previously described, the presence of sRNA severely inhibits the incorporation of ^{14}C-leucine into the PCA-stable fraction of post-microsomal pellet. Can this be explained by a mechanism involving the removal of ^{14}C-leucine, already incorporated into post-microsomal pellet protein, by the sRNA? The previous evidence (See page 94.) indicated that this did not happen; the following experiment was carried out to resolve the question finally. After preincubation of post-microsomal pellet with ^{14}C-leucine for 110 minutes, one can anticipate that the protein portion of the post-microsomal pellet will be highly labelled. If a large excess of non-radioactive leucine is then added (to prevent further uptake of ^{14}C-leucine directly from the free leucine present in the incubation medium) along with sRNA, any labelling of the sRNA which occurs must be due to a specific transfer of ^{14}C-leucine from the post-microsomal protein. The results of such an experiment are included in Table 18, as the experiment presented there was carried out, using the same preparations; the same controls, therefore, serve for both experiments. As can be seen in the lower part of the Table, after 110

minutes of preincubation the protein (PCA-stable) portion of post-microsomal pellet is, indeed, highly labelled but no transfer of this label to sRNA occurs on the addition of sRNA with nonactive leucine in large excess. Obviously, therefore, post-microsomal protein cannot transfer ^{14}C-leucine directly to sRNA, as previously surmised. This is consonant with the finding (Table 15) that RNA species other than cell sap RNA depress the incorporation into post-microsomal protein when added at the beginning of incubation with ^{14}C-leucine.

4. Spectrum of Transfer of Various Amino Acids to sRNA by Post-microsomal Pellet.

In Part I, it was suggested that there are three systems present in post-microsomal pellet, one being the activating enzyme ability, one an energy-dependent incorporation of amino acids and the third an energy-independent incorporation. Lysine was an amino acid which could be activated by the post-microsomal pellet enzymes and which was incorporated into the protein fraction by a non-ATP-dependent mechanism. The relationship of this incorporation to the activating enzyme ability has been investigated. If there is a link between these two pathways, post-microsomal pellet enzymes will be able to transfer lysine to sRNA in the absence of ATP. Table 19 shows that this is not the case. Although the amino acid is incorporated by a non-ATP-dependent process in both post-microsomal pellet and cell sap, the transfer of the amino acid to sRNA is completely dependent on the presence of ATP.

Table 19.

The Effect of the Presence of sRNA on the Incorporation of Leucine and Lysine by Post-microsomal Pellet and pH 5 Enzyme from 3 Hour Cell Sap.

1 mg. aliquots of post-microsomal or pH5 enzyme protein were incubated for 10 mins. at 37°C with 1 umole ATP, 50 ug. sRNA (where noted) and 1 uC ^{14}C-DL-leucine or 1 uC ^{14}C-L-lysine in a total volume of 1 ml. Campbell buffer. The results are expressed as total counts incorporated (in mumoles) per min. per mg. protein.

Amino Acid used	Conditions during Incubation	mumoles incorporated/min./mg. Protein	
		Post-microsomal Pellet	pH 5 Enzyme
Leucine	- ATP	0.05	0.05
	+ ATP	1.5	0.5
	- ATP + sRNA	0.1	0.01
	+ ATP + sRNA	1.9	1.3
Lysine	- ATP	1.1	0.5
	+ ATP	1.7	1.1
	- ATP + sRNA	0.8	0.6
	+ ATP + sRNA	2.5	1.4

Table 20 gives data obtained in a similar experiment using glutamic acid and glycine. It has previously been shown that no activating enzymes detectable by ^{32}PP-ATP exchange exist in post-microsomal pellet for these amino acids (Table 10). However, the results presented in Table 20 show that both of these amino acids can be transferred to sRNA by post-microsomal pellet enzymes. Glycine, like lysine, is incorporated into post-microsomal protein by a non-ATP-dependent reaction. Here again, however, the transfer to sRNA is energy-dependent. It can be seen, that even after 10 minutes, and even more so after 120 minutes of incubation, the presence of sRNA inhibits this non-ATP-dependent incorporation just as it has previously been shown to inhibit energy-dependent incorporation of leucine.(See page 94.)

General Conclusions.

The conclusions reached on the ability of post-microsomal activating enzymes to transfer amino acids to sRNA can be summarised as follows:-

1. Post-microsomal pellet contains no detectable RNA which is able to accept activated amino acids of the sRNA type.
2. When sRNA is added to a post-microsomal pellet preparation, this sRNA can receive amino acids, activated by the activating enzymes present in the post-microsomal pellet.
3. On prolonged incubation post-microsomal pellet inactivates sRNA, probably, by interfering with the acceptor trinucleotide.

Table 20.

The Transfer of ^{14}C-Leucine, Glutamic acid and Glycine to sRNA by Post-microsomal Pellet and its Effects on the Incorporation of Glycine into Post-microsomal Protein.

The conditions of incubation were as given for Table 12, 50 ug. sRNA being added where noted.

Amino Acid used.	ATP	mumoles incorporated/min./mg. protein x 10^{-2}			
		10 min. Incubation			
		Hot PCA soluble		Hot PCA stable	
		+ sRNA	- sRNA	+ sRNA	- sRNA
Leucine	+	217	32	0	19
Glutamic Acid	+	216	63	26	2
Glycine	+	174	57	20	34
	-	45	84	20	31
		120 min. Incubation			
Glycine	+	21	47	24	53
	-	51	65	20	48

4. Amino acids, for which no amino acid activating enzymes have been demonstrated in post-microsomal pellet, can be transferred to sRNA by post-microsomal pellet enzymes.

5. Amino acids which can be incorporated into post-microsomal protein by a non-ATP-dependent mechanism, nevertheless require the presence of ATP for their transfer to sRNA by post-microsomal pellet activating enzymes.

6. The presence of sRNA, or indeed any RNA, whether it is an amino acid acceptor or not, reduces the incorporation of amino acids into the protein fraction of the post-microsomal pellet. This depression occurs whether the type of amino acid incorporation is ATP-dependent or not.

7. When an amino acid is once incorporated into post-microsomal protein, it cannot subsequently be transferred directly to sRNA.

Part 3.

The Capacity of Post-microsomal Pellet to Catalyse the Incorporation of ^{14}C-leucine into Ribosomal Protein.

Post-microsomal pellet can activate amino acids and transfer them to sRNA as shown in Parts 1 and 2 of this section. Can it complete the process of protein "synthesis" and transfer the amino acids to ribosomal protein?

As post-microsomal pellet contains more activating enzymes than cell sap pH 5 enzyme on the basis of equal protein content, it was first necessary to investigate the question - "Is the incorporation of leucine into ribosomal protein critically influenced by the level of the activating enzymes present?" This problem was first investigated by adding increasing amounts of a pH 5 enzyme preparation to a ribosomal incorporating system. The results of this experiment are given in Fig. 23. The incorporation of ^{14}C-leucine into ribosomes obviously does not vary proportionately with the amount of pH 5 enzyme added, indeed, above about 2 mgs. of protein there is a marked decrease in the incorporation. This decline is probably due to a dilution of the ^{14}C-leucine by the amino acids already attached to the sRNA of the pH 5 enzyme preparation rather than to an inhibitory effect on the activating enzymes. When post-microsomal pellet is used, this complication does not occur, however, as this fraction does not contain any active sRNA.

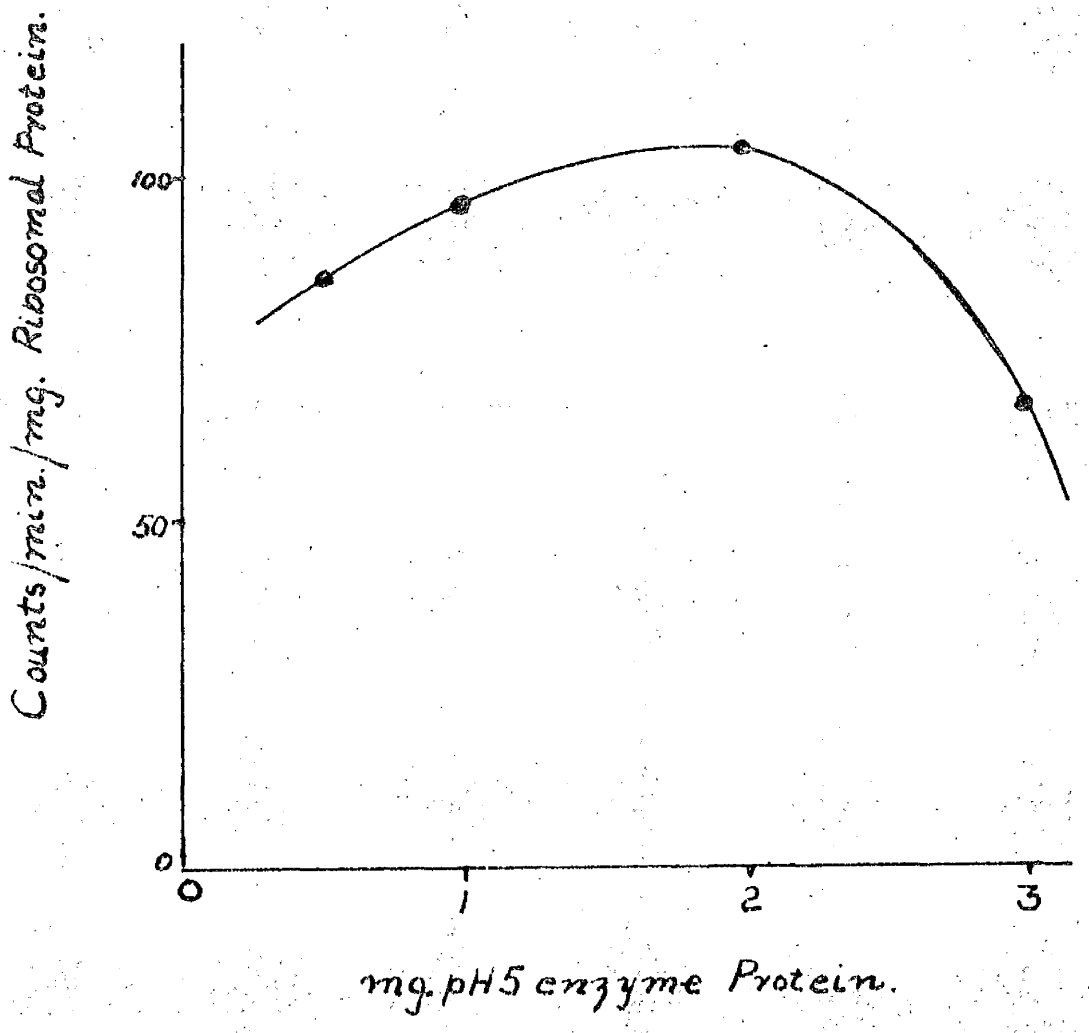
The next experiment carried out was one in which the

Fig. 23.

1.5 mg. ribosomal protein was incubated with 5 umoles ATP, 0.25 umoles GTP and 0.72 uC ^{14}C-DL-leucine for 20 minutes at 37°C. The amounts of 3 hour cell sap pH 5 enzyme noted in the Fig. were also added. The total incubation mixture was in 1 ml. Medium A with the Mg^{++} ion concentration boosted to 9 umoles. The results are expressed as cts./min./mg. ribosomal protein minus the count incorporated into the pH 5 enzyme when it was incubated in the absence of added ribosomes.

Fig. 23.

The Effect of Varying the Amount of pH 5 Enzyme Present in the Ribosome Incorporating System.



effect of replacing pH 5 enzyme with post-microsomal pellet in a ribosomal incorporating system was investigated. Ribosomes were incubated with (a) pH 5 enzyme (b) post-microsomal pellet and (c) a combination of both these fractions. The effect of the addition of sRNA to the ribosome-post-microsomal system was also explored. To circumvent any difficulties due to concentration effects, various concentrations of post-microsomal pellet and pH 5 enzyme were used.

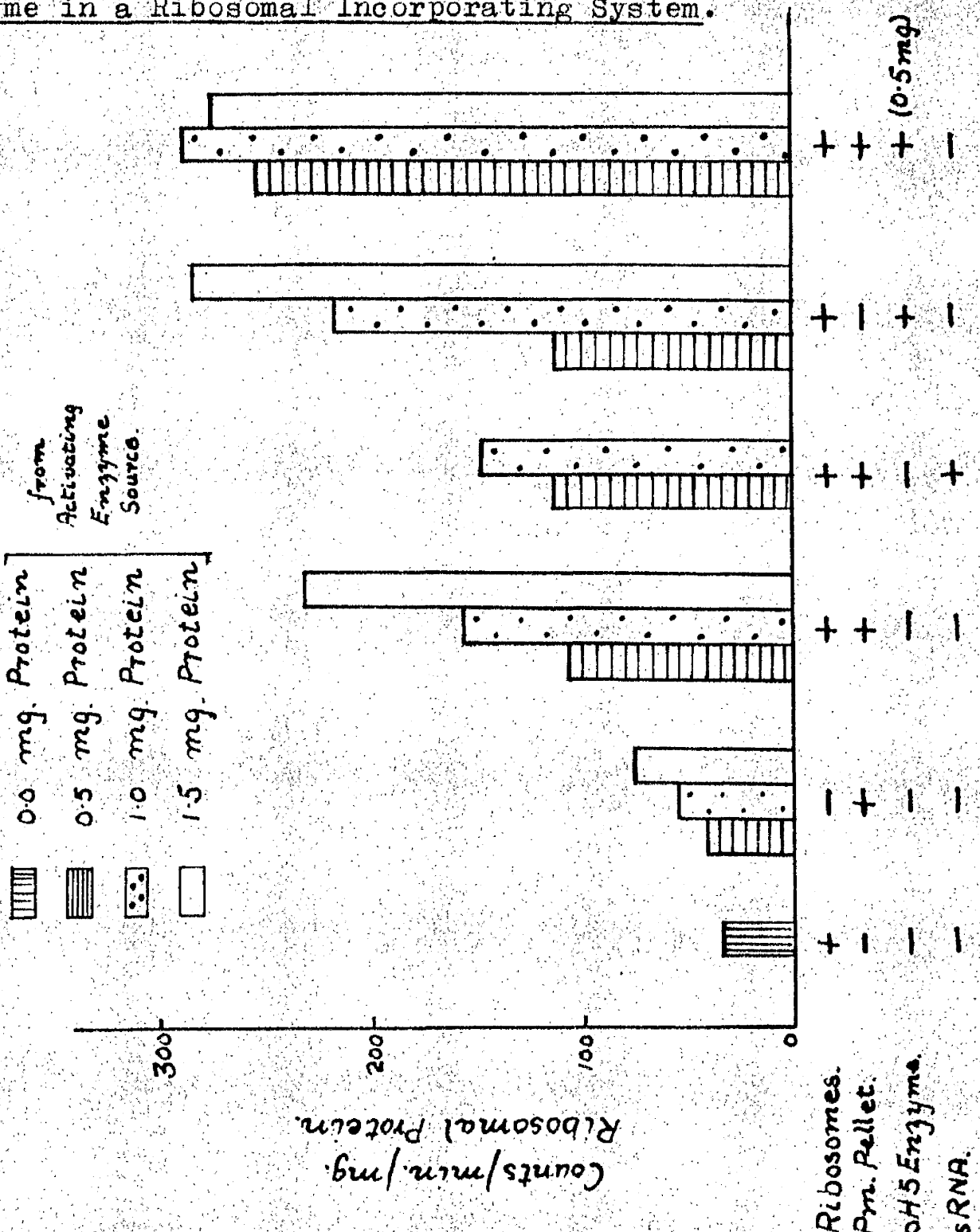
The results obtained in this experiment are given in Fig. 24. Post-microsomal pellet can obviously catalyse the incorporation of ^{14}C-leucine into ribosomal protein, although not so efficiently as pH 5 enzyme, when expressed per mg. of protein of the activating enzyme source. This difference cannot be explained by the fact that post-microsomal pellet does not contain any sRNA, as the addition of sRNA prepared from 3 hour cell sap by phenol extraction does not stimulate the incorporation of leucine into ribosomal protein by post-microsomal pellet. The results obtained when post-microsomal pellet and pH 5 enzyme from cell sap are both added to the system are interesting. When the sample contained 0.5 mg. pH 5 enzyme protein and 0.5 mg. post-microsomal protein, the counts obtained were very similar to those obtained for 1 mg. pH 5 enzyme protein (253 cts./min./ mg. as compared with 220 cts./min./mg.). This slight difference can be explained by the fact that post-microsomal pellet incorporates some ^{14}C-leucine into its own protein under these

Fig. 24.

2 mg. ribosomal protein was incubated with the amounts of post-microsomal or 3 hour cell sap pH 5 enzyme protein indicated in the Fig. In some samples both post-microsomal and pH 5 enzyme protein were added. 5 umoles ATP, 0.25 umoles GTP, 0.72 uC ^{14}C-DL-leucine and, in some cases, 30 ug. sRNA were added and incubation was allowed to proceed for 20 minutes at 37°C. The results are expressed as cts./min./mg. ribosomal protein in all cases including those samples which were incubated without the addition of ribosomes.

Fig. 24.

The Capacity of Post-microsomal Pellet to Substitute for pH 5 Enzyme in a Ribosomal Incorporating System.



conditions. The picture obtained when 1.5 mg. of pH 5 enzyme protein is compared with a mixture of 1.0 mg. post-microsomal protein and 0.5 mg. pH 5 enzyme protein is essentially similar. However, when 1.5 mg. post-microsomal protein and 0.5 mg. pH 5 enzyme are used, the activity obtained is slightly less than that in the previous case i.e. a mixture of 1.0 mg. post-microsomal protein and 0.5 mg. pH 5 enzyme. This suggests that the system is saturated with activating enzymes when 1.0 mg. post-microsomal protein and 0.5 mg. pH 5 enzyme are present and, consequently, the addition of a further 0.5 mg. post-microsomal protein has no effect. These findings indicate that the activating enzymes are not the limiting part of the incorporating system.

From these observations, it is evident that post-microsomal pellet can replace cell sap in the ribosome incorporating system. Are the post-microsomal pellet-ribosome system and the pH 5 enzyme-ribosome system identical in time course and in other respects?

Post-microsomal pellet can incorporate amino acids into its own protein for a period of at least two hours, but its ability to transfer leucine to sRNA is lost after only 10 minutes of incubation, probably due to breakdown of the sRNA by post-microsomal enzymes. In the pH 5 enzyme - ribosome system after 10 minutes of incubation the rate of incorporation of leucine into ribosomes decreases rapidly

(Fig. 25) and after 60 minutes the activity of the ribosomal protein may even decrease slightly. Does the time course of the transfer of amino acids to ribosomes by post-microsomal pellet follow this pattern? Aliquots of ribosomal protein were incubated with pH 5 enzyme or post-microsomal pellet for various times and the radioactivity of the ribosomal protein determined (Table 21). Control samples in which each fraction was incubated alone were also included. In the samples incubated for 20 minutes, pH 5 enzyme and post-microsomal pellet have the same capacity to transfer leucine to ribosomes. In the samples incubated for 40 minutes, however, some of the label seems to be lost from the ribosomal protein which was incubated with post-microsomal pellet. This may again be due to degradation by enzymes present in post-microsomal pellet.

If the post-microsomal pellet contains any factors not present in cell sap it would be expected that, after the incorporation plateau has been attained with a cell sap-ribosome system, the addition of post-microsomal pellet could cause a further stimulation of incorporation. Therefore, the effects of the addition of various factors after a preliminary period of incubation were explored (Fig. 26). The standard ribosome-pH 5 enzyme system was incubated for 20 minutes followed by the addition of (a) more ribosomes (b) pH 5 enzyme or (c) post-microsomal pellet as indicated as Fig. 26. Additional ATP and GTP were present in every case to ensure

Fig. 25.

The incubation mixture contained 17 mg. ribosomal protein, 10 mg. pH 5 enzyme from 3 hour cell sap protein, 30 umoles ATP, 1.5 umoles and 4.32 uC ^{14}C-DL-leucine in a total volume of 6 ml. Medium A. 0.4 ml. samples were removed at the times shown and treated in the usual way. The results are expressed as cts./min./mg. protein (ribosomal).

Fig. 25.

The Time Course of the Incorporation of ^{14}C-leucine into Ribosomal Protein Catalysed by pH 5 Enzyme from 3 Hour Cell Sap.

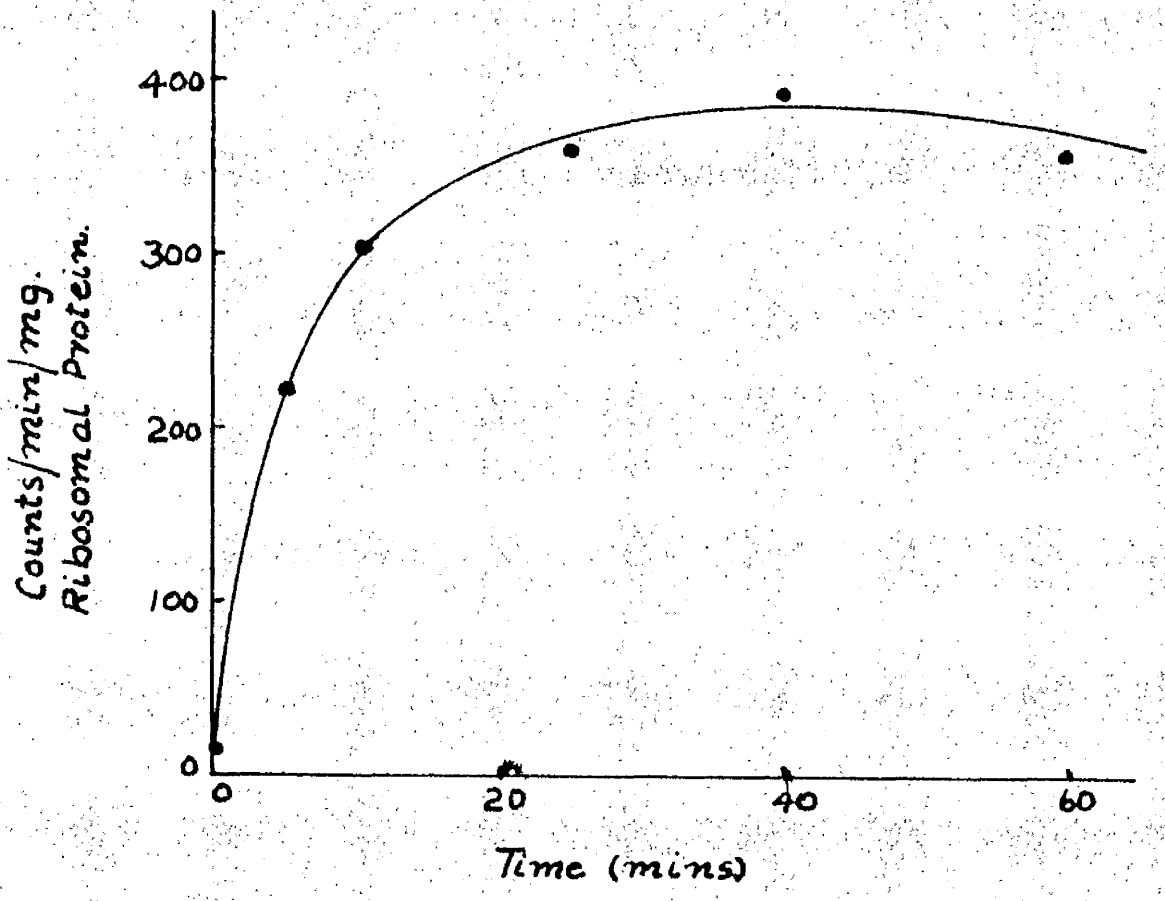


Table 21.

Time Course of the Incorporation of ^{14}C-leucine into Ribosomes by pH 5 Enzyme and Post-microsomal Pellet.

0.78 mg. of ribosomal protein was incubated at 37°C in the presence or absence of 1 mg. post-microsomal protein or 0.5 mg. pH 5 enzyme protein from 3 hour cell sap, as indicated. Incubation was continued for 20 minutes or 40 minutes in the presence of 5 umoles ATP, 0.25 umole GTP and 0.72 uC ^{14}C-DL-leucine.

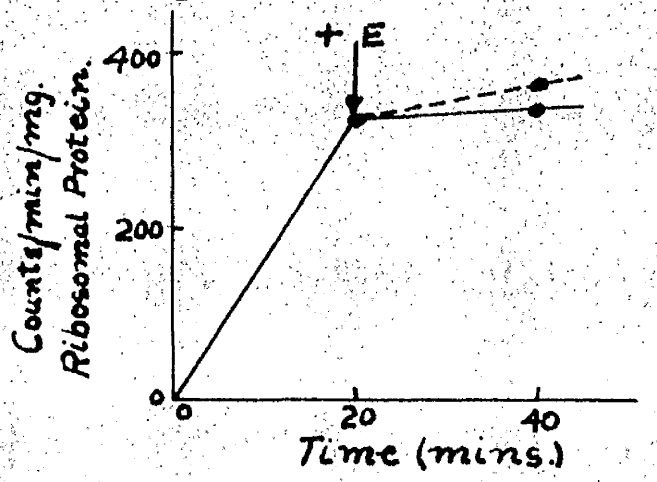
Ribosomes	pH 5 Enzyme	Post-microsomal pellet	Duration of Incubation mins.	Cts./min./mg. Ribosomal protein
+	-	-	20	41
-	+	-	20	31
-	-	+	20	48
+	+	-	20	320
	+	-	40	330
+	-	+	20	318
	-	+	40	256
	-	+ + sRNA	20	281

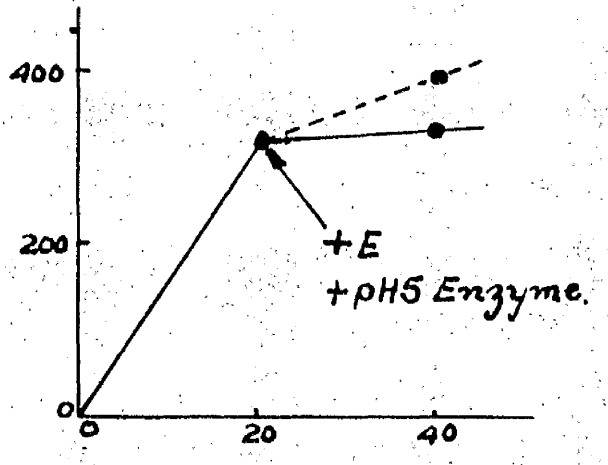
Fig. 26.

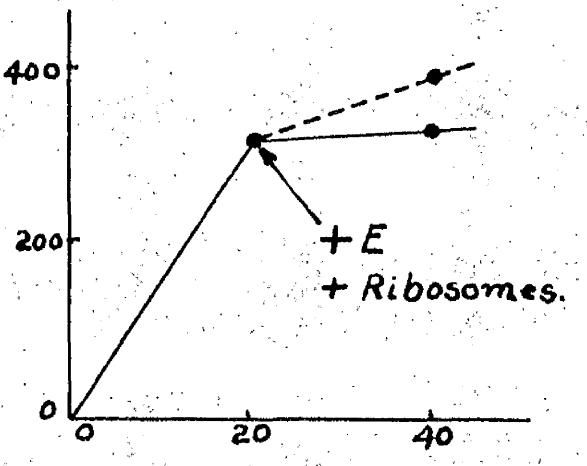
The Effect of the Addition of Various Factors to a Ribosome Incorporating System after an Initial Period of Incubation.

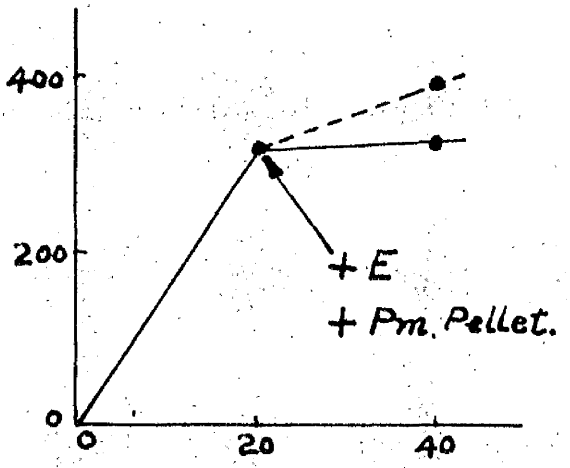
The conditions used in the initial period of incubation were identical to those used in Table 21, the ribosome- pH 5 enzyme system being used. After 20 minutes of incubation, the samples were chilled and the additions shown in the Fig. made. E stands for 5 umoles ATP + 0.25 umoles GTP. The amounts of ribosomes, pH 5 enzyme and post-microsomal pellet added were the same as added to the initial incubation system. 30 ug. sRNA was added where noted. Incubation was continued for a further 20 minutes after these additions were made. In the Fig. duration of incubation is plotted against cts./min./mg. ribosomal protein. The curve obtained in the presence of these additions (dotted line) is superimposed on the curve obtained in their absence (continuous line).

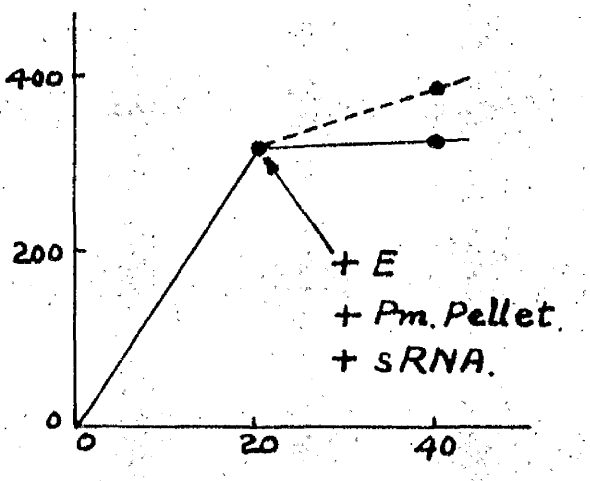
Fig. 26.

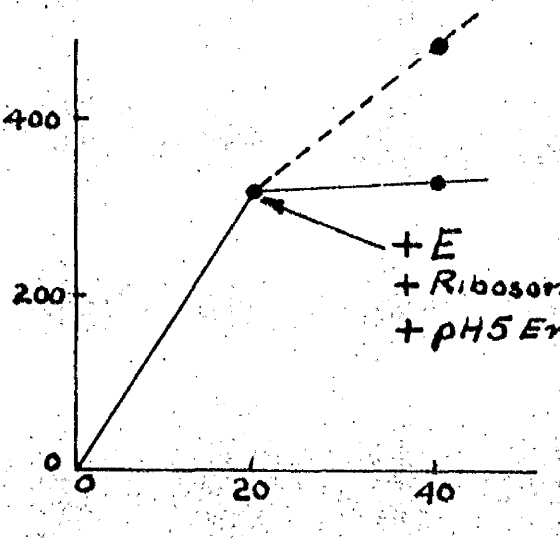

+ E
Counts/min/mg. Ribosomal Protein.
400
200
0
0
20
40
Time (mins.)


400
200
0
+ E
+ pH5 Enzyme.
0
20
40


400
200
0
+ E
+ Ribosomes.
0
20
40


400
200
0
+ E
+ Pm. Pellet.
0
20
40


400
200
0
+ E
+ Pm. Pellet.
+ sRNA.
0
20
40


400
200
0
+ E
+ Ribosomes.
+ pH5 Enzyme.
0
20
40

the energy requirement was not the limiting factor for the incorporation. Incubation for a further period of 20 minutes followed these additions. In such an experiment, the addition of only ATP and GTP does not affect the incorporating system, the activity obtained being the same as in a sample incubated for 40 minutes without any additions. The addition of ribosomes, pH 5 enzyme or post-microsomal pellet (with or without sRNA) during incubation does not cause any stimulation of activity greater than that attributable to the endogenous incorporation into the protein of the fraction. However, when both ribosomes and pH 5 enzyme are added together, there is a stimulation of the total activity of the ribosomal protein of about 50%.

From these results it can be deduced that post-microsomal pellet does not have any capacity in the ribosome incorporating system that cell sap does not possess. We may also conclude that the decline in the rate of incorporation of leucine into ribosomes after a prolonged period of incubation is not due to an effect on either the ribosomes of the activating enzymes as the addition of these factors alone, produces no effect on the incorporation. Rather, some inactivation of both fractions must occur, possibly due to the production of some inhibitor. The fact that the addition of fresh ribosomes and activating enzymes together after the initial incubation period does not reconstitute the system to its original rate gives some confirmation of this theory.

Conclusions.

Post-microsomal pellet does appear to be able to replace pH 5 enzyme from cell sap in a ribosomal incorporating system. The addition of exogenous sRNA is not required in this system, probably indicating that there is sufficient sRNA released from the ribosome preparation to catalyse the transfer of amino acids to ribosomal protein.

Although post-microsomal pellet has a greater activating enzyme content than pH 5 enzyme per mg. protein, its capacity to transfer aminoacids to ribosomes is slightly inferior to that of an equal quantity of pH 5 enzyme protein. This indicates that the activation of the amino acid cannot be the rate-limiting step of the incorporation reaction and that some factor necessary for maximum incorporation of leucine into ribosomes may be absent from post-microsomal pellet. Alternatively, there may be some factor present in post-microsomal pellet which inhibits the incorporation system to a small extent. From our present knowledge, no conclusions can be reached concerning this problem, although the latter explanation seems the more probable.

SECTION XVI.

SECTION III.

Introduction.

Having investigated the involvement of post-microsomal pellet in the "classical" system of protein biosynthesis, the points at which post-microsomal pellet is at variance with this system were next explored.

In Section II, we showed that post-microsomal pellet contains activating enzymes which can replace pH 5 enzyme in the "classical" system, presumably receiving an adequate supply of sRNA from that adhering to the ribosome preparation. However, this is only one facet of the activity of post-microsomal pellet and appears to be quite independent of the other systems of amino acid incorporation into the post-microsomal pellet itself. Unlike pH 5 enzyme, which post-microsomal pellet replaces in the "classical" system, post-microsomal pellet can incorporate ^{14}C-leucine into its own protein possibly by two different mechanisms, one involving ATP as an energy source, the other apparently independent of the presence of an energy source. In this section, we will consider the nature of these incorporation processes and also some attempts to separate the various activities of post-microsomal pellet. The general characteristics of the ATP-dependent-system have been discussed in the introduction to this thesis and were :-

(a) The incorporation continues for at least 2 hours of incubation.

(b) The incorporating system has an optimum pH of 7.6 to 7.9.

(c) The incorporation is insensitive to ribonuclease.

(d) Preincubation increases the capacity of post-microsomal pellet to incorporate amino acids.

Experimental Methods.

The only additional methods used in this Section are the following:-

Preparation of Post-microsomal Protein Labelled with ^{14}C-leucine for the Preparation of Dinitrophenol Derivatives.

About 15 mg. of post-microsomal protein were incubated for 2 hours at 37°C with 10 umoles ATP and 10 uC ^{14}C-DL-leucine in 10 ml. Campbell buffer. The reaction was stopped by the addition of 10 ml. 0.6N PCA at 0°C, and the precipitate formed was separated and washed four times with 10 ml. 0.3N PCA. The RNA was extracted by hydrolysis in 3 ml. 0.3N KOH at 37°C for 1 hour. The tubes were chilled and the protein reprecipitated by the addition of 6 ml. 0.6N PCA giving a final PCA concentration of 0.3N. The precipitate was washed twice with 5 ml. 0.3N PCA and dissolved in 2 ml. N NaOH.

Preparation of Dinitrophenol Derivatives.

The method used was that of Rhinesmith et al., (1956).

The pH of the solution of the protein obtained as above was adjusted to $\leqslant 9$ by the addition of N HCl. The solution was washed into the cell of a radiometer titrigraph with distilled water to a total volume of about 10 ml. and the pH brought to exactly 9 by titration with N NaOH. Once the pH had stabilised, 50 ul. fluorodinitrobenzene (B.D.H.) was added and the reaction was allowed to proceed at 37°C

for 2½ to 3 hours, the pH being kept constant at 9 by titration with N NaOH. The reaction mixture was then removed from the radiometer cell, chilled and the protein precipitated by the addition of 1 volume of 0.6N PCA. The precipitate of dinitrophenyl (DNP) protein obtained was separated by centrifugation and washed four times with 0.1N HCl to remove mineral salts, and once with ethanol and three times with ether (peroxide free) to remove any excess fluorodinitrobenzene or dinitrophenol formed during the reaction. The final product was a dry, yellow powder of DNP protein.

Hydrolysis of DNP Protein and Separation of DNP Amino Acids.

The dry powder obtained as above was transferred to a hydrolysis tube. The last few grains were transferred in 1 ml. 6N HCl (Made with glass distilled water) and the tube was sealed. The protein was hydrolysed by heating at 110°C for 18 hours in a metal bomb tube.

The hydrolysate so formed was made up to 3 ml. with distilled water, and 2 ml. of this was used to separate the DNP amino acids. The amino acids which had reacted with the fluorodinitrobenzene were the original N-terminal amino acidsof the protein chain. They were separated from the non-terminal amino acids by extracting four times with 4 ml. of ether. Each extract was washed separately with 1 ml. of water to remove any residual salt, which would interfere with the subsequent counting of the sample. The same 1 ml. of water was used repeatedly, as it quickly became saturated with DNP amino

acids, some of which are water soluble, thus giving a minimal loss of material. The ether extracts were evaporated to about 1 ml. and the DNP amino acids were extracted from this into 1 ml. N NaOH.

Aliquots of the total hydrolysate, the DNP amino acids and the residual material left after extraction of the DNP amino acids were plated onto lens paper discs and counted as described in Section II. Of the total leucine incorporated by post-microsomal protein, the percentage present in the N-terminal position of the protein could be determined by this technique.

Results.

The Site of Incorporation of ^{14}C-leucine.

The initial experiment carried out in this study was an investigation of the type of reaction involved in the labelling of post-microsomal protein when incubated with ^{14}C-leucine and an energy source. If leucine is being attached to the protein, purely in a terminal position, as for example by an exchange reaction, incubation in the presence of "cold" leucine after an initial period of incubation with ^{14}C-leucine will cause a rapid decline in the labelling of the protein due to replacement of the ^{14}C-leucine by non-radioactive leucine.

Post-microsomal pellet was incubated with ATP and ^{14}C-leucine. A twenty-fold excess of "cold" leucine was added to one sample after 1 hour of incubation and to an identical sample after 2 hours of incubation; thereafter, both samples were incubated for a further hour. Fig. 27 gives the result of this experiment. From this, it is clear that the addition of "cold" leucine and incubation for a further hour causes a reduction in the labelling of the post-microsomal protein by about 30%. As suggested above, this indicates that at least part of the leucine incorporated is located in a terminal position.

We, therefore, proceeded to investigate by chemical means the proportion of the leucine incorporated into the terminal positions of the protein chain. The N-terminal end was studied. Post-microsomal pellet was incubated with

Fig. 27.

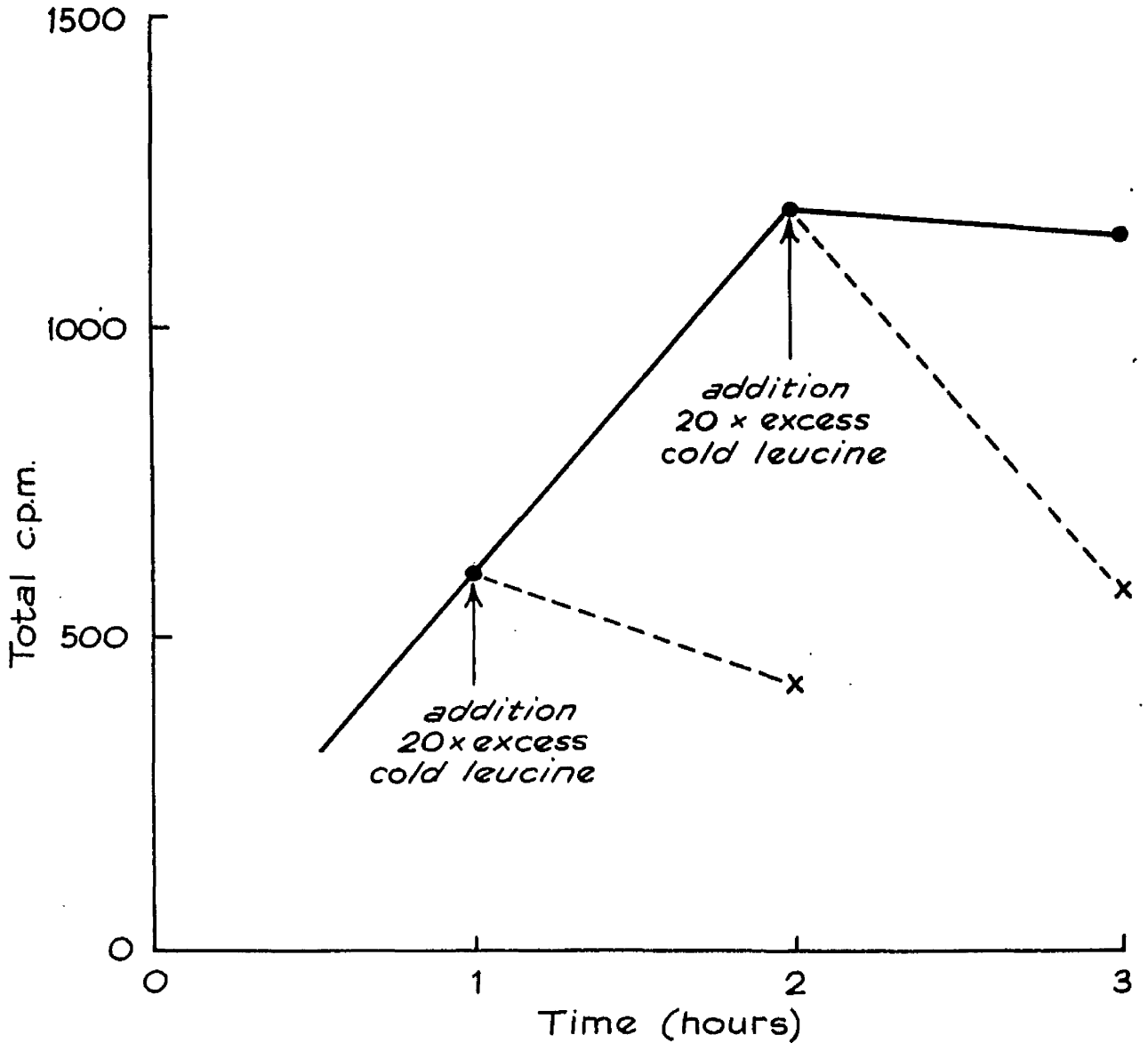
1 mg. post-microsomal protein was incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine in 1 ml. Campbell buffer for 3 hours at 37^{o}C. A twenty-fold excess of ^{12}C-leucine was added where indicated and incubation was continued.

The broken line represents the activity remaining after the addition of the unlabelled leucine.

The continuous line represents leucine uptake without dilution.

Fig. 27.

The Effect of Isotope Dilution on Leucine Uptake by Post-microsomal Pellet.



^{14}C-leucine and ATP under the standard conditions of incubation. The protein was recovered by the usual procedure, except that the washes with lipid solvents were omitted in order to ensure that the protein could be redissolved in NaOH for treatment with fluorodinitrobenzene (FDNB). After incubation for about 3 hours with FDNB, the dinitrophenyl (DNP) protein formed was hydrolysed and the DNP amino acids which were, of course, N-terminal in position in the protein chain were separated from the other amino acids. As leucine was the only radioactive amino acid involved, the proportion of the total incorporated radioactivity in the N-terminal position represented the leucine appearing in this position. In the original incubation, some samples containing additional amino acids were included. It was hoped that this addition of amino acids would cause more of the leucine to be incorporated into an internal position in the protein chain. The results of this experiment are given in Table 22.

When incubation is carried out in the absence of added amino acids, only about 28% of the leucine is incorporated into an N-terminal position. The addition of a complete amino acid mixture markedly inhibits the total incorporation of ^{14}C-leucine. Contrary to expectation a greater percentage, about 43%, of this leucine was incorporated in an N-terminal position. This result is difficult to explain. The marked decrease in incorporation may be due to some kind of non-specific incorporation in which leucine is diluted by the presence of other

Table 22.

The Distribution of Leucine Incorporated into Post-microsomal Pellet.

About 15 mg. of post-microsomal protein was incubated with 10 umoles ATP and 10 uC ^{14}C-DL-leucine for 2 hours at 37^{o}C in 1 ml. Campbell buffer. To some samples, a complete amino acid mixture containing 1.5 umoles of each amino acid was also added.

In the percentage column, the duplicates from 2 experiments are given, to show the agreement from experiment to experiment.

	No Added Amino Acids		+ Added Amino Acids	
	Cts./min.	% Total cts./min.	Cts./min.	% Total cts./min.
Total Hydrolysate	3164	100	703	100
N-terminal Leucine	835	26,30 28	264	38,47 43
Internal and C-terminal Leucine	2043	60,64 62	335	54,32 43
% Total counts recovered		90		86

amino acids which serve the same purpose.

Thus, the previous finding that about 30% of the ^{14}C-leucine incorporated is removed on incubation with non-active leucine may be explained by the fact that about 28% of the leucine is attached to the N-terminal end of the protein chain.

The ATP-dependence of the Incorporating System of Post-microsomal Pellet.

In Section II, we showed that some amino acids, notably leucine and methionine, were incorporated into post-microsomal protein by a reaction which was dependent on the presence of ATP, whereas others, particularly lysine, seemed not to require the presence of ATP for incorporation.

We have since found that, by altering the medium used for the preparation of post-microsomal pellet, we can obtain a post-microsomal pellet which will incorporate ^{14}C-leucine by a reaction which is independent of the presence of ATP. Table 23 gives the result of such an experiment. Obviously, post-microsomal pellet prepared in the first three of these media incorporates leucine by a reaction which is dependent on the presence of ATP. However, that prepared in the fourth medium seems to incorporate leucine by a reaction which is independent of the presence of ATP. This effect seems to involve the ions present in the medium, but is somewhat complex. The salient points can be summarised as follows:- Post-

Table 23.

The Effect of the Preparation Medium on the Incorporation of ^{14}C-leucine by Post-microsomal Pellet.

1 mg. post-microsomal pellet protein prepared in the medium shown below was incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine for 2 hours at 37°C in a total volume of 1 ml. Campbell buffer with only 0.0125M KCl instead of the normal 0.025M KCl.

Preparation Medium	PCA-stable cts./min./mg. protein	
	+ ATP	- ATP
Sucrose/phosphate pH 7.8	715	110
Sucrose/phosphate + 0.001M $MgCl_2$ + 0.03M $KHCO_3$ pH 7.8	471	119
Sucrose/phosphate + 0.001M $MgCl_2$ + 0.03M $KHCO_3$ + 0.0125M KCl pH 7.8	752	134
Sucrose/phosphate + 0.001M $MgCl_2$ + 0.03M $KHCO_3$ + 0.025M KCl pH 7.8 i.e. Campbell buffer	557	518

microsomal pellet prepared in sucrose/phosphate, pH 7.8 with no ions added has a high capacity to incorporate ^{14}C-leucine by an ATP-dependent reaction. The presence of $MgCl_2$ and $KHCO_3$ causes the formation of a post-microsomal pellet with lower activity. However, when KCl (0.0125M) is added as well as $MgCl_2$ and $KHCO_3$, the post-microsomal pellet obtained has the same activity as that prepared in sucrose/phosphate, pH 7.8 with no added ions. But if the concentration of KCl in the preparation medium exceeds 0.0125M, the ATP-dependence of the system is lost. These findings are difficult to explain; Petermann and Hamilton (1961) have shown the existence of complex interactions between Mg^{++} ions, $KHCO_3$ and KCl and the stability and protein-binding capacity of ribonucleoprotein particles prepared from rat liver. The explanation of our findings may lie in such a field.

An alternative explanation of the high incorporation of ^{14}C-leucine in the absence of ATP might be that when post-microsomal pellet is prepared in a medium of high KCl concentration, there may be, by some unknown means, a retention of ATP in the pellet. To test this possibility post-microsomal pellet was prepared in Campbell buffer and dialysed overnight against 4 litres of sucrose/phosphate, pH 7.8. If there was any ATP present in the preparation, this treatment would be expected to reduce the level to a very low value and incorporation would thus become ATP-dependent. The results shown in Table 24 indicate that dialysis has no effect on the ATP-

Table 24.

The Effect of Dialysis on the Incorporating Capacity of Post-microsomal Pellet.

1 mg. post-microsomal protein prepared in Campbell buffer and dialysed overnight against 4 l. of sucrose/phosphate, pH 7.8 was incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine for 2 hours at 37^{o}C in a total volume of 1 ml. Campbell buffer. Undialysed post-microsomal pellet was used as a control.

	Total Cts./min./mg. Protein	
	Dialysed Post-microsomal pellet	Undialysed post-microsomal pellet
+ ATP	959	970
- ATP	934	915

dependence of the incorporating system.

Dialysis, possibly, does not remove all of the ATP present. Removal of the ATP by treatment with bacterial alkaline phosphatase was therefore attempted. This enzyme is known to hydrolyse ATP completely. The results of an experiment in which post-microsomal pellet was incubated in the presence and absence of ATP, with the addition of bacterial alkaline phosphatase, are given in Table 25. If the leucine incorporated in the absence of added ATP made use of endogenous ATP, the addition of bacterial alkaline phosphatase would be expected to suppress this activity completely. However, from Table 25, it can be seen that the incorporation in the presence and absence of alkaline phosphatase is identical when no ATP is added. When ATP is added to the incubation medium, the presence of alkaline phosphatase reduces the level of the activity to almost the same value as that obtained in the absence of ATP. This indicates that the alkaline phosphatase is effectively removing the ATP and hence the ATP-dependent incorporation but not the non-ATP-dependent incorporating activity.

Again, we can conclude from this experiment that there are two pathways of incorporation active in post-microsomal pellet, one involving an ATP-dependent reaction the other independent of the presence of ATP.

In Fig. 28 the time course of the incorporation in the presence and absence of ATP is presented. Over the 2 hour

Table 25.

The Effect of the Addition of Bacterial Alkaline Phosphatase to the Post-microsomal Pellet Incorporating System.

1 mg. post-microsomal protein prepared in Campbell buffer was incubated with 1 uC ^{14}C-DL-leucine in 1 ml. Campbell buffer for 2 hours at 37°C. 1 umole ATP and 10 ug. bacterial alkaline phosphatase were added where noted.

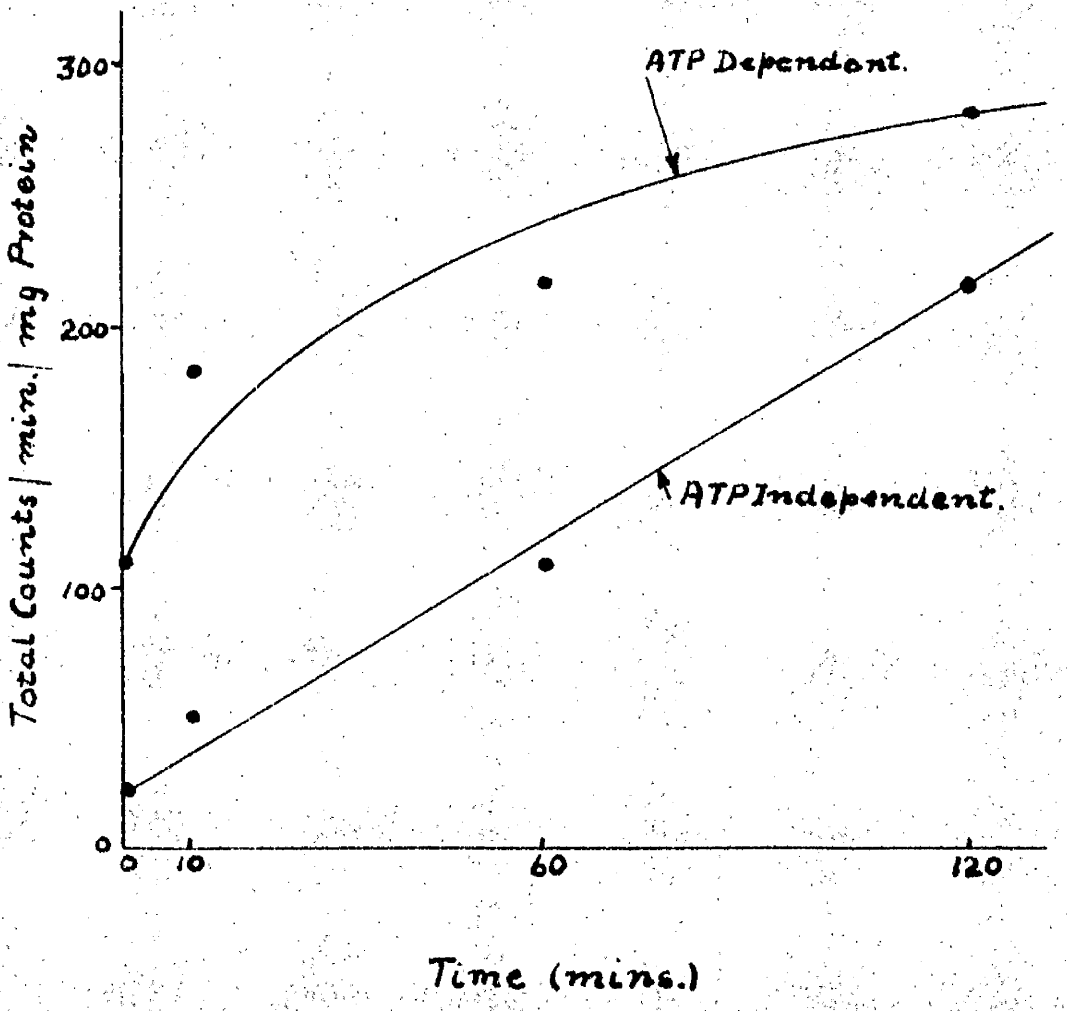
The results are a mean of 2 experiments.

	PCA stable cts./min./mg. protein	
	- Alkaline Phosphatase	+ Alkaline Phosphatase
- ATP	237	272
+ ATP	675	335

Fig 28.

The Time Course of the ATP-Dependent and Non-ATP-dependent Incorporation of ^{14}C-leucine into Post-microsomal Pellet.

1 mg. post-microsomal protein prepared in Campbell buffer was incubated under the standard conditions in the presence or absence of 1 umole ATP, for the times noted. The ATP-dependent incorporation shown is the total incorporation in the presence of ATP less the incorporation in its absence.



period of incubation, the non-ATP-dependent incorporation increases at a fairly steady rate whereas the rate of the ATP-dependent reaction decreases with time. If incubation were prolonged, it appears probable that the ATP-dependent reaction would be overtaken by the non-ATP-dependent incorporation.

However, the main conclusion to be drawn is that the non-ATP-dependent reaction is not due purely to a non-specific adsorption of leucine since:-

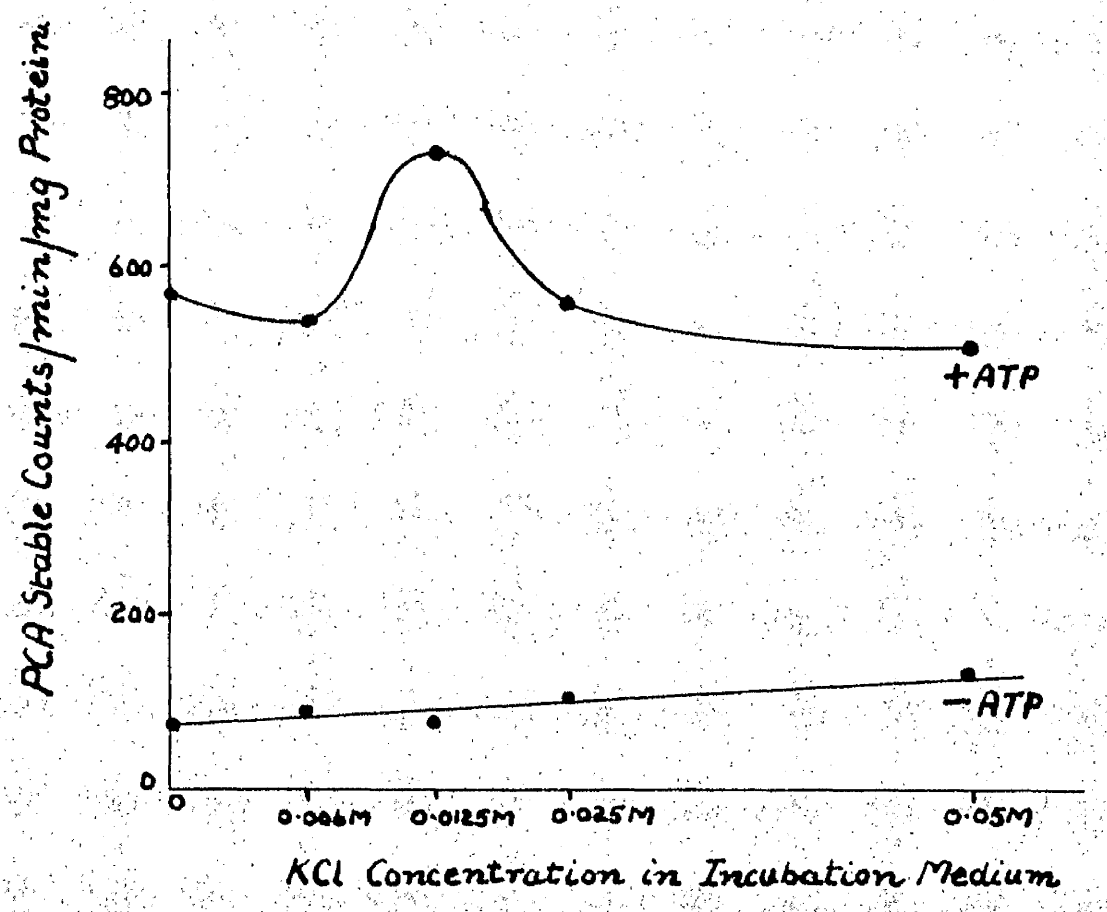
1. An unincubated sample shows very little uptake of leucine.
2. The incorporation proceeds linearly with time.

In Table 23, we showed that by increasing the KCl concentration of the preparation medium we could produce a post-microsomal pellet which could incorporate leucine by a reaction which was not dependent on the presence of ATP. Is this effect of the KCl concentration confined to the preparation medium or is it also significant in the incubation medium? To investigate this, post-microsomal pellet was prepared in sucrose/phosphate, pH 7.8 and incubated under the standard conditions but with varying concentrations of KCl in the medium. In Fig. 29 the activity obtained in the post-microsomal protein is plotted against the concentration of KCl in the incubation medium in the presence and absence of ATP. The ATP-dependence of the incorporation does not seem to be greatly affected by the variations in the KCl concentration in the medium used for incubation. The activity of the samples incubated in the presence of ATP showed a large peak of

Fig. 29.

The Effect of the KCl Concentration of the Incubation medium on the ATP-dependent Incorporation of Leucine by Post-microsomal Pellet.

1 mg. post-microsomal protein prepared in sucrose/phosphate, pH 7.8 was incubated with 1 uC ^{14}C-DL-leucine with or without 1 umole ATP at 37°C for 2 hours. The incubation medium was that of Campbell except that the KCl concentration was varied as shown. The points plotted are a mean of 4 experiments.



incorporation at a KCl concentration of 0.0125M. This peak was reproducible in four experiments and its significance is probably due to the stability of the ribonucleoprotein at this ionic concentration and to the relative concentrations of $MgCl_2$, $KHCO_3$ and KCl. The activity of the samples incubated in the absence of ATP increases slightly as the concentration of KCl is increased but shows no marked peak. Possibly, if a very large concentration of KCl had been used, the non-ATP-dependent incorporation would have reached the level of the ATP-dependent incorporation.

Thus, the KCl concentration of the incubation medium affects the level of incorporation obtained but does not have a gross effect on the ATP-dependence of the incorporating system. Does the addition or removal of the other ions present in the incubation medium have any effect on the ATP-dependence of the incorporation? Post-microsomal pellet was prepared in sucrose/phosphate, pH 7.8 and incubated in this same medium with the addition of $MgCl_2$, $KHCO_3$ and KCl as noted in Table 26. In the upper part of the Table the effect of the addition of each ion separately is shown. In no case does the incorporation approach that obtained when all three ions are added at the same time. $KHCO_3$ is the only compound which will support incorporation to any extent when added alone. This is not due to a pH effect, since the phosphate buffer present is sufficient to prevent any change of pH. The non-ATP-dependent incorporation of ^{14}C-leucine in the presence of only $KHCO_3$

Table 26.

The Effect of Varying the Ionic Composition of the Incubation Medium on the Incorporating Capacity of Post-microsomal Pellet.

1 mg. post-microsomal protein, prepared in sucrose/phosphate, pH 7.8, was incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine for 2 hours at 37°C in a total volume of 1 ml. sucrose/phosphate, pH 7.8. The incubation medium also contained the inorganic salts indicated below.

Two series of experiments were carried out; in the first, the salts were added singly and in the second, in pairs.

$MgCl_2$ 10 uM	$KHCO_3$ 30 uM	KCl 25 uM	PCA stable cts./min./ mg. protein	
			+ ATP	- ATP
-	-	-	35	26
+	-	-	33	26
-	-	+	39	26
-	+	-	147	53
+	+	+	621	36
-	-	-	122	127
+	+	-	548	106
-	+	+	168	95
+	-	+	125	131
+	+	+	581	130

is 36% of the ATP-dependent incorporation; in the presence of all three ions it is only 6%, due to a rise in the ATP-dependent uptake. However, the total incorporation supported by $KHCO_3$ when added by itself is so small in comparison with that when $MgCl_2$ and KCl are also added that this effect on the ATP-dependence is not of importance. In the lower part of Table 26, the effect of adding the compounds in pairs is shown. These figures show that the presence of KCl is not essential in the incubation medium which is in agreement with the results shown in Fig. 29. On the other hand, the presence of $MgCl_2$ and $KHCO_3$ is necessary. In this experiment there was a fairly high incorporation in the absence of added ions which was not dependent on the presence of ATP. However, the true incorporation does seem to depend on the presence of ATP.

Attempts to Separate Post-microsomal Pellet into Fractions with Different Activities.

The first attempt to separate post-microsomal pellet into fractions was carried out by taking 1 hour cell sap and centrifuging it for 1 hour at 105,000g giving a pellet and a supernatant fraction. This pellet will hereafter be called Pm I. The supernatant was decanted and centrifuged for a further 1 hour at 105,000g again giving a pellet (Pm II) and a supernatant. The process of removal of the supernatant and re-centrifugation was repeated once more giving a third pellet (Pm III) and a final supernatant. In Table 27 the yields and

Table 27.

An Attempt to Separate Post-microsomal Pellet into Three Fractions by Differential Centrifugation.

Three fractions were obtained from 1 hour cell sap by centrifuging for 1 hour, 2 hours and 3 hours at 105,000g. 1 mg. of protein of each fraction and of total post-microsomal pellet prepared in sucrose/phosphate by the usual procedure were incubated under the conditions described in Table 12.

A. The Leucine Incorporating Ability of Each Fraction.

	Yield mg. protein/ Spinco tube	PCA-stable cts./min./ mg. protein		
		+ ATP	- ATP	ATP dependent (by difference)
Pm I	1.5	393	282	111
Pm II	0.7	218	158	60
Pm III	1.5	223	160	63
Post-microsomal Pellet	4.4	642	119	523

B. The Activating Enzyme Content of Each Fraction.

	% Exchange per mg. Protein		
	No Added Amino Acids	+ Complete Amino Acid Mixture	+ Leucine
Pm I	1	18	9
Pm II	4	44	21
Pm III	4	26	15
Post-microsomal Pellet	3	34	17

activities of each of the precipitated fractions are shown. A control sample of normal post-microsomal pellet is also included. Pm II is the smallest fraction; Pm I and Pm III are similar in amount and roughly twice as much as Pm II (Table 27 A). The total protein recovered in these three fractions is somewhat less than that in the post-microsomal pellet obtained from one 3 hour centrifugation at 105,000g, presumably indicating that the final supernatant after three 1 hour centrifugations contains a small amount of protein. In part A of Table 27, the ^{14}C-leucine incorporating activity of each of the subfractions of post-microsomal pellet is also recorded. Pm I is rather more active than either of the other fractions but this incorporation is largely independent of the presence of ATP. None of the subfractions is as active as total post-microsomal pellet and the incorporating activity of the total pellet is much more dependent on the presence of ATP than the incorporation of any of the subfractions. The sum of the ATP-dependent incorporation of ^{14}C-leucine achieved by the three subfractions does not approach that of total post-microsomal pellet. This indicates that some link must exist between the three separated fractions when they are present together in total post-microsomal pellet which enables it to incorporate ^{14}C-leucine by an ATP-dependent reaction.

The activating enzyme activity of each fraction was also investigated and is recorded in Table 27 B. The pattern here is different from the pattern of incorporation. Pm II and Pm III

being much more active per mg. of protein than Fm I. The value obtained for Fm II may be rather high since large amounts of this suspension were added to the incubation medium and therefore, contamination with supernatant enzymes may have occurred to a significant extent.

Once again, the independence of the ^{14}C-leucine incorporating activity and the activating enzyme activity present in post-microsomal pellet is evident. Thus, Fm I was the most active of the three subfractions in ability to incorporate ^{14}C-leucine but was the least active in activating enzyme activity. Despite these data, we have not succeeded in completely separating the activities present in post-microsomal pellet, each fraction obtained possessing some of both of the activities found in total post-microsomal pellet.

A second attempt to separate post-microsomal pellet into fractions was made. On this occasion, the post-microsomal pellet was first prepared in the usual way and then subjected to fractionation (previously, each fraction was isolated directly from the homogenate). The separated pellet was precipitated at pH 5 by the usual procedure and the precipitate dissolved in sucrose/phosphate, pH 7.8. Any insoluble material was then separated by centrifugation at 2,000 r.p.m. for 10 minutes. The leucine incorporating ability and the capacity to transfer leucine to sRNA in the supernatant fraction and the particulate fraction so obtained was compared with that of the total post-microsomal pellet (Table 28). The activating enzymes are

Table 28.

Separation of Post-microsomal Pellet into Two Fractions after Isolation.

Post-microsomal pellet prepared by the usual procedure in sucrose/phosphate, pH 7.8 was separated into two fractions by centrifugation for 10 minutes at 2,000 r.p.m.. 1 mg. of protein of each fraction was incubated for 2 hours at 37°C with 1 umole ATP and 1 uC ^{14}C-DL-leucine in a total volume of 1 ml. Campbell buffer (KCl = 0.0125M). The ability of each fraction to transfer leucine to sRNA was tested by incubating 1 mg. with 1 umole ATP, 1 uC ^{14}C-DL-leucine and 50 ug. sRNA for 10 minutes at 37°C in 1 ml Campbell buffer (KCl = 0.0125M).

	Transfer to sRNA		Total cts./min./mg. protein	
	+ sRNA	- sRNA	+ ATP	- ATP
2,000 r.p.m. Supernatant	357	-	390	136
2,000 r.p.m. Sediment	383	-	1038	1683
Total Post-microsomal Pellet	392	125	-	-

obviously not confined to any one fraction. Thus, both the soluble and the particulate fractions have the same capacity to transfer leucine to sRNA as total post-microsomal pellet. On examining the ^{14}C-leucine incorporation, the 2,000 r.p.m. sediment has a very high capacity to incorporate leucine into a form which is stable to hot PCA but the reaction is not dependent on the presence of ATP.

This experiment gives some indication that separation of the various activities of post-microsomal pellet may be possible by simple centrifugation but much more work is required before any definite conclusions about the separate systems can be reached.

Conclusions.

There are two systems present in post-microsomal pellet which will incorporate ^{14}C-leucine into a form which is stable to treatment with hot PCA, one involving an ATP-dependent process and the other not dependent on the participation of ATP. Both are progressive with time of incubation, and thus appear to be enzyme catalysed reactions. However, neither reaction can be equated to true protein synthesis since, in the ATP-dependent system:-

1. After an initial period of incubation with ^{14}C-leucine the addition of "cold" leucine and further incubation causes a fall of about 30% in the labelling of the post-microsomal pellet.

2. The addition of a complete amino acid mixture (less leucine) causes a marked inhibition of the incorporation of leucine.

3. About 30% of the leucine incorporated is in an N-terminal position.

The very fact that the other system does not require a source of energy rules it out from being true protein synthesis. The activities of post-microsomal pellet may be inter-convertible; thus, the ATP-dependence of the incorporation is lost when the KCl concentration of the preparation medium is increased.

SECTION IV.

SECTION IV.

Introduction.

Studies on the Intracellular Site of Origin of Post-microsomal Pellet.

The earlier studies of McLean (1962) on post-microsomal pellet showed that the quantity varied with dietary conditions. Moreover, the amount of pellet recovered per g. of liver could be varied by changes in the homogenisation medium (See Section I). For this reason it was thought worth exploring to determine whether the post-microsomal pellet could be a breakdown product of other sub-cellular structures.

In Section I, we showed that the preparation of post-microsomal pellet from a medium which did not contain Mg^{++} led to an increase in the yield of post-microsomal protein. As the yield of RNA was not affected to the same extent we judged that this increase was not due to ribosomal breakdown. Where does this additional material originate?

In this section is reported an investigation of this problem. We have treated nuclei, mitochondria and microsomes with EDTA in an attempt to obtain an active post-microsomal pellet. The effect of sonication of microsomes has also been examined. Finally, both deoxycholate and pyrophosphate have been used in an attempt to disrupt microsomes with the production of an active post-microsomal pellet.

Experimental Methods.

The methods used in this section are the same as those used in the previous sections with the undernoted changes and additions.

Media used for the Preparation of Fractions.

All the media used in this section are outlined below. Three of these were used in Section I but they are described again here to facilitate the drawing of comparisons.

1. Campbell buffer.

0.01M $MgCl_2.6H_2O$, 0.03M $KHCO_3$, 0.025M KCl, 0.02M potassium phosphate buffer, pH 7.8 (KH_2PO_4:K_2HPO_4 = 1:9), and 0.35M sucrose.

2. Sucrose/phosphate, pH 7.8

0.02M potassium phosphate as in (1) and 0.35M sucrose.

3. Sucrose/phosphate, pH 3.4

Same as (2), but adjusted to pH 3.4 by the addition of N HCl.

4. Sucrose/EDTA, pH 3.4

0.35M sucrose and 0.001M EDTA.

5. Sucrose/EDTA, pH 7.8.

Same as (4), but adjusted to pH 7.8 by the addition of N NaOH.

6. Sucrose/phosphate/EDTA, pH 3.4

Same as (4) but with the addition of 0.02M potassium phosphate as in (1).

7. Sucrose/phosphate/EDTA, pH 7.8

Same as (6) but adjusted to pH 7.8 by the addition of N NaOH.

8. Sucrose/$CaCl_2$

0.25M sucrose and 0.0018M $CaCl_2$.

Preparation of Nuclei.

The method used was essentially that of Wilczok and Charazy (1960).

Rats were killed by a blow on the head, the livers rapidly excised and washed with ice cold suspending medium. They were blotted dry, weighed and a 1 in 10 homogenate in sucrose/$CaCl_2$ prepared, using a Potter-Elvejhem homogeniser (1936). The homogenate was filtered roughly to remove the fibrous tissue and centrifuged at 500 r.p.m. for 5 minutes in an M.S.E. refrigerated centrifuge to remove any whole cells and large cell debris. The supernatant was layered on an equal volume of 0.34M sucrose containing 0.0018M $CaCl_2$. This was centrifuged at 2,000 r.p.m. for 10 minutes giving a pellet of crude nuclei. To purify the nuclei the pellet was resuspended in sucrose/$CaCl_2$ and re-separated. This procedure was repeated until clean nuclei were obtained as determined by microscopic examination after staining with crystal violet.

Preparation of Mitochondria.

The method of Schneider and Hogeboom (1950) was used.

The rats were killed by a blow on the head, the livers rapidly excised and washed with ice cold 0.25M sucrose and

roughly weighed. A 1 in 9 homogenate was made using a Potter-Elvejhem homogeniser. The homogenate was strained to remove fibrous tissue and centrifuged at 2,000 r.p.m. for 10 minutes in an M.S.E. refrigerated centrifuge to remove whole cells, cell debris and nuclei. The supernatant was recentrifuged at 5,000g in the Rotor 40 of the Spinco, Model L ultracentrifuge for 10 minutes to sediment the mitochondria. The pellet so obtained was suspended and resedimented several times, until the supernatant was clear. Quite pure mitochondria could thus be obtained.

Treatment of Fractions with EDTA.

Nuclei and Mitochondria prepared as described above, and microsomes prepared in Campbell buffer or sucrose/phosphate, pH 7.8 (as described in Section I) were resuspended in sucrose/phosphate/EDTA, pH 3.4 and allowed to stand for 15 minutes. The large particles were then sedimented under the same centrifugation conditions as used in their original preparation i.e. 10 minutes at 2,000 r.p.m. for nuclei; 10 minutes at 5,000g for mitochondria; and 1 hour at 105,000g for microsomes. The supernatant obtained from this separation was centrifuged for 3 hours at 105,000g. No pellet was obtained from nuclei or mitochondria under these conditions, but a pellet was obtained from the microsome fraction. The pellets obtained were suspended in Campbell buffer for use in incubation experiments.

Sonication of Microsomes.

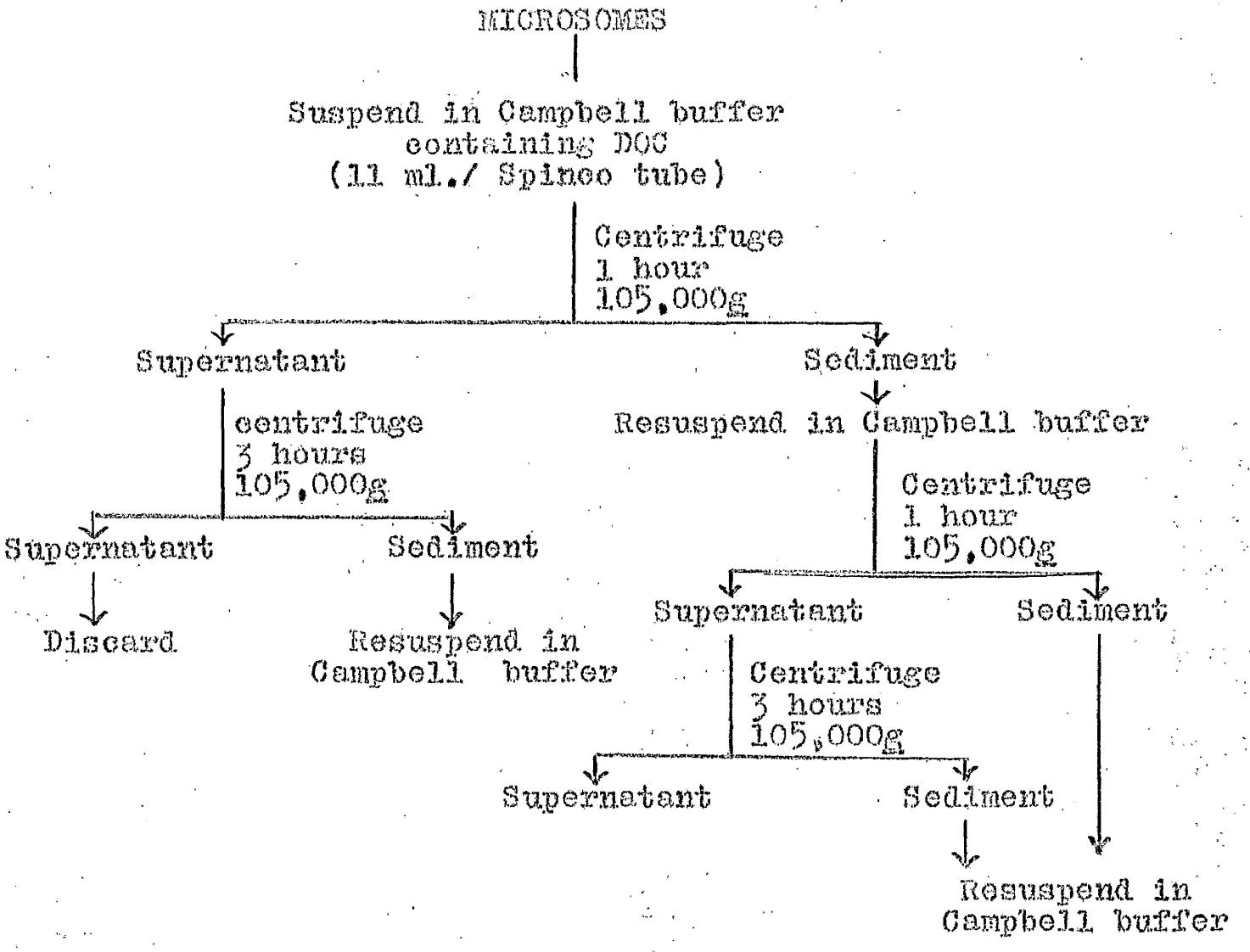
Microsomes, prepared by the usual procedure, were resuspended in medium to give a protein concentration of between 5 and 15 mg. per ml.. Thirty three ml. of such a suspension were sonicated for 5 minutes using an ultrasonic drill at the maximum number of K.cycles/sec.. The sonicate was centrifuged for 1 hour at 105,000g and the supernatant recentrifuged for 3 hours at 105,000g. The sedimented fractions were suspended in buffer and used in subsequent incorporation experiments.

Treatment of Microsomes with Deoxycholate.

Microsomes, prepared by the usual procedure, were resuspended in Campbell buffer containing sodium deoxycholate (DOC) ranging in concentration from 0.1 to 1%. Eleven ml. of this medium was added to each Spinco tube and the microsomal pellet suspended in it by brief homogenisation. After standing for 10 minutes, the microsomes were separated by centrifugation for 1 hour at 105,000g. The supernatant obtained was recentrifuged for 3 hours at 105,000g giving a sizeable pellet. The microsome pellet obtained from the 1 hour centrifugation was resuspended in Campbell buffer and centrifuged for 1 hour at 105,000g followed by removal of the supernatant and a further centrifugation of this supernatant for 3 hours at 105,000g. This procedure is summarised in Fig. 30. All the fractions sedimented were resuspended in Campbell buffer and subsequently used in an incorporation experiment.

Fig. 30.

The Treatment of Microsomes with Deoxycholate.



Treatment of Microsomes with Pyrophosphate.

Elevel ml. of a solution of tetrasodium pyrophosphate in Campbell buffer with no Mg^{++} was added to each Spinco tube of microsomes previously isolated by the standard procedure. The concentration of pyrophosphate used varied from 0.005% to 0.1%. The microsome pellet was homogenised briefly in this medium and after standing for 15 minutes was separated by centrifugation at 105,000g for 1 hour. The pellet obtained was resuspended in Campbell buffer and recentrifuged for 1 hour at 105,000g giving a pellet and supernatant. This supernatant was centrifuged at 105,000g for 3 hours to obtain a further pellet. Once more, each pellet was suspended in Campbell buffer for use in incubation experiments.

Results.

Treatment of sub-cellular Fractions with EDTA.

As mentioned in Section I, post-microsomal pellet is quantitatively a very small sub-cellular fraction, 5 g. of liver producing only about 2 mg. post-microsomal protein when preparation is carried out in Campbell buffer. However, the yield of post-microsomal pellet can be increased three-fold by preparing it from a homogenate made in either sucrose/phosphate, pH 7.8 or sucrose/phosphate/EDTA, pH 3.4. These two media differ from Campbell buffer in that they do not contain any $MgCl_2$, $KHCO_3$ or KCl. The pH of the sucrose/phosphate/EDTA medium was arrived at by chance - when a solution of sucrose and phosphate of the concentrations used in Campbell buffer is made up and 0.001M EDTA is added the pH of the resultant medium is 3.4. As this medium was shown to have a large effect on the properties of post-microsomal pellet, its use at this pH was continued. Note, however, that the pH of a homogenate of rat liver prepared in this buffer is about 6.

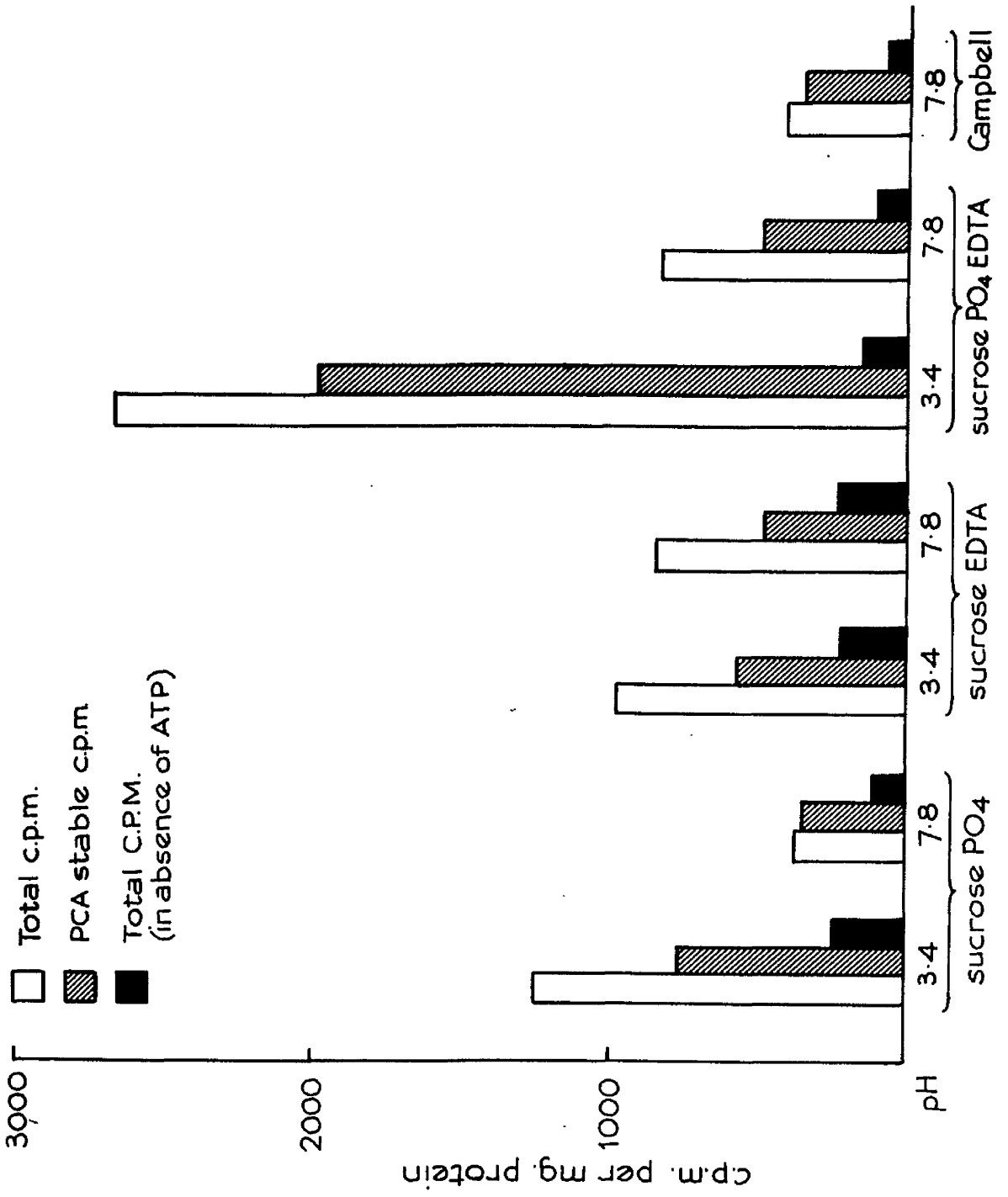
The effect of preparation in these buffers on the ability of post-microsomal pellet to incorporate amino acids is given in Fig. 31. Obviously, the material prepared in Campbell buffer is the least active and that prepared in sucrose/phosphate /EDTA, pH 3.4 is, by far, the most active. The activities of the fractions prepared in the various other buffers are scattered between these two levels. In every case, however,

Fig. 31.

About 1 mg. portions of post-microsomal protein prepared in the medium noted below the Fig. were incubated for 2 hours at 37°C with 1 umole ATP and 1 uC ^{14}C-DL-leucine in a total volume of 1 ml. Gamphell buffer.

Fig. 31.

The Activity of Post-microsomal Peller Prepared in Various Media.



the fraction prepared in buffer at pH 3.4 is more active than that prepared in the same buffer at pH 7.8. It must be remembered that incubation in every case, was carried out in Campbell buffer at pH 7.8.

The large activity of the fraction prepared in sucrose/phosphate/EDTA, pH 3.4 is not due to a selective concentration of the active portion of the post-microsomal pellet. The yield of pellet in sucrose/phosphate/EDTA, pH 3.4 is, in fact, somewhat greater than in Campbell buffer, as previously demonstrated (Table 4). Where does the additional material come from? and what causes the great stimulation in activity of post-microsomal pellet prepared in sucrose/phosphate/EDTA, pH 3.4?

In an attempt to answer these questions, a variety of cell fractions was treated with sucrose/phosphate/EDTA, pH 3.4 in the hope of obtaining particles which will incorporate amino acids by a similar mechanism to that of post-microsomal pellet. If such are obtainable then it is a fair conclusion that this cell fraction is the origin of the post-microsomal pellet.

1. Treatment of Nuclei with EDTA.

Nuclei prepared from rat liver were treated with sucrose/phosphate/EDTA, pH 3.4 and the resultant suspension centrifuged at 105,000g for 1 hour. The supernatant obtained was recentrifuged for 3 hours at 105,000g but no pellet was obtained. Nuclei similarly treated with sucrose/phosphate, pH 7.8 were used as

a control. No 3-hour pellet was obtained in this case, either. On incubation of the 1 hour pellets obtained under the standard conditions used for amino acid incorporation into post-microsomal pellet, some activity was obtained in the fraction treated with sucrose/phosphate/EDTA, pH 3.4; however, this incorporation was not dependent on the presence of ATP (Table 29).

The separation scheme used in this experiment did not, however, separate whole nuclei from any particles broken off by the presence of EDTA i.e. whole nuclei would be included in the 1 hour precipitate. A further experiment was therefore, carried out, in which the whole nuclei were reisolated by centrifugation at 2,000 r.p.m. for 10 minutes after treatment with sucrose/phosphate/EDTA, pH 3.4, followed by the isolation at 105,000g, as above, of any fragments which had been produced. In this experiment, no pellet was obtained from the nuclei treated with EDTA by centrifugation for 1 hour at 105,000g. However, a very small pellet with some ability to incorporate amino acids was obtained from the control sample which had been treated with sucrose/phosphate, pH 7.8 (Table 30). The nuclei reisolated from either medium did not show any ability to incorporate amino acids under these conditions. The small amount of active material obtained in these experiments is probably due to the contamination of the original nuclei preparation with traces of cytoplasm.

As these experiments had proved quite unfruitful in obtaining a large pellet with amino acid incorporating ability,

Table 29.

The Effect of the Treatment of Nuclei with EDTA.

Nuclei, prepared as described in the methods section were resuspended in sucrose/phosphate/EDTA, pH 3.4 and centrifuged for 1 hour at 105,000g. 1 mg. of protein of the fraction so obtained was incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine for 2 hours at 37°C in a total volume of 1 ml. Campbell buffer.

Suspending Medium	Speed and Duration of Spin	Total cts./min./mg. protein		TCA stable cts./min./mg. protein	
		+ ATP	- ATP	+ ATP	- ATP
Sucrose/ Phosphate pH 7.8	1 hr. 105,000g	46	46	33	28
Sucrose/ Phosphate/ EDTA pH 3.4	1 hr 105,000g	120	109	91	102

Table 30.

The Effect of the Treatment of Nuclei with EDTA.

Nuclei, prepared as described in the methods section, were resuspended in sucrose/phosphate/EDTA, pH 3.4 and separated into two fractions by centrifugation. 1 mg. of protein of each fraction was incubated with 1 uC ^{14}C-DL-leucine with or without 1 umole ATP for 2 hours at 37°C in a total volume of 1 ml. Campbell buffer.

Suspending Medium	Speed and Duration of Spin	Total cts./min./mg. protein		PCA stable cts./min./mg. protein	
		+ ATP	- ATP	+ ATP	- ATP
Sucrose/ Phosphate pH 7.8	2,000 rpm 10 mins.	50	38	47	41
	105,000g 1 hour	-	-	336	-
Sucrose/ Phosphate/ EDTA pH 3.4	2,000 rpm 10 mins.	34	29	29	28
	105,000g 1 hour	No pellet was obtained			

this investigation was abandonned.

2. Treatment of Mitochondria with EDTA.

From nuclei, we turned to the effect of sucrose/phosphate EDTA, pH 3.4 on a mitochondrial preparation. A similar experiment to that carried out with nuclei was performed. Mitochondria, prepared from a rat liver homogenate, were suspended in sucrose/phosphate/EDTA, pH 3.4 and then reisolated. The supernatant obtained was centrifuged at 105,000g for 3 hours, but no pellet was obtained. Incubation of the whole mitochondria under the standard incubation conditions showed there to be no activity present in mitochondria treated with EDTA that was not present in a control sample treated only with 0.25M sucrose or sucrose/phosphate, pH 7.8 (Table 31).

3. Treatment of Microsomes with EDTA.

The last fraction that was treated with sucrose/phosphate/EDTA, pH 3.4 in an attempt to obtain an active particle was a microsome preparation. An identical experiment to those carried out with nuclei and mitochondria was performed. Microsomes were prepared in Campbell buffer, resuspended in sucrose/phosphate/EDTA, pH 3.4, reprecipitated by centrifugation at 105,000g for 1 hour. Any post-microsomal particles produced by the EDTA would remain in the supernatant and could be isolated by centrifugation at 105,000g for 3 hours. In fact, a post-microsomal fraction was obtained in this way. The ability of each fraction so obtained to incorporate amino

Table 31.

The Effect of the Treatment of Mitochondria with EDTA.

Mitochondria, prepared as described in the methods section, were resuspended in sucrose/phosphate/EDTA, pH 3.4 and re-isolated by centrifugation at 2,000 rpm for 10 minutes. The resulting supernatant was centrifuged at 105,000g for 3 hours but no pellet was obtained. 1 mg. portions of the mitochondrial protein were incubated with 1 uC ^{14}C-DL-leucine with or without 1 umole ATP in 1 ml. Campbell buffer for 2 hours at 37°C.

Suspending Medium	Speed and Duration of Spin	Total cts./min./mg. protein		PCA stable cts./min./mg. protein	
		+ ATP	- ATP	+ ATP	- ATP
0.25M Sucrose	2,000 rpm 10 mins.	104	66	69	-
Sucrose/phosphate pH 7.8	2,000 rpm 10 mins.	99	81	76	-
Sucrose/phosphate EDTA pH 3.4	2,000 rpm 10 mins.	68	105	87	-

acids is given in Table 32. Obviously, the reisolated microsome fraction has no ability to incorporate leucine under these conditions. However, the fraction obtained after 3 hours centrifugation has a very high capacity to incorporate amino acids. Nevertheless, microsomes treated with Campbell buffer instead of EDTA also show this behaviour. This liberation of an active fraction is thus not due to the presence of EDTA but may be due to the washing out of some cytoplasmic contaminant including post-microsomal pellet material from the microsome preparation.

4. Conclusions.

No post-microsomal pellet-like material could be obtained from nuclei, mitochondria or microsomes by treatment with sucrose/phosphate/EDTA, pH 3.4. A pellet was obtained from microsomes but the production of this fraction was not dependent on the presence of EDTA. The question was therefore raised whether this activity was merely due to cytoplasmic contamination. To explore this, an additional experiment was carried out in which the microsomes were prepared in Campbell buffer or in sucrose/phosphate/EDTA, pH 3.4. Some of these microsomes were washed by resuspension in 0.35M sucrose and resedimented before suspension in Campbell buffer and reseparation of a 1 hour fraction and of a 3 hour fraction. This procedure is summarised in Fig. 32. Such an operation would, presumably remove any cytoplasmic contamination of the microsomes. The ability of the fractions

Table 32.

The Effect of the Treatment of Microsomes with EDTA.

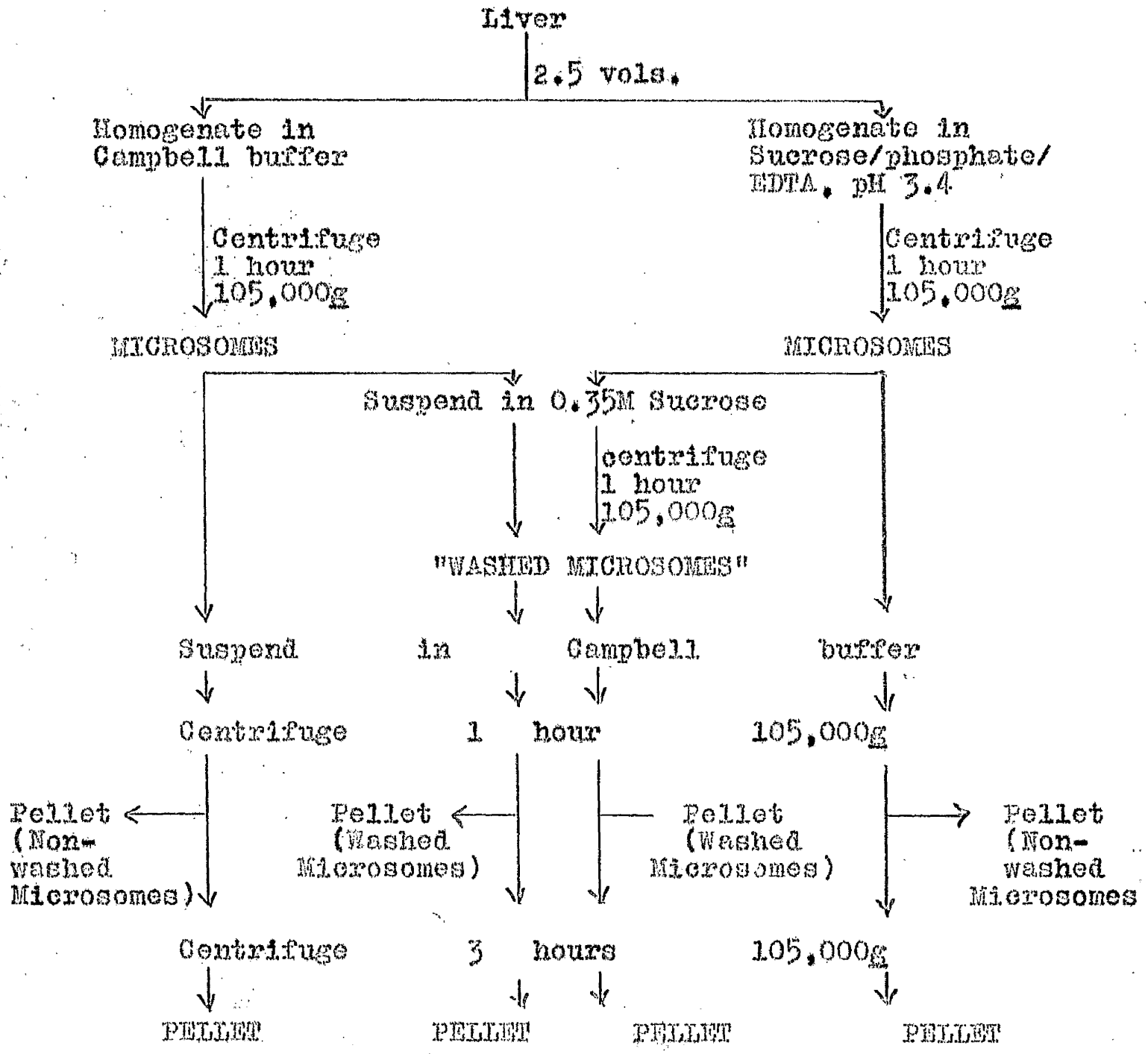
Microsomes, prepared as described in Fig. 6 in Campbell buffer, were resuspended in the medium shown in the Table and the suspension separated into three fractions by centrifugation for 1 hour and then 3 hours at 105,000g. 1 mg. portions of the precipitated fractions were incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine for 2 hours at 37^{o}C in 1 ml. Campbell buffer.

Preparation Medium	Suspension Medium	Speed and Duration of Spin	PCA stable cts./min./ mg. protein + ATP
Campbell Buffer	Campbell Buffer	105,000g 1 hour	37
		105,000g 3 hours	479
	Sucrose Phosphate EDTA pH 3.4	105,000g 1 hour	73
		105,000g 3 hours	613

Fig. 32.

Preparation of Subfractions from Washed and Non-washed Microsomes.

This Fig. gives a flow sheet for the preparation of the fractions used in the experiment for which the results are reported in Table 33.



so formed, to incorporate amino acids was studied and the results obtained are presented in Table 33. The fractions prepared from the microsomes prepared in sucrose/phosphate/ EDTA, pH 7.4 show little capacity to incorporate amino acids, but the 3-hour pellet from the microsomes prepared initially in Campbell buffer shows some activity, whether the microsomes were washed with sucrose or not. Obviously, therefore, the activity of this fraction cannot be due solely to contamination with cytoplasmic material, as this material would have been largely removed by the preliminary wash with sucrose.

Sonication of Microsomes.

In an attempt to obtain a larger quantity of active material from microsomes, we treated a microsome preparation with ultrasonic vibrations. After this treatment, the microsomes were resedimented by centrifugation at 105,000g for 1 hour and any particles produced were separated by centrifugation at 105,000g for 3 hours. Table 34 gives the ability of each of these fractions to incorporate leucine. It can be seen that sonication releases a fraction from microsomes, sedimentable by centrifugation at 105,000g for 3 hours which has a high capacity to incorporate amino acids under the conditions in which post-microsomal pellet can carry out incorporation.

Table 33.

The Effect of Washing Microsomes with Sucrose before Fractionation

1 mg. of protein of each fraction isolated as described in Fig. 32 was incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine in 1 ml. Campbell buffer for 2 hours at 37°C.

Preparation Medium	Suspension Medium	Speed and Duration of Spin	Sucrose Wash	PCA stable cts./ min./mg. protein + ATP
Campbell Buffer	Campbell Buffer	105,000g 1 hour	-	72
			+	42
		105,000g 3 hours	-	240
			+	362
Sucrose/ phosphate/ EDTA pH 3.4		105,000g 1 hour	-	51
			+	53
		105,000g 3 hours	-	184
			+	85

Table 34.

The Effect of Sonication on Microsomes.

Microsomes, prepared by the normal procedure were resuspended in Campbell buffer and sonicated for 5 minutes. The resulting suspension was centrifuged for 1 hour and then 3 hours at 105,000g. 1 mg. portions of each fraction were incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine for 2 hours at 37°C in 1 ml. Campbell buffer.

Sonication	Procedure for Reisolation of Fractions	Total cts./min./ mg. protein + ATP	PCA stable cts./ min./ mg. protein + ATP
+	105,000g 1 hour	418	599
	105,000g 3 hours	2360	1790
	Final Supernatant	96	46
-	105,000g 1 hour	96	74
	105,000g 3 hours	-	140
	Final Supernatant	40	58

Characteristics of the Incorporation Reaction.

Having obtained a fraction which is able to incorporate ^{14}C-leucine under the standard conditions used for incorporation by post-microsomal pellet, it was of interest to study the characteristics of this incorporation.

First, the time course of the incorporation was investigated by incubating aliquots of protein from the 3 hour pellet ffom sonicated microsomes for varying times under the standard conditions of incubation. In Fig. 32 the counts incorporated into the protein of the fraction are plotted against the duration of the incubation. Obviously, this fraction is capable of incorporating amino acids over a period of at least 4 hours. This is similar to the pattern in post-microsomal pellet (Fig. 4) although incorporation was only followed for 2 hours in the latter case.

Table 35 shows the effect of the addition of ribonuclease to the incubation mixture and also of the removal of ATP. Similar to its action on the post-microsomal pellet system, ribonuclease has no effect on the incorporating system, although the RNA present is reduced from 100ug. to 40 ug. On the other hand, the removal of ATP from the system does not inhibit incorporation, as it does in the post-microsomal pellet system under these conditions. Although the incorporation by 3 hour pellet from sonicated microsomes is independent of the presence of ATP it appears to be enzymic, since no incorporation occurs in unincubated samples.

Fig. 33.

The Time Course of the Incorporation of Leucine into the 3 Hour Pellet from Sonicated Microsomes.

1 mg. protein from the 3 hour pellet from sonicated microsomes was incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine in 1ml. Campbell buffer at 37°C for the times noted.

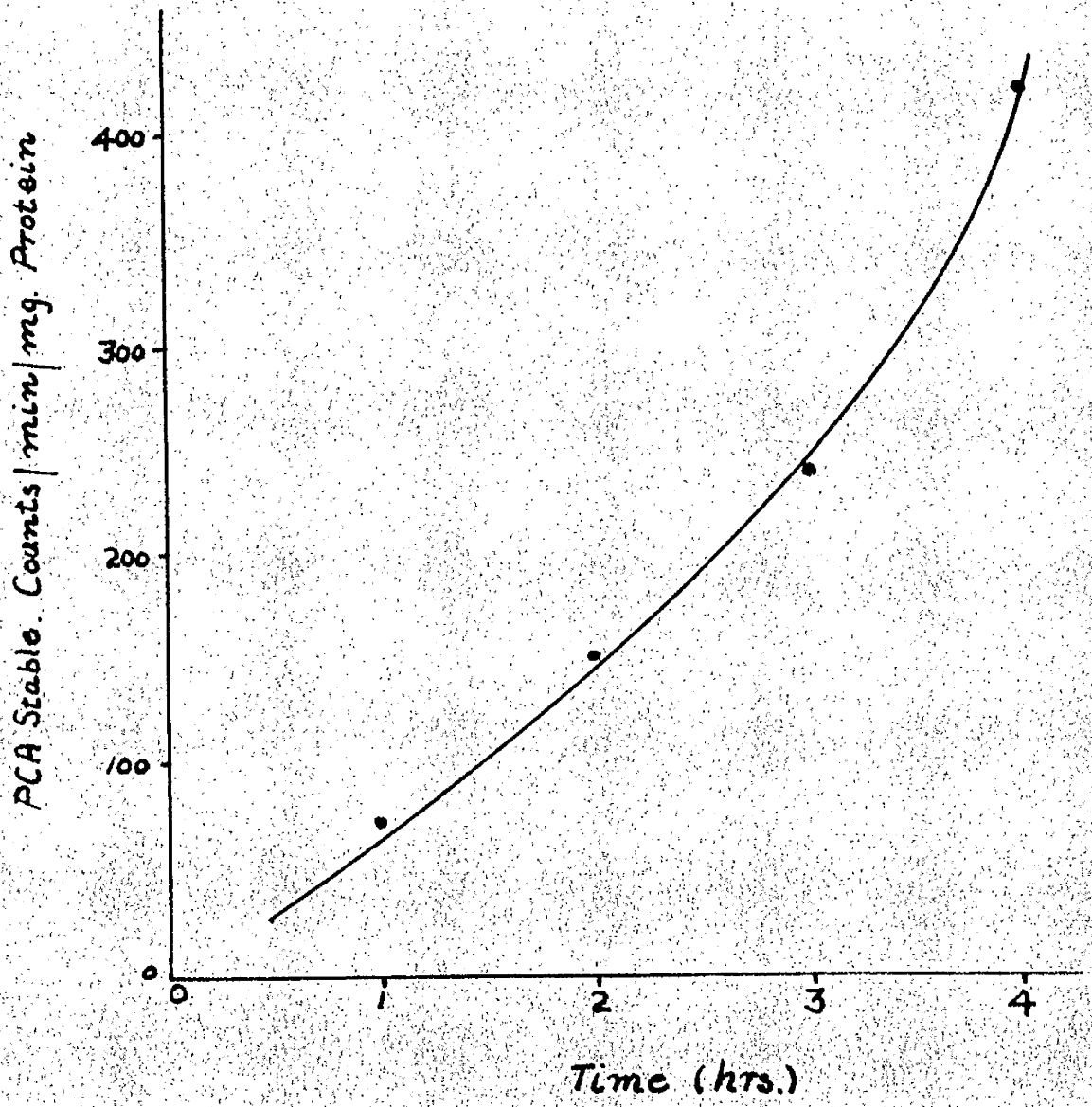


Table 35.

The Effect of ATP and Ribonuclease on the Ability of 3 Hour Pellet from Sonicated Microsomes to Incorporate ^{14}C-leucine.

Microsomes were prepared and resuspended in Campbell buffer. After sonication, the 3 hour pellet was separated by the usual procedure. 1 mg. portions of the pellet protein were incubated under the conditions given below in a total volume of 1 ml. Campbell buffer, 1 uC ^{14}C-DL-leucine, 1 umole ATP and 200 ug RNAase were added where noted.

Addition to Incubation		Temperature of Incubation	PCA stable cts./ min./mg protein	ug. RNA recovered
ATP	RNAase			
+	-	0°C	17	135
+	-	37°C	589	110
+	+	37°C	477	44
-	-	37°C	518	113
-	+	37°C	469	43

The effect of the addition of a large excess of unlabelled leucine to the post-microsomal pellet incorporating system has been investigated (Fig. 27). A similar experiment was therefore performed with the 3 hour pellet from sonicated microsomes. 1 mg. portions of the protein of this fraction were incubated under the standard incubation conditions. At various time intervals a twenty fold excess of non-radioactive leucine was added and incubation continued for a further period. If the ^{14}C-leucine was being incorporated into a terminal position purely by an exchange reaction or some other such mechanism, this addition of cold leucine would be expected to lead to a marked diminution in the labelling of the protein of the fraction. Fig. 34 gives the result obtained with the 3 hour pellet from sonicated microsomes. Obviously, no significant amount of the leucine incorporated is being removed by this procedure, and, in fact, some of the leucine present initially in a form unstable to treatment with hot PCA is converted to a hot-PCA-stable form, after the addition of the nonactive leucine.

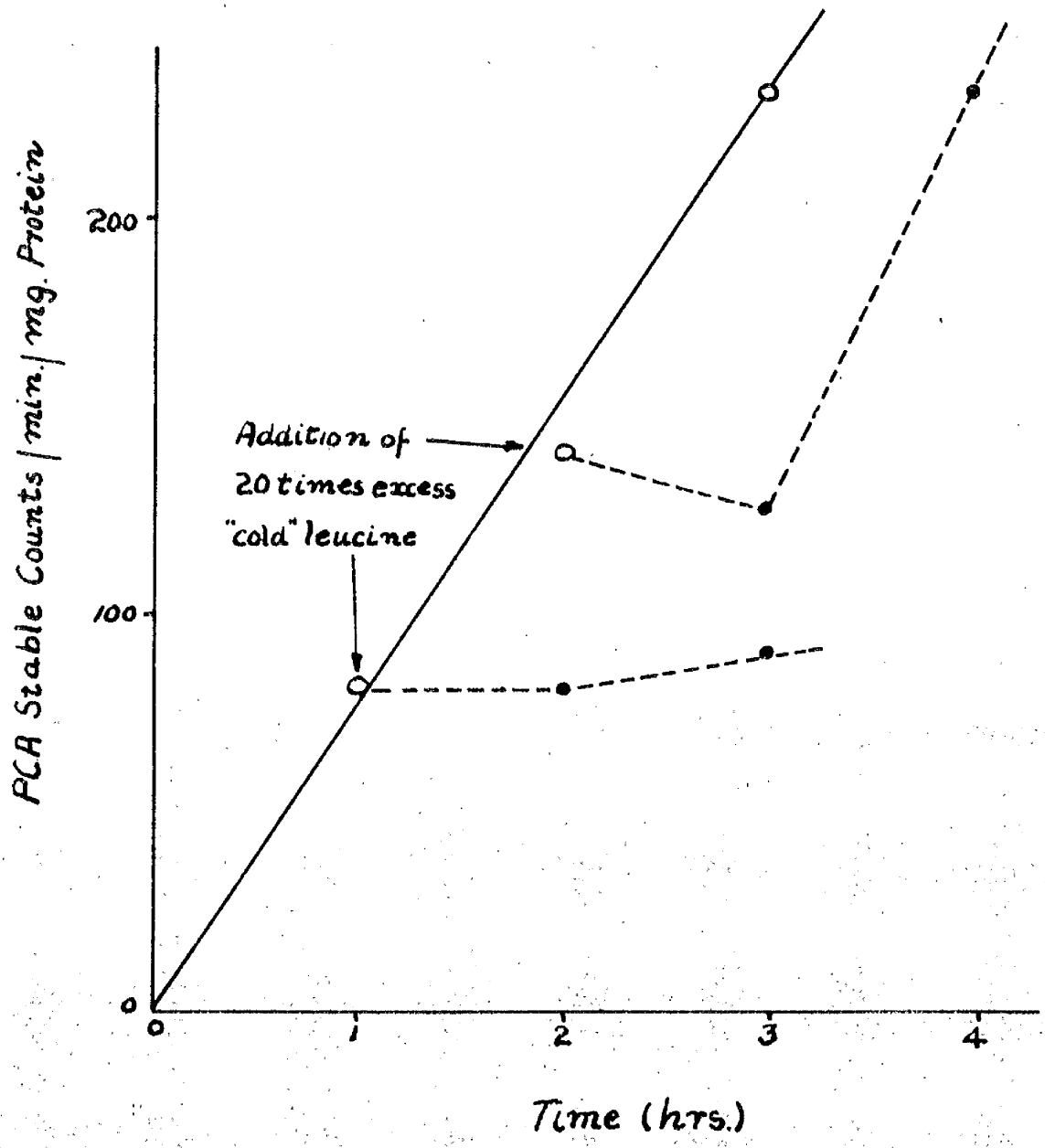
This finding indicates that the incorporation system present in 3 hour pellet from sonicated microsomes is different from that of post-microsomal pellet since in the latter case, 30% of the leucine incorporated initially was removed by subsequent incubation with nonactive leucine (Fig. 27).

A further highly characteristic property of the natural post-microsomal pellet system is its content of activating

Fig. 34.

The Effect of Isotope Dilution on Leucine Uptake by the 3 Hour Pellet from Sonicated Microsomes.

The conditions of the experiment were the same as those described for Fig. 27.



enzymes (See Section II.). Does this sub-fraction of the microsomes also contain activating enzymes? Untreated microsomes have little demonstrable activating enzyme activity (Table 10). However, it seemed quite possible that sonication might release some enzymes which may be sedimentable by centrifugation for 3 hours at 105,000g. Table 36 gives the result of an experiment carried out to investigate this possibility. As suggested, neither untreated microsomes nor the 1 hour pellet from sonicated microsomes showed any activity greater than that due to the content of endogenous amino acids. However, the 3 hour pellet did possess an enzyme which could activate leucine. The question of activating enzymes was therefore further explored. The spectrum of amino acids activated by post-microsomal pellet is highly characteristic (Table 10). It was therefore of interest to study the spectrum of amino acid activation of 3 hour pellet from sonicated microsomes. Table 37 gives the result of such an experiment. The activating enzyme spectrum follows very closely that of cell sap except in its content of phenylalanine activating enzyme which is much higher. It is noteworthy that phenylalanine activating enzyme was the only one found to be present in an untreated microsome preparation (Table 10). This spectrum is very different from that of post-microsomal pellet.

Although the incorporation of amino acids by 3 hour pellet from sonicated microsomes is unaffected by a preliminary wash of the microsomes, it seems highly probable from the above

Table 36.

The Activating Enzyme Content of Sonicated Microsomes.

The conditions of incubation were the same as those described in Table 10.

Fraction	% Exchange		
	No Added Amino Acid	Leucine Added	Difference
pH 5 enzyme	5	10	5
Untreated Microsomes	8	8	0
1 hour pellet from Sonicated Microsomes	8	8	0
3 hour pellet from Sonicated Microsomes	7	14	7

Table 37.

The Activating Enzyme Activity of 3 Hour Pellet from Sonicated Microsomes for Various Amino Acids. (Per mg. Protein)

The conditions of incubation were the same as those described in Table 10.

Amino Acid Added	% Exchange		
	Post-microsomal pellet	pH 5 enzyme from 3 hour cell sap	3 Hour Pellet from Sonicated Microsomes
Leucine	100	47	48
Methionine	66	42	41
Lysine	57	24	21
Phenylalanine	25	4	17

result, that the activating enzyme content is due purely to contamination with cell sap, except perhaps in the case of phenylalanine.

An experiment was carried out, therefore, in which microsomes were prepared in Campbell buffer and washed with the same medium before sonication. A control sample of non-washed microsomes was processed, also. In Table 38 is reported the activating enzyme content, for leucine, of the various fractions isolated by this procedure. As suggested, washing of microsomes prior to sonication completely removes the activating enzymes from the 3 hour pellet obtained by centrifugation after sonication.

These experiments on the nature of the incorporation process in the 3 hour pellet from sonicated microsomes can be summarised as follows:-

1. Incorporation into a hot-PCA-stable form will continue for at least 4 hours of incubation.
2. Incorporation is insensitive to ribonuclease.
3. Incorporation is independent of the presence of ATP.
4. Incorporation is not reversed by the addition of non-radioactive leucine during incubation.
5. The incorporating ability cannot be removed by simple washing (resuspension and centrifugation) of the microsomes before sonication.

Table 38.

The Effect of Washing on the Activating Enzyme Content of Sonicated Microsomes.

Microsomes were prepared as usual in Campbell buffer and resuspended and resedimented before the usual sonication procedure. The incubation conditions were the same as those described for Table 10.

Fraction		% Exchange		
		No added Amino Acid	Leucine Added	Difference
Post-microsomal Pellet		4	16	12
Microsomes Unwashed Sonicated	1 hr.	6	5	0
	3 hr.	6	15	9
Microsomes Washed Sonicated	1 hr.	7	9	2
	3 hr.	4	5	1

6. There are activating enzymes present.

7. The spectrum for the activation of various amino acids is very similar to that of cell sap.

8. The activating enzymes can be removed from the preparation by washing the microsomes prior to sonication.

Thus, the characteristics of the amino acid incorporation process present in this fraction are somewhat different from those of natural post-microsomal pellet. In the latter fraction, there is a system which requires the presence of ATP for the incorporation of leucine. It may be, however, that this fraction from sonicated microsomes is the source of that part of post-microsomal pellet which can incorporate amino acids in the absence of an energy source. The further point that the addition of "cold" leucine has a different effect on the post-microsomal system than on the system from sonicated microsomes is not significant, as the investigation carried out with post-microsomal pellet was on the ATP-dependent incorporation system and not on the incorporation which will occur in the absence of ATP. The activating enzyme content of this 3 hour pellet from sonicated microsomes is not equivalent to that of post-microsomal pellet, the spectra of activities for various amino acids being quite different.

Thus, we may conclude that this fraction from sonicated microsomes may be the source of part of the post-microsomal pellet but not of the total fraction.

To determine whether the addition of this fraction to

post-microsomal pellet had any effect on the ATP-dependence of the incorporating system the following experiment was carried out:-

Post-microsomal pellet and 3 hour pellet from sonicated microsomes were prepared by the usual procedures and incubated separately, and together, in the standard incubation system. The results obtained are reported in Table 39. Each fraction shows an ability to incorporate leucine; post-microsomal pellet requiring the presence of ATP and the microsome fraction incorporating leucine in the absence of ATP, as expected. However, when the fractions are incubated together the incorporation obtained was not additive and there was no effect on the ATP-dependence of the post-microsomal system. Apparently, there is some inhibition occurring, which may be an effect on either one of the systems or on both. As both fractions are sedimented by centrifugation at 105,000g. for 3 hours the inhibition cannot be traced to either fraction by separating them by centrifugation. Thus, the only conclusion that can be reached from this experiment is that post-microsomal pellet and 3 hour pellet from sonicated microsomes cannot replace each other as an incorporating system.

An Investigation of the Procedure used for Sonication.

Although the relative distribution of activities obtained in each of the experiments using sonicated microsomes was quite

Table 39.

The Effect of the Addition of 3 Hour Pellet from Sonicated Microsomes to the Post-microsomal Incorporating System.

1 mg. of post-microsomal protein was incubated with or without 1 mg. protein from the 3 hour pellet from sonicated microsomes. The incubation mixture also contained 1 umole ATP and 1 uC ^{14}C-DL-leucine in a total volume of 1 ml. Campbell buffer. Incubation was continued for 2 hours at 37°C.

	PCA stable cts./min./ incubation tube	
	+ ATP	- ATP
Post-microsomal Pellet	290	107
3 hour pellet from Sonicated Microsomes	146	139
Post-microsomal pellet + 3 hour pellet from sonicated microsomes	316	187

reproducible, the absolute level of activity obtained was rather variable from experiment to experiment. We therefore embarked on an investigation of this effect. Although we have already shown that the washing of microsomes does not remove the incorporating ability of the 3 hour fraction (Table 33), it may remove part of it which is due to cytoplasmic contamination. For instance, we have just shown that the activating enzyme content of the 3 hour fraction can be removed by a preliminary wash of the microsomes. Thus, variations in the degree of cytoplasmic contamination of this fraction may account for the different levels of activity obtained.

To test this, microsomes, prepared by the usual procedure, were resuspended in 0.35M sucrose and resedimented prior to the usual sonication procedure. Table 40 records the capacity of each fraction obtained to incorporate amino acids. It can be seen that washing of the microsomes with sucrose before sonication serves to increase the incorporating activity of the fractions obtained rather than decrease it, as might be expected from the above argument. Thus, contamination with cell sap material cannot be the cause of the variability inactivity.

As various media had been used in the preparation of microsomes in the previously described experiments on the sonication of microsomes the effect of varying the medium used for the preparation of the microsome fraction and also, for resuspension before sonication was studied in an effort to resolve this problem of variable activity. Fig. 35 gives the

Table 40.

The Effect of Washing of Microsomes on the Leucine Incorporating of the 3 Hour Pellet obtained from them.

Microsomes were prepared from a 2.5 homogenate in sucrose/phosphate/EDTA, pH 3.4. They were resuspended in 0.35M sucrose and resedimented by centrifuging for 2 hours at 105,000g. Thereafter the pellets were suspended in Campbell buffer and sonicated. The 3 hour pellet was then separated by the usual procedure. 1 mg. of protein of each fraction obtained was incubated for 2 hours at 37°C with 1 umole ATP and 1 uC ^{14}C-DL-leucine in 1 ml. Campbell buffer.

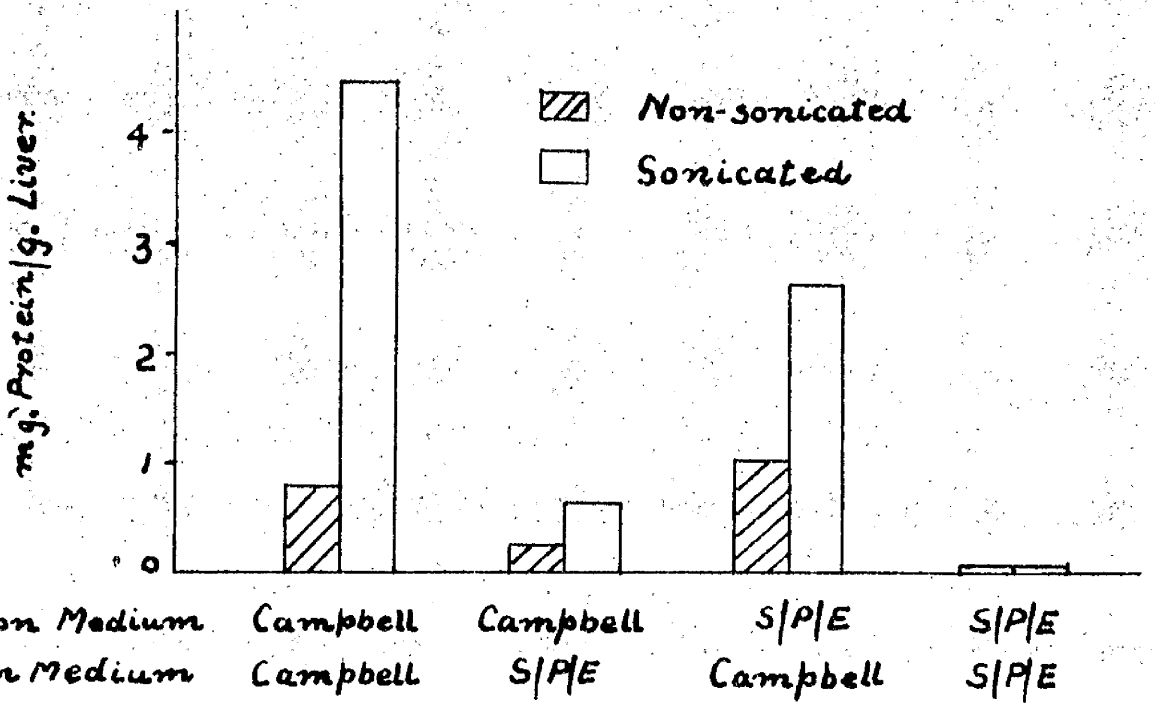
Wash	Duration of centrifugation after Sonication	ATP	PCA stable cts./min./mg. protein	ug. RNA recovered
-	1 hour	+	63	134
	3 hours	+	114	119
		-	87	117
+	1 hour	+	70	126
	3 hours	+	228	145
		-	397	126

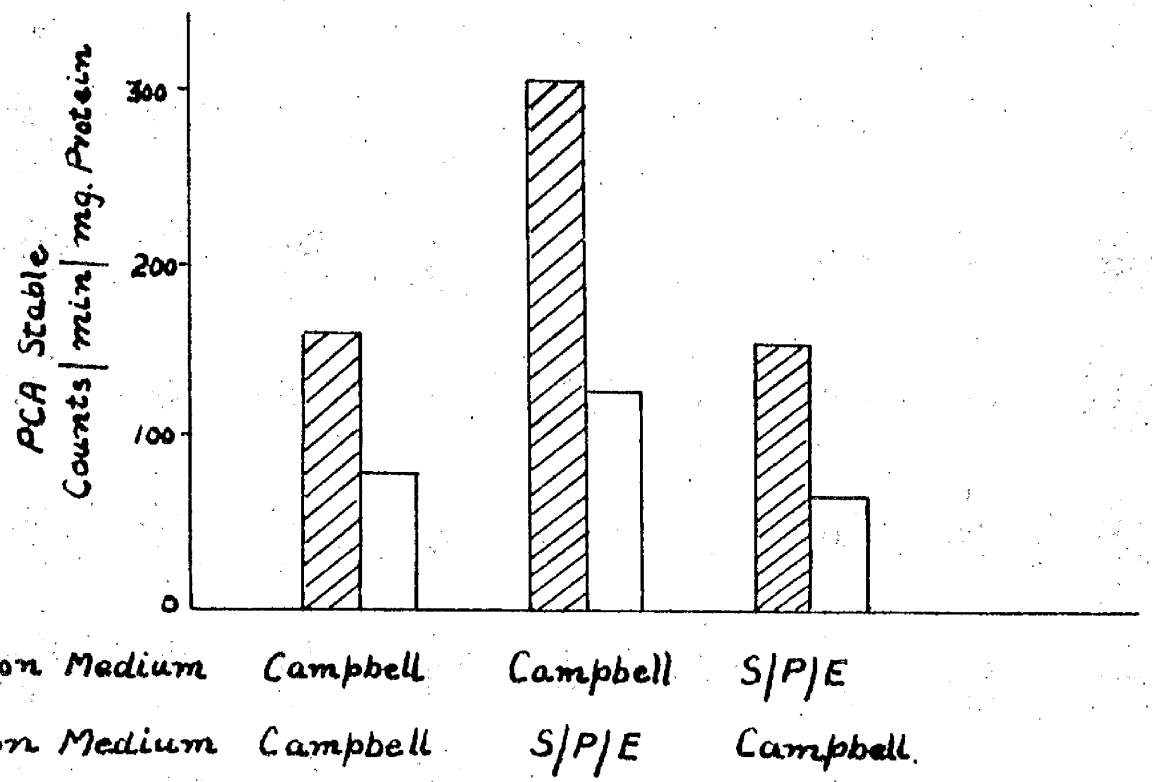
Fig. 35.

The Effect of the Preparation Medium and the Medium used for Resuspension on the Yield and the Activity of the 3 Hour Pellet obtained from Sonicated Microsomes.

Microsomes were prepared in the medium indicated and re-suspended in the appropriate medium. The suspensions were adjusted to equal protein concentrations and sonicated under the usual conditions. Control samples were carried out which were treated identically except that the sonication step was omitted. The 3 hour pellet was prepared from this suspension by the usual procedure. In the upper part of Fig. 35 the yield of 3 hour pellet material from each buffer is shown, and in the lower part, the ability of each pellet to incorporate leucine into a hot-PCA-stable form under the standard incubation conditions is presented.

Fig. 35.


mg. Protein/g. Liver
4
3
2
1
0
Non-sonicated
Sonicated
Preparation Medium
Campbell
Campbell
S/P/E
S/P/E
Suspension Medium
Campbell
S/P/E
Campbell
S/P/E


PCA Stable Counts/min/mg. Protein
300
200
100
0
Preparation Medium
Campbell
Campbell
S/P/E
Suspension Medium
Campbell
S/P/E
Campbell

result of an experiment in which microsomes were prepared in Campbell buffer or sucrose/phosphate/EDTA, pH 7.4 and re-suspended in one or other of these media before sonication. The upper portion of the diagram gives the yield of 3 hour pellet from microsomes both sonicated and non-sonicated. It can be seen that the buffer used in the preparation of the microsomes is not critical, but that resuspension in Campbell buffer before sonication leads to a much greater yield of pellet material, irrespective of the preparation medium. In every case, the amount of material obtained from sonicated microsomes is much greater than that obtained from non-sonicated microsomes. However, when microsomes are prepared in sucrose/phosphate/EDTA, pH 7.4 and resuspended in this medium for sonication all the post-microsomal material is solubilised as evidenced by the absence of a precipitate after 3 hours centrifugation at 105,000g.

The lower half of Fig. 35 gives the ability of each fraction to incorporate amino acids. In each case, the pellet from the non-sonicated microsomes is more active than the equivalent one from sonicated microsomes. The most active pellet is that obtained from microsomes prepared in Campbell buffer and resuspended in sucrose/phosphate/EDTA, pH 7.4. However, the yield of this fraction was very small. The total activity of each fraction per g. liver (i.e. the activity obtained per mg. protein multiplied by the yield in mg. per g. liver) is summarised in Table 41. This is probably a better method of representing the

Table 41.

Total Activity of the 3 Hour Pellet obtained after Sonication of Microsomes which had been Prepared in Various Buffers.

The conditions of the experiment were exactly as described for Fig. 35. The total activity can be defined as the counts obtained per mg. 3 hour pellet protein multiplied by the yield of this protein per g. liver.

Preparation Medium	Suspension Medium	Total Activity per g. Liver	
		Sonicated Microsomes	Non-sonicated Microsomes
Campbell Buffer	Campbell Buffer	319	116
Campbell Buffer	Sucrose/ Phosphate/ EDTA pH 3.4	76	74
Sucrose/ Phosphate/ EDTA pH 3.4	Campbell Buffer	149	161
Sucrose/ Phosphate/ EDTA pH 3.4	Sucrose/ Phosphate/ EDTA pH 3.4	No pellet was obtained	

activities. Obviously, preparation of microsomes in Campbell buffer followed by resuspension in the same buffer for sonication is the best combination of buffers, when yield and activity are examined together. Thus, some of the variation in activity obtained can be explained by the fact that in some experiments microsomes were prepared in sucrose/phosphate/EDTA, pH 3.4 and in others in Campbell buffer.

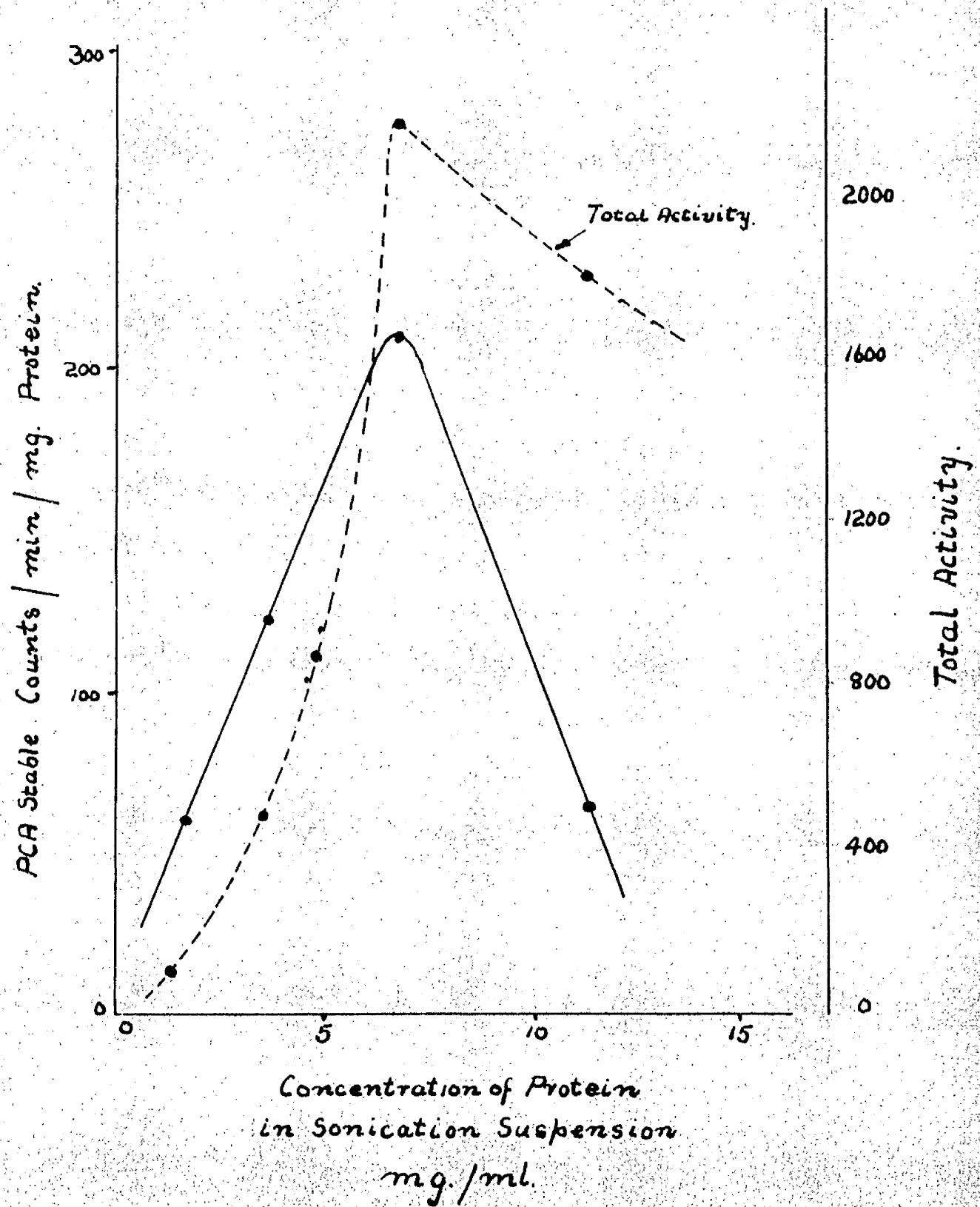
As a further investigation of the causes of the variability of the activity of the 3 hour pellet from microsomes the effect of the protein concentration in the suspension used for sonication was studied. Previously, this concentration had been adjusted to between 5 and 15 mg. per ml. This restriction may not be precise enough however. Microsomes were prepared in Campbell buffer by the usual method and resuspended in Campbell buffer. Portions of the suspension obtained were adjusted to a protein concentration of from 2 mg. per ml. to 13 mg. per ml.. Thirty-three ml. of each suspension were sonicated by the usual method and the sonicate divided into a 1 hour pellet and a 3 hour pellet by centrifugation at 105,000g. In Fig. 36 the capacity of the 3 hour pellet so obtained to incorporate amino acids is plotted against the protein concentration of the suspension used for sonication. There is obviously an optimum concentration of protein in the sonication suspension at about 7 mg. per ml. Below this level, some of the material appears to be inactivated or is not dislodged from the microsomes. Above this level,

Fig. 36.

Microsomes were prepared in Campbell buffer and resuspended in this buffer. Equal volumes of this suspension, adjusted to give the protein concentrations shown, were sonicated and the 3 hour pellet obtained by the usual procedure. The ability of the pellets so obtained to incorporate ^{14}C-leucine into a PCA stable form under the standard conditions of incubation is represented by the continuous line in the Fig. The total activity, i.e. the cts. incorporated per mg. protein multiplied by the mg. protein obtained in the 3 hour pellet from equal amounts of microsomes is shown by the broken line.

Fig. 36

The Effect of the Protein Concentration of the Microsome Suspension used for Sonication on the Activity of the 3 Hour Pellet Obtained.



much more material was obtained, but it was much less active, presumably due to dilution of the active system by non-active fragments of the microsomes. On considering the total activity i.e. the counts incorporated per mg. pellet protein multiplied by the yield of pellet per g. liver, the pattern is similar, but the decline in activity above a protein concentration of 7 mg./ml. is not so marked. A possible explanation of this decline is that there is a maximum amount of active material produced at about 7 mg./ml. Above this level additional material is produced by sonication which is, however, inactive in the incorporating system and which may, indeed, cause some inhibition of the active system.

Thus, it would seem likely that the wide variation in the activity obtained from preparation to preparation in this section is due, at least in part, to the protein concentrations used in the sonication suspensions in each experiment.

Treatment of Microsomes with Deoxycholate and Pyrophosphate.

Having obtained some indication of the presence of a post-microsomal pellet-like material in microsomes, we turned to the usual chemical methods of disruption of microsomes, i.e. treatment with deoxycholate (DOC) and pyrophosphate, in an attempt to obtain a fraction with all the characteristics of post-microsomal pellet from microsomes.

Microsomes were prepared in Campbell buffer by the normal method, resuspended in the same buffer containing various concentrations of DOC and reisolated by centrifugation at 105,000g for

1 hour. The solubilised portion was centrifuged for 3 hours at 105,000g and a very large pellet obtained. The ability of this fraction to incorporate amino acids was studied and the results obtained are presented in Table 42. Although up to 90% of the microsomal protein was solubilised by this technique and a large amount of this material appeared in the 3 hour pellet, none of the fractions obtained showed any incorporating activity for leucine.

An identical experiment was carried out using pyrophosphate instead of deoxycholate. Again, the same result was obtained (Table 43). None of the fractions isolated showed any capacity to incorporate leucine into protein.

This concludes the investigations on the site of origin of post-microsomal pellet. Of all the techniques used (treatment with sucrose/phosphate/EDTA, pH 7.4, sonication, treatment with deoxycholate and treatment with pyrophosphate) only sonication of microsomes produced a fraction that showed any activity in incorporating amino acids. This incorporation system has some of the features of the post-microsomal pellet system but differs in some other properties. Thus, the incorporation of amino acids by the fraction from sonicated microsomes is independent of ATP whereas that of post-microsomal pellet is dependent on the presence of ATP under the same conditions of incubation. Also, the spectrum of amino acids activated by this microsome fraction is quite different from that of post-microsomal pellet.

Table 42.

Treatment of Microsomes with Varying Concentrations of Deoxycholate.

The fractions were obtained as described in Fig. 30 using the concentrations of deoxycholate shown in this Table. 1 mg. of protein of the fractions obtained was incubated with 1 umole ATP and 1 uC ^{14}C-DL-leucine for 2 hours at 37°C in a total volume of 1 ml. Campbell buffer.

Concentration of Deoxycholate used	Duration of Centrifugation after Treatment	PCA stable cts./ min./mg. protein + ATP	ug. RNA recovered
None	1 hour	9	163
	3 hours	108	106
0.1%	1 hour	71	560
	3 hours	62	133
0.25%	1 hour	57	662
	3 hours	73	346
1.0%	1 hour	29	1210
	3 hours	44	229

Table 43.

Treatment of Microsomes with Varying Concentrations of Pyrophosphate.

The fractions were prepared as described in the methods section (page 131) using the concentrations of pyrophosphate shown in this Table. The conditions of incubation were as described in Table 42.

Treatment of Microsomes	Duration of Centrifugation after Treatment	PCA stable cpm./min./mg. protein + ATP
None	1 hour	19
	3 hours	37
Washed with Campbell Buffer	1 hour	18
	3 hours	70
0.005% pyrophosphate	1 hour	18
	3 hours	30
0.03% pyrophosphate	1 hour	29
	3 hours	70
0.1% pyrophosphate	1 hour	19
	3 hours	79

All in all the results obtained seem to imply that post-microsomal pellet, as prepared from normal homogenates, may contain some fragment from disrupted microsomes, thus accounting for the small ATP-independent incorporation of ^{14}C-leucine, but much of the post-microsomal pellet activity cannot be reproduced by disrupting microsomes or other cell fractions. Consequently, it seems likely that a particle with the characteristics of post-microsomal pellet may well exist <u>in vivo</u> in the intact cell.

GENERAL DISCUSSION.

DISCUSSION.

Before entering into any discussion of our results on the properties and functions of post-microsomal pellet, a short account of the published literature may enable us to put some of our findings into perspective.

Very few reports of investigations of naturally occurring subcellular fractions which are smaller than microsomes prepared from mammalian tissues occur in the literature. The first references to a post-microsome or "ultramicrosome" fraction were made by Chantrenne (1947) and Barnum and Huseby (1948) when they separated a fraction with a high RNA content which sedimented at a slower rate than microsomes. However, the scheme of differential centrifugation used by these workers indicates that the fraction they obtained would, in fact, consist of much larger particles than the post-microsomal fraction, as we know it.

Petermann *et al.* (1953; 1954), Palade and Siekevitz (1956; 1956a; Siekevitz and Palade, 1958; 1958a), Goldthwait (1959) and Hoagland and Askonas (1963) have investigated fractions prepared by a similar method to ours. However, no two groups have used the same conditions of homogenisation or the same sequence of steps in differential centrifugation. A summary of the various procedures is given in Table 44.

It can be seen that the preparative method of Goldthwait resembles ours quite closely; his post-microsomal pellet is

Table 44.

Summary of the Conditions used by Various Workers to Prepare a Post-microsomal Pellet.

In each case, the pellet from the final centrifugation shown has been investigated by the workers noted.

Preparation Medium	Centrifugation Scheme	Reference
0.35M sucrose 0.02M potassium phosphate 0.03M $KHCO_3$ 0.025M KCl 0.01M $MgCl_2$ pH 7.8 OR 0.35M sucrose 0.02M potassium phosphate pH 7.8	Homogenised in 2.5 vols. 5 mins. 18,000g 1 hour 105,000g 3 hours 105,000g	This Thesis
0.88M sucrose	Homogenised in 40 vols. 30 mins. 20,000g 1 hour 105,000g 4 to 5 hours 105,000g	Peterman et al. 1953; 1954
0.88M sucrose	Homogenised in 10 vols. 30 mins. 20,000g 1 hour 105,000g 15 hours 105,000g	Palade and Siekevitz, 1956 Siekevitz and Palade, 1958
0.25M sucrose	Homogenised in 2 vols. 15 mins. 25,000g 1 hour 105,000g 3 hours 105,000g	Goldthwait, 1959
0.15M sucrose 0.1M tris 0.0025M KCl 0.005M $MgCl_2$ 0.005M 3 mercapto-ethanol	Homogenised in 2 vols. 15,000g 1 hour 105,000g 12 to 13 hours 105,000g	Hoagland and Ankonas, 1963

likely to be very similar to ours, therefore. That of Hoagland and Askonas is again quite similar, but we would expect their procedure to give a pellet containing our material and also some additional material. These authors quote some analysis figures for RNA in their fraction. The RNA content of both the post-microsomal pellet (fraction X in their terminology) and the pH 5 fraction from the final supernatant are higher than our corresponding figures. Indeed, Hoagland and Askonas obtain a greater total recovery of RNA per gram liver than we do, thus making the comparison of analytical figures quite inconclusive. The preparations of Petermann et al. (1953; 1954) and Palade and Siekevitz (1956) have been obtained by very similar procedures from 0.88M sucrose and thus will have similar characteristics to one another. In some experiments, Palade and Siekevitz separated their post-microsomal pellet into two parts, one the sediment of a 3 hour centrifugation at 105,000g (PM 1) which is thus produced by a procedure almost identical to that of Petermann et al. and a second one obtained by a further centrifugation at 105,000g for 15 hours (PM 2). Analysis of these fractions showed them to have RNA/protein ratios of 0.1 and 0.06, respectively; PM 1 had about three times the amount of phospholipid of PM 2 but even that present in PM 1 is very small compared with that of the microsome fraction. Comparison with our values (Table 5) shows our post-microsomal pellet to have about the same RNA/protein ratio as PM 1 but a much lower amount of phospholipid than

this fraction. Therefore, our fraction is likely to contain much less membranous material than the PM 1 of Palade and Siekevitz. Our post-microsomal pellet might be thought to be similar to PM 2 but the RNA/protein ratio of our fraction is much too high.

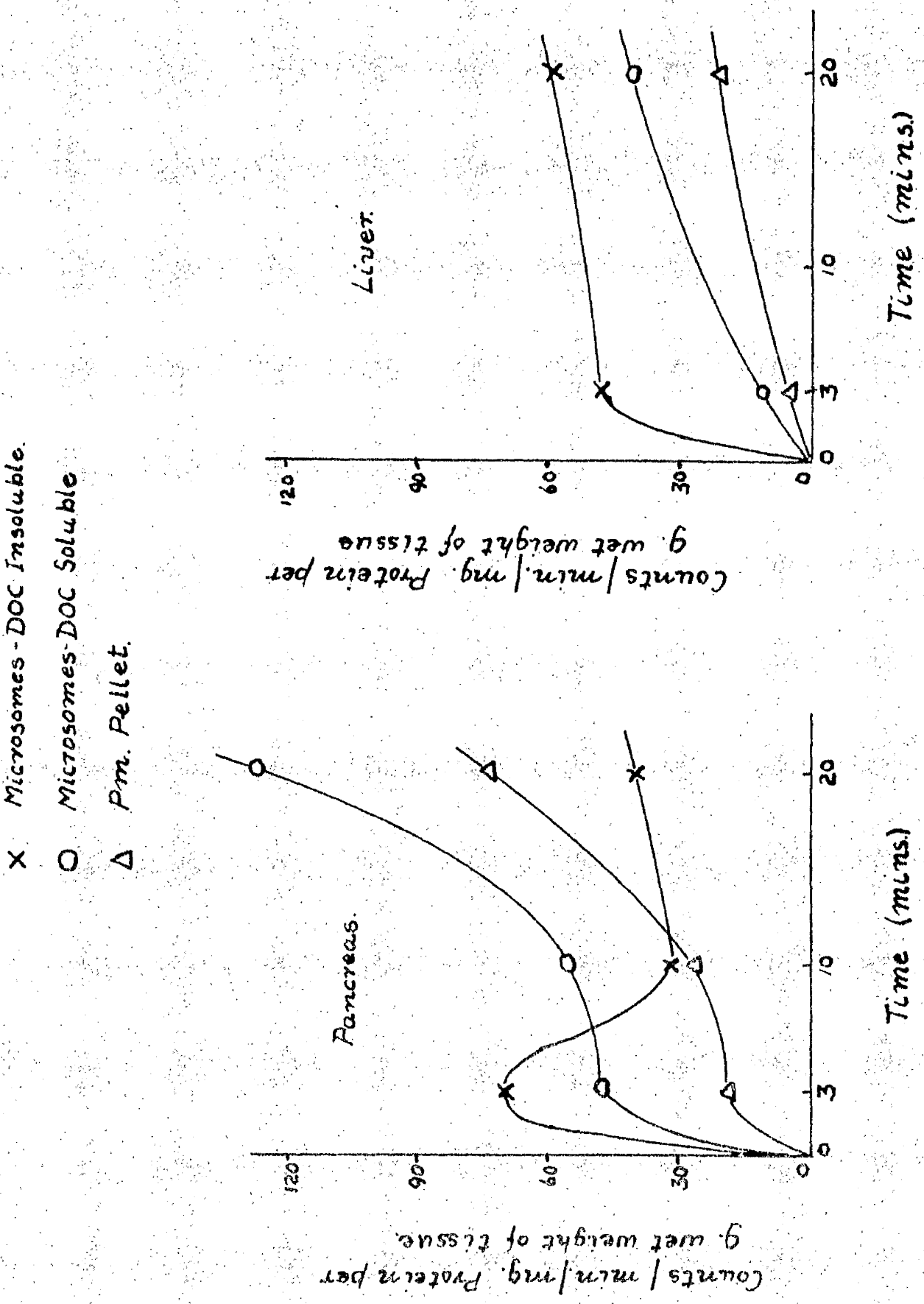
Although there are obvious difficulties involved in drawing comparisons between these various post-microsomal fractions, a short account of the properties of the fractions isolated by each of these groups of workers may provide some useful pointers.

Petermann et al. (1953;1954) examined their pellet and demonstrated the presence of six distinct boundaries with sedimentation constants of 107 S, 90 S; 72 S, 49 S, 38 S and 27 S in their preparation. Recent evidence on the instability of isolated ribosomes might lead us to expect that these particles represent breakdown products of the ribosomes produced artificially in the course of cell disruption. Nevertheless, it is interesting to note, from Petermann's findings, that the amounts of these particles are different in regenerating liver from the amounts in normal liver. Thus, the amount of the 49 S component fell by about 30% during the first two days after partial hepatectomy and remained at this low level for several days during regeneration only returning to normal by about the twelfth day. The 38 S and 27 S components showed changes in the opposite direction. On the second day after partial hepatectomy the concentration of these components

per gram of liver pulp doubled, returning to normal by about the seventh day of regeneration. Thus, these components appear to have some function in the process of cell division and consequently may play some part in cell metabolism, rather than being the products of ribosome disruption.

Palade and Siekevitz (1956; 1956a; Siekevitz and Palade, 1958; 1958a) have carried out electron microscopy and *in vivo* incorporation experiments using post-microsomal pellet material prepared from guinea pig liver and pancreas and from rat liver. They found a marked difference in the fractions prepared from these tissues. Thus post-microsomal pellet (PM 1 and PM 2) is heterogeneous, containing abundant small smooth vesicles, numerous free particles and some amorphous material (Palade and Siekevitz, 1956). On the other hand, pancreatic post-microsomal pellet appears to be homogeneous consisting entirely of free ribonucleoprotein particles (Siekevitz and Palade, 1958). In Fig. 37 the abilities of liver and pancreatic post-microsomal pellet to incorporate amino acids *in vivo* are compared with those of two subfractions of microsomes. (These data are taken from Siekevitz and Palade, 1958a). Post-microsomal pellet from both these tissues can incorporate amino acids, but that from the pancreas is much more active. In fact, pancreatic post-microsomal pellet has a greater specific activity than the ribonucleoprotein particles attached to the reticulum (i.e. the DOC insoluble fraction) twenty minutes after injection of the tracer amino acid. Again, these

Fig. 37. In vivo Incorporation of Leucine into Cell Fractions.



(From Siekevitz and Palade, 1958a.)

experiments indicate that post-microsomal pellet may have some part to play in cell metabolism since it has an incorporating pattern distinct from those of the microsome sub-fractions.

The RNA of the post-microsomal fraction has been studied by Goldthwait (1959) using Ecteola columns. Goldthwait, like several other groups of workers (Bosch et al., 1960; Hoagland et al., 1958) found that RNA prepared by phenol extraction from the 1 hour supernatant could be separated into four peaks on Ecteola. The first and last of these peaks represented only oligonucleotides and other UV absorbing contaminants but the other two seemed to contain true RNA (RNA I and RNA II). By centrifugation of the supernatant used in this separation for 3 hours at 105,000g, Goldthwait obtained two fractions, one of which contained mostly RNA I and the other, the precipitate from the centrifugation, RNA II, which can thus be equated to post-microsomal RNA. The properties of RNA II as reported by Goldthwait can be summarised as follows:- It has a sedimentation coefficient of about 4 S, and a nucleotide analysis reveals the presence of a high guanine and low uracil content. Rechromatography of RNA II on Ecteola enables a portion of it to be eluted exactly where RNA I is normally recovered. AMP is not incorporated into RNA II but ^{14}C-leucine can be incorporated. However, if RNA II is first extracted by phenol from post-microsomal pellet and then incubated with ^{14}C-leucine no incorporation takes place, Such phenol prepared RNA II does not stimulate a pH 5 enzyme-microsome incorporating

system to any extent. This is in agreement with the finding of Hoagland et al. (1958) that 50% of the RNA of the supernatant from a 1 hour centrifugation at 105,000g can be sedimented by a further centrifugation for 3 hours at 105,000g, without altering the total transferring activity of the RNA remaining in the supernatant. In conclusion, Goldthwait suggests that RNA II (post-microsomal RNA) is a form of acceptor RNA (sRNA) associated with particles which makes it sedimentable. This acceptor RNA loses its activity when it is separated from the particles by extraction with phenol, unlike soluble RNA which retains its activity after extraction with phenol.

The most recent paper published about a post-microsomal pellet fraction is that of Hoagland and Askonas (1963). These workers claim that there is a messenger RNA present in post-microsomal pellet (X fraction in their terminology) as evidenced by the one thousand-fold stimulation of a cell sap-microsome incorporating system on the addition of X. Supporting evidence for the presence of messenger RNA in X is that RNA extracted from X and freed from sRNA will stimulate incorporation when added to an incorporating system. Also, X-RNA is labelled more rapidly with ^{32}P in vivo than sRNA or ribosomal RNA.

A discussion of the results obtained with these post-microsomal pellet preparations as compared with ours will not be presented at this point but will be included in the

discussion of the biological significance of post-microsomal pellet.

Summary of our Main Findings on Post-microsomal Pellet.

Our investigations fall into two groups (1) the physical properties of post-microsomal pellet and (2) the biochemical properties. We will, therefore, summarise our findings under these main headings.

1. Physical Properties.

Chemical composition. Post-microsomal pellet represents about 6 mg. of material (i.e. RNA + protein + phospholipid) per gram of liver. It has an RNA/protein ratio of 0.1 to 0.2 and a very low content of phospholipid.

Ultracentrifugation. Analysis of post-microsomal pellet in the analytical ultracentrifuge revealed the presence of four peaks of material with S values of 57, 32, 21 and a major peak of 5.

Electrophoresis. Electrophoresis on cellulose acetate strips revealed the presence of at least four protein components.

2. Biochemical properties.

The enzymic activities of post-microsomal pellet can be divided into three categories as follows:-

(a) Enzymes involved in the "classical" system of protein biosynthesis.

(b) Enzymes involved in the incorporation of ^{14}C-leucine into post-microsomal pellet by an ATP-dependent reaction.

(c) Enzymes involved in the incorporation of amino acids into post-microsomal pellet by a reaction which does not require ATP.

First, we will review the evidence obtained for the existence of these pathways in post-microsomal pellet.

1. Post-microsomal pellet catalyses ^{32}PP-ATP exchange in the presence of amino acids.

2. Post-microsomal pellet will transfer amino acids to sRNA.

3. Post-microsomal pellet will replace cell sap pH 5 enzyme in a ribosome incorporating system.

4. Post-microsomal pellet can incorporate ^{14}C-leucine and methionine into a form which is stable to hot-PCA on incubation in the presence of ATP.

5. Post-microsomal pellet can incorporate ^{14}C-lysine and glycine into a form which is stable to hot-PCA in the absence of ATP from the incubation medium

Considerable evidence hasbeen accumulated to indicate that these pathways are not interdependent. These findings can be summarised as follows:-

a. Activating enzymes are present in post-microsomal pellet for leucine, methionine, lysine and phenylalanine. Of these amino acids, leucine and methionine are incorporated into post-microsomal pellet by an ATP-dependent reaction, lysine does not require the presence of ATP for incorporation and phenylalanine is not incorporated to any extent. Thus, the presence of activating enzymes does not inevitably mean ATP-

dependent incorporation.

b. Similarly, although post-microsomal pellet can incorporate ^{14}C-lysine and glycine into a hot-PCA-stable form in the absence of ATP, transfer of these amino acids to sRNA requires the presence of ATP.

c. Washing of post-microsomal pellet removes most of the leucine activating enzyme; the ATP-dependent incorporation is unaffected and there is a stimulation of the non-ATP-dependent incorporation.

d. ^{14}C-leucine, once it has been incorporated into post-microsomal pellet, cannot be directly transferred to sRNA. Thus, the ^{14}C-leucine is not attached to the post-microsomal pellet at the site of the activating enzymes in the form of an amino acyl-adenylate.

e. Incubation of post-microsomal pellet in the presence of bacterial alkaline phosphatase, which hydrolyses ATP, suppresses the ATP-dependent incorporation but has no effect on the incorporation which is not dependent on the presence of ATP.

Each facet of the activity of post-microsomal pellet can be fitted into some previously established picture. However, the biological significance of post-microsomal pellet as a total sub-cellular fraction is more difficult to assess. First, we will deal with the relationship of each system which is active in post-microsomal pellet to current concepts of protein biosynthetic processes.

The Place of Post-microsomal Pellet in the "Classical" System of Protein Biosynthesis.

As post-microsomal pellet has been shown to contain activating enzymes and to be able to replace pH 5 enzyme in the presently accepted system of protein biosynthesis it has some claim to inclusion in this system.

Post-microsomal pellet and pH 5 enzyme have some similarities and some differences. The main differences can be summarised:-

1. Post-microsomal pellet is sedimentable whereas pH 5 enzyme consists of soluble material.
2. The activating enzymes of post-microsomal pellet are three to five times more active than the corresponding ones in pH 5 enzyme.
3. Post-microsomal pellet does not contain any detectable sRNA but pH 5 enzyme does.
4. Post-microsomal pellet can inactivate sRNA during incubation.
5. Post-microsomal pellet can incorporate ^{14}C-leucine into a hot-PCA-stable form when incubated with only ATP and leucine. Under similar conditions, pH 5 enzyme incorporates leucine into a form which is entirely soluble in hot-PCA (i.e. into sRNA).

The similarities between post-microsomal pellet and pH 5 enzyme are:-

1. Both fractions will catalyse the incorporation of ^{14}C-leucine into ribosomal protein to the same extent per mg. of protein (Some doubts exists as to whether post-microsomal pellet must be supplemented with additional sRNA before

incorporation will proceed at a maximum rate. This varies from experiment to experiment, presumably due to a variable amount of contamination of the preparations with soluble material including sRNA .)

2. Once a ribosome system is saturated with activating enzymes from pH 5 enzyme the addition of post-microsomal pellet does not stimulate incorporation, indicating that the activating enzymes in both fractions are exactly equivalent in function.

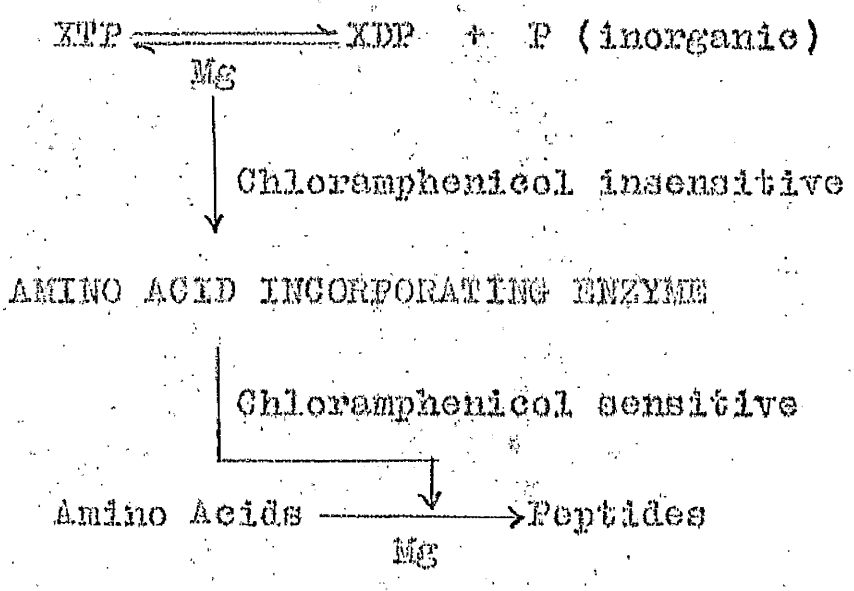
Thus, post-microsomal pellet can replace pH 5 enzyme in a ribosome incorporating system the main differences which affect this system being that post-microsomal pellet contains no detectable sRNA and is, in fact, able to degrade sRNA.

2. Incorporation of Amino Acids by Post-microsomal Pellet.

Before discussing our findings, it is desirable to briefly review the findings of other workers in the field of amino acid incorporation by mechanisms different from the currently accepted pathways.

Much of this work has been done with bacterial preparations, the main investigator being Beljanski. In bacteria, the initial site of protein synthesis seems to be in the cytoplasmic membrane (Butler et al., 1958; Wachsmann et al., 1960; Beljanski and Ochoa, 1958; 1958a). This process is not dependent on the presence of activating enzymes and is not inhibited by RNAase, both findings indicating that the mechanism is different from the"classical" one. Beljanski has isolated an enzyme ("Amino Acid Incorporating Enzyme") in

highly purified form from the supernatant fraction of A.faecalis. This enzyme markedly stimulates the in vitro incorporation of amino acids into the cytoplasmic membrane fraction of an A.faecalis lysate (Beljanski and Ochoa, 1958). He has studied the properties of this enzyme in great detail and found it to catalyse two distinct reactions:-



(Beljanski and Ochoa, 1958a; Beljanski et al., 1962). These reactions are, however, mutually dependent (Beljanski, 1959; 1960). Also, Beljanski (1960a) has reported the presence of four separate kinases in his enzyme preparation each one catalysing the exchange of inorganic phosphate between a specific trinucleotide and dinucleotide.

Nisman et al.(1960; Nisman and Fukuhara, 1960a and b; Wachsmann et al., 1960) have shown the presence of similar enzymes in E.coli and B.megaterium. In these systems, as in the A.faecalis system of Beljanski, amino acids can be incorporated into protein in the absence of activating enzymes and

the incorporation is stimulated by the presence of "amino acid incorporating enzyme". Nisman et al. (1958) have also studied other fractions from an E.coli lysate. They find that the activating enzymes present in the soluble fraction can be sedimented by centrifugation at 105,000g for 2 hours. Both this pellet and the supernatant obtained have enzymes of the "Beljanski" type also i.e. they will catalyse the formation of peptides with the simultaneous release of inorganic phosphate (Nisman et al., 1960). The same group of workers have isolated yet another fraction from E.coli by treating the supernatant from a 3 to 4 hour centrifugation at 105,000g with 1 volume of ethanol and isolating the precipitate formed. This fraction is also able to incorporate amino acids by a mechanism which is very similar to those previously discussed (Nisman et al., 1960).

The properties of the incorporating system from A.faecalis described by Beljanski can be summarised as follows:-

1. All amino acids tested were incorporated into the protein of the fraction by this system.

2. ATP is not an essential addition to the incubation medium.

3. Incorporation is inhibited by the presence of 2.4.dinitrophenol, presumably indicating that the lack of requirement for ATP is due to the provision of energy by oxidative phosphorylation.

4. The reaction is stimulated by the presence of an enzyme prepared from the soluble fraction - "the amino acid incor-

porating enzyme".

5. The addition of an amino acid mixture and a mixture of trinucleotides stimulates incorporation.

6. The incorporation is inhibited by chloramphenicol.

Beljanski reckons that true protein synthesis is taking place since (a) the reaction is stimulated by the addition of an amino acid mixture (b) a small net synthesis of protein is measurable (c) incorporation cannot be reversed by the addition of an excess of unlabelled amino acids after an initial period of incubation (Beljanski and Ochoa, 1958) and (d) the incorporation is not into the terminal portion of the protein chain (Beljanski, 1960b).

The system involving the cytoplasmic membrane fractions of E.coli and B.megaterium, as described by Nisman and his coworkers, is very similar. The factors influencing this incorporation can be summarised as follows:-

1. The incorporation is stimulated by the presence of a mixture of di- or trinucleotides including ATP. This stimulation is even more marked when an amino acid mixture is also added.

2. sRNA stimulates incorporation, especially when added in the presence of RNAase. Thus this stimulation is probably due to an increase in the supply of nucleotides rather than an effect of sRNA as such.

3. DNA stimulates incorporation.

Nisman and Fukuhara (1959a) have shown that the systems from E.coli will synthesise the specific protein β-galactos-

idase under these conditions, presumably indicating that this system is an expression of a true protein synthesising mechanism also.

In these systems of Nisman, although the above additions do cause a stimulation of incorporation of amino acids, it is of interest to us that a marked incorporation does occur in the absence of any addition.

The foregoing schemes have all been investigated in bacterial systems. However, Beljanski and Ochoa (1958a) have shown that the amino acid incorporating enzyme purified by them from A.faecalis stimulates the incorporation of amino acids into rat liver microsomes just as efficiently as the conventional pH 5 enzyme system. (Note, however, that Campbell (1960) could not repeat this experiment.) Similarly, Zalta and Beljanski (1961) have shown the presence of both of the activities of the "amino acid incorporating enzyme" in rat liver pH 5 enzyme. Zalta has also produced particles from rat liver microsomes using surface active agents which can incorporate amino acids without the addition of ATP or soluble enzymes (Zalta, 1958; 1960; Zalta et al., 1959; 1960). This incorporation reaction is not sensitive to RNAase, and no activating enzymes could be detected, indicating that the mechanism is different from the "classical" one. The energy for the reaction comes from an exchange reaction of the type postulated by Beljanski, according to Zalta. The possibility of transpeptidation by proteolytic enzymes was ruled out by

the use of inhibitors. About 80% of the labelling is not in a terminal position (Zalta et al., 1960a).

Cohn (Cohn and Butler, 1958; Cohn, 1959) has isolated a similar microsomal fraction from rat liver microsomes using Lubrol W and perfluorooctanoate. Again, in this system, activating enzymes could not be detected in the microsomal particles and the addition of pH5 enzyme has no effect on the incorporation.

Another factor which is present in rat liver soluble fraction is S-protein, first described by Sachs (1957) and later purified to some extent by Rendi and Hultin (1960). This fraction has a very low activating enzyme content and no sRNA, detectable by the most sensitive methods but can catalyse the transfer of amino acids to ribosomal protein if supplemented with only ATP, GTP, PEP and PK. S-protein can also catalyse the transfer of amino acids to added sRNA and will transfer amino acids to microsomes from sRNA in the presence of GSH. Thus, part of the activity of S-protein seems to be similar to that of the tranfer enzyme of the classical system. Thus, it will transfer amino acids from sRNA to microsomes by a mechanism which is stimulated by GSH. However, in the absence of activating enzymes and sRNA S-protein will catalyse the incorporation of amino acids into microsomal protein indicating the presence of an additional system. It is of interest to note that S-protein does not incorporate amino acids into its own substance.

This concludes our resumé of the non-conventional

mechanisms of protein biosynthesis which have been described in the literature. Obviously, there is a large amount of evidence pointing to the existence of these pathways.

The following is an account of the properties of the post-microsomal incorporating system which will enable us to ascertain the merits of this system for inclusion in the above category of atypical protein-synthesising mechanisms. (Some of these investigations were carried out by McLean (1962) but are included here for the sake of completeness.)

1. Post-microsomal pellet will incorporate leucine, methionine and lysine to a much greater extent than phenylalanine, glycine, alanine or glutamic acid.

2. The incorporation of leucine and methionine is dependent on the presence of ATP whereas lysine is incorporated in the absence of ATP.

3. GTP, CTP, UTP separately, or together, do not support the incorporation of any of the amino acids mentioned above.

4. Incorporation of leucine continues throughout a 2 hour period of incubation.

5. Incorporation of leucine occurs into two forms one stable to hot-PCA and one completely soluble in hot-PCA.

6. Incorporation of leucine is completely insensitive to ribonuclease.

7. The addition of RNA prepared by phenol extraction from several sub-cellular fractions markedly inhibits the incorporation of leucine.

8. The addition of an amino acid mixture, even in the presence of ATP, GTP, CTP and UTP inhibits incorporation of leucine.

9. The incorporation of leucine is dependent on the presence of $MgCl_2$ and $KHCO_3$.

10. The incorporation of leucine is inhibited by GSH.

11. Chloramphenicol has only a small inhibitory effect on the incorporation of leucine.

This system cannot claim to be true protein synthesis since (a) 30% of the leucine incorporated is present in the N-terminal position in the protein chain (b) addition of "cold" leucine after a period of incubation with ^{14}C-leucine causes a diminution of the labelling (c) all amino acids are not incorporated and (d) the addition of an amino acid mixture inhibits incorporation.

The main characteristics of all the mechanisms mentioned previously were the absence of activating enzymes and the insensitivity of the incorporation to ribonuclease. Thus, immediately, we may say that post-microsomal pellet cannot be placed in this category, as it contains large amounts of activating enzymes. However, we have produced much evidence for the independence of the incorporating system and the activating enzyme activity.

Comparison with the Beljanski and Nisman systems shows many differences between these bacterial systems and the post-microsomal incorporating system. The main difference

is the stimulatory effect of amino acids and trinucleotides on the bacterial system whereas these additions inhibit the post-microsomal system. Also, post-microsomal incorporation cannot be equated to true protein synthesis whereas both Beljanski and Nisman claim that their preparations are producing protein.

The S-protein system has some similarities to post-microsomal pellet. Thus, both fractions catalyse the transfer of free amino acids to microsomes. Previously, we had assumed that in post-microsomal pellet this was due to the activating enzymes present. This does not exclude the possibility that the incorporating system of post-microsomal pellet is also involved in this transfer by a similar mechanism to that of S-protein. Nevertheless, S-protein and post-microsomal pellet differ in the fact that S-protein cannot incorporate amino acids into its own substance.

A comparison with Zalta's system involving microsomal fragments from rat liver shows that these fragments are different from post-microsomal pellet in several respects. However, there is a marked similarity between them and the fraction obtained by us from the sonication of microsomes which could incorporate leucine by a reaction which was not dependent on the presence of ATP but also contained activating enzymes.

An interesting paper by Suttie (1962) may throw some light on the problem of the incorporation of amino acids by

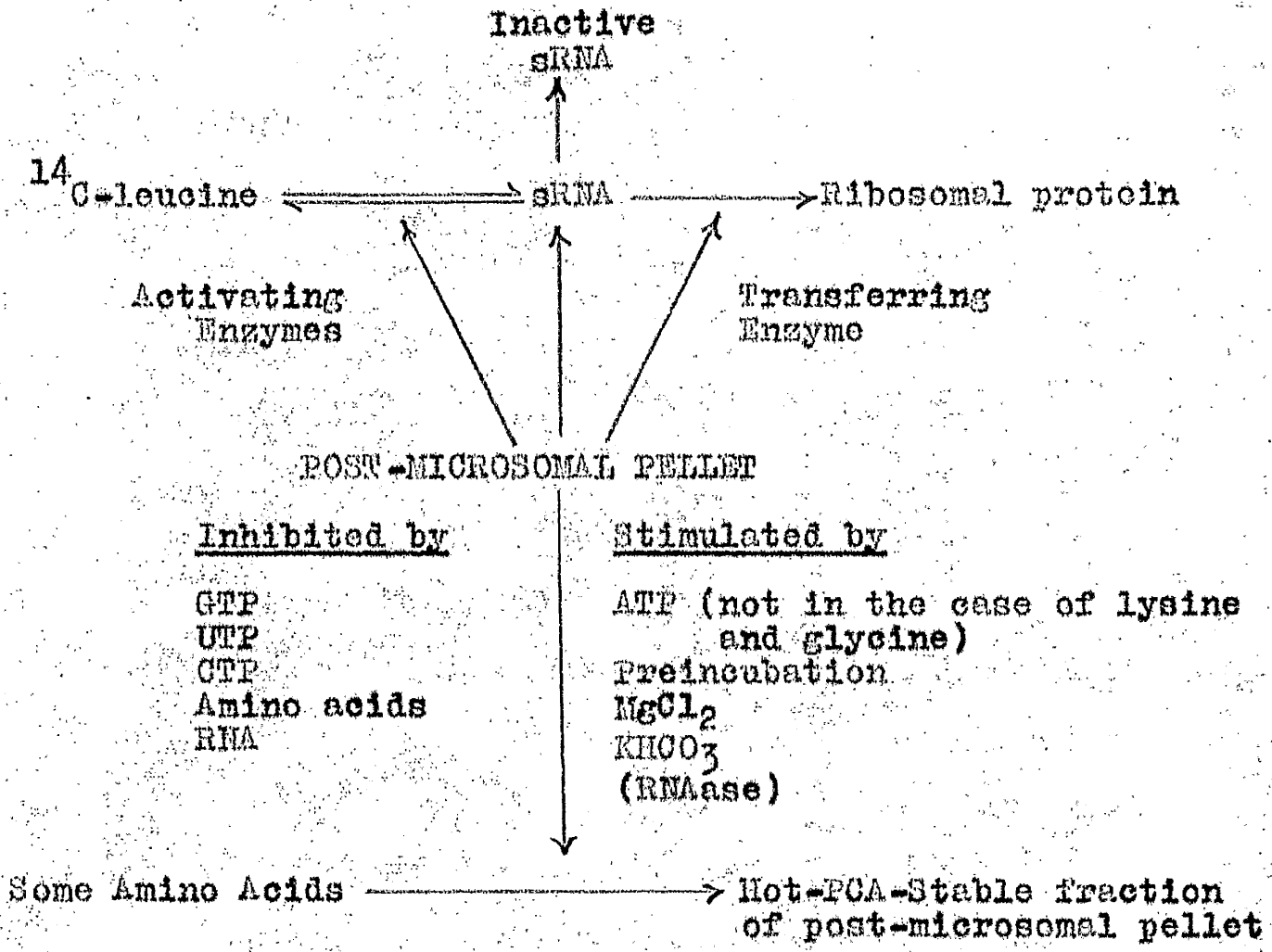
post-microsomal pellet. He used a mitochondrial preparation from rat liver. Two routes of incorporation were shown to be present, one by the normal process of activation and transfer by way of sRNA involving oxidative phosphorylation, and the other proceeding independent of the supply of energy from oxidative phosphorylation or added ATP. This second pathway has been shown to be due to transpeptidation.

Without attempting to explain the mechanisms involved, Fig. 38 gives a summary of the reactions catalysed by post-microsomal pellet and the various factors affecting these processes. All of our findings are not included in this but all of the main points are represented.

In the upper part of the figure, the relationship of the post-microsomal system to the "classical" system of protein synthesis is shown. Thus, we know that post-microsomal pellet can activate amino acids and transfer them to sRNA; also, post-microsomal pellet inactivates sRNA on incubation; lastly, post-microsomal pellet can transfer amino acids to ribosomal protein, presumably by way of the activating enzymes and sRNA. In the lower part of the diagram the incorporating activity of post-microsomal pellet is shown. Only the ATP-dependent system is indicated since the system which is not dependent on the presence of ATP has not been investigated to any extent.

Fig. 38.

Reactions Catalysed by Post-microsomal Pellet.



Technical Implications of Post-microsomal Pellet Activity.

The use of pH 5 enzyme from 1 hour cell sap in incorporating systems is common practice. In view of our findings on the properties of post-microsomal pellet which is, of course, included in 1 hour cell sap pH 5 enzyme post-microsomal pellet may cause some complications in these incorporating systems. Probably the most important activity of post-microsomal pellet in this respect is its ability to inactivate sRNA (Fig. 22). This may be one explanation for the inability of *in vitro* protein synthesising systems to continue incorporating amino acids for more than about 20 minutes.

A second point at which the activities of post-microsomal pellet may lead to the drawing of erroneous conclusions has been brought to notice by the recent experiments of Hoagland and Askonas (1963). Thus, these workers show a large stimulation of incorporation when they add post-microsomal pellet to a microsome incorporating system. Certainly, they do first saturate the system with cell sap pH 5 enzyme and from our results in Fig. 24 we would not expect this stimulation of incorporation on the addition of post-microsomal pellet. However, this stimulation obtained by Hoagland may be explicable in terms of the activating enzyme content and incorporating capacity of post-microsomal pellet rather than by a mechanism involving messenger RNA.

The Biological Significance of Post-microsomal Pellet.

The true biological significance of this fraction is subject to some doubt. Arguments can be produced for and against the natural occurrence of post-microsomal pellet and no conclusion can be reached.

Post-microsomal pellet represents 6 mg. per 5 grams of liver; post-microsomal RNA = 2% of the total cellular RNA. Thus, we may ask "What possible function can so small a fraction containing a variety of enzymes carry out in the cell?" It has a rich supply of activating enzymes and can replace pH 5 enzyme in the classical system of protein biosynthesis. However, 60% of these enzymes can be removed by a simple "washing" procedure involving resuspension. What significance can this have in the living cell? It can incorporate amino acids into a form which is stable to extraction with hot PCA but this reaction cannot be equated to true protein synthesis and seems to be independent of amino acid activation. Can this reaction have any function in the cell?

However, Siekevitz and Palade (1958a) (Fig. 37) and ourselves (Fig. 5) have shown that post-microsomal pellet does become labelled to a significant extent in an *in vivo* incorporation experiment. The pancreatic post-microsomal pellet of Siekevitz and Palade reaches a higher level of activity than the ribonucleoprotein particles of the microsomes some twenty minutes after the injection of the tracer amino acid. In this case, it seem probable that the labelling of

the post-microsomal pellet is due to the release of label (or of labelled ribonucleoprotein particles) from the microsomes. Although in the post-microsomal pellet from liver the labelling does not exceed that of the microsome fraction, it is possible that the label which is present in the post-microsomal fraction has arisen from the release of material initially incorporated into the microsomes. If this is true, what causes this release of material from the microsomal fraction and why does it accumulate in the post-microsomal pellet?

The characteristics of post-microsomal pellet change with the dietary condition of the animal before death. Thus, post-microsomal pellet prepared from an animal receiving a diet with a very low protein content and fasting for 18 hours before death, has a much greater capacity to incorporate ^{14}C-leucine into a hot-PCA-stable form than that from an animal on an adequate protein diet and similarly treated (McLean, 1962). The post-microsomal pellet from this latter group of animals has a much higher RNA/protein ratio than that from the animals fed a low protein diet. Munro and Clark (1960) have shown that when animals are fed a high protein diet and then fasted, there is a marked loss of RNA from the endoplasmic reticulum. Presumably, therefore, this increased amount of RNA in the post-microsomal pellet under these conditions is due to an accumulation of this "breakdown" RNA. The lower incorporating capacity of the

post-microsomal pellet with an increased amount of RNA can be explained by our finding of inhibition of incorporation by RNA. Thus, once again, post-microsomal pellet seems to be involved in microsomal activities.

Petermann et al. (1953; 1954) observed changes in the relative proportions of the 49 S, 38 S and 27 S particles of their post-microsomal preparation during liver regeneration. These particles have remarkably similar sedimentation coefficients to those obtained for the particles present in our post-microsomal preparation, namely 57 S, 32 S, 21 S and a major peak of 5S. Petermann et al. did not separate a 5 S particle but they did mention the presence of a large peak of material with an S value of about this order. They could not separate this fraction in their system, however, as its peak was distorted by some residual sucrose in the preparation. During liver regeneration, the cells are proliferating very rapidly; thus there will be a rapid synthesis of endoplasmic reticulum to cope with the increased requirement for protein synthesis. Once again, therefore, changes can be observed in post-microsomal pellet synchronous with changes in the synthetic activity of the endoplasmic reticulum.

From this examination of the conditions which affect post-microsomal pellet, both in quantity and in activity, we can conclude that there is some connection between the behaviour of the microsomal fraction and the post-microsomal

pellet. As post-microsomal pellet contains extremely small amounts of phospholipid, we can probably narrow down this link to the ribosome fraction of the microsomes rather than the lipid-rich membrane portion. It is therefore relevant to examine briefly some of the properties of free ribosomes which have been established.

The point which immediately springs to notice is that particles with similar S values to those of our post-microsomal pellet exist in ribosome preparations. (Although most of this work has been done with preparations from bacteria, enough evidence has been accumulated to suggest that the picture is very similar in mammalian preparations.) In a cell free extract from E.coli, 70 S, 50 S and 30 S particles could all be identified (Tissières, 1961). It is of interest that all of these particles seem to occur naturally as well as being induced by decreasing the Mg^{++} ion concentration of a 70 S particle suspension to disrupt the ribosomes. Possibly, the 57 S and 32 S particles of our post-microsomal pellet represent similar ribosome fragments. Two RNA components have been obtained from ribosomes using phenol and detergents and these have S values of 15 to 18 and 23 to 32 (Timasheff et al., 1958; Hall and Doty, 1959). Once again, we may identify our 21 S particle with one of these. There remains the 5 S particle of post-microsomal pellet to be identified. Recently, several workers have shown the release of 4 S particles from

ribosomes. For example, Aronson and McCarthy (1961) have demonstrated the release of about forty eight 4 S units from one 70 S ribosome.

What role do these smaller fragments play in ribosome function? The synthesis of ribosomes has been explored by Roberts (1961) and many other workers. The following sequence of events has been suggested as a likely method of formation. First, several 4 S RNA components are joined to give the 18 S and 28 S RNA particles. From this, small ribonucleoprotein particles are formed which then aggregate to form the larger particles. Roberts (1961) also suggests that there is a constant circulation of ribosomal material between the small and the large particles. The relative amounts of these sub-components of the ribosomes vary during the growth cycle of bacteria, the smaller particles being present to a greater extent during a rapid period of growth. (The work of Petermann on mammalian particles in regenerating liver, previously reported, may indicate similar changes in mammalian tissue.)

All these data fit into our picture very well and we therefore tentatively suggest that post-microsomal pellet represents small ribosome sub-components which are involved in the ribonucleoprotein metabolism of the cell.

However, there are several findings which do not fit this concept. We may list these as follows:-

1. Post-microsomal pellet does not incorporate amino-acids

under the conditions which enable ribosomes to carry out synthesis i.e. pH 5 enzyme addition does not cause incorporation to increase.

2. Post-microsomal pellet contains large amounts of activating enzymes, whereas ribosomes contain very small amounts.

3. Post-microsomal pellet will incorporate amino acids into a hot-TCA-stable form in conditions under which ribosomes show no incorporating ability.

4. The RNA/protein ratio of post-microsomal pellet is only about a tenth of that of a ribosome preparation.

The first of these observations can be explained by the fact that only ribosomes in the 70 S form are able to synthesise proteins. Indeed, recent experiments have suggested that the 70 S particles must be in the form of a "polysome" of five 70 S particles before protein synthesis can proceed (Wettstein et al., 1962).

The activating enzyme content of post-microsomal pellet is more difficult to explain. However, Petermann (1961) has shown that there is an increase in protein binding capacity when ribosomes are dissociated into sub-components. Thus, we suggest that the small ribonucleoprotein particles of post-microsomal pellet have a large protein binding capacity and therefore bind soluble proteins including activating enzymes. It may be significant that they seem to have a greater affinity for activating enzymes than other soluble proteins, as evidenced by the concentration of the activating

enzymes in post-microsomal pellet. Similarly we may be able to explain the ability of post-microsomal pellet to incorporate amino acids in terms of binding. However, the fact that the amino acid is incorporated into a form which is stable to treatment with hot TCA and that only 30% is in a form which will react with FDNB makes it impossible to explain this phenomenon in terms of simple binding of the amino acid. Nevertheless, it must be remembered that a considerable part of the incorporated amino acid can easily be removed by exchange and also that an amino acid mixture seems to reduce the incorporation of one particular amino acid, indicating a lack of specificity of the reaction.

There remains the problem of the chemical composition of post-microsomal pellet which is a real problem. We may explain the low RNA/protein ratio in terms of protein binding once again but this seems highly unlikely on quantitative grounds (nine tenths of the protein would have to be present in a non-specific-bound form). If this was the case, obviously the S values would not correspond to those of ribosomal fragments.

Nevertheless, the most possible position that we can ascribe to post-microsomal pellet in cell metabolism from our present knowledge is that of ribosomal subcomponents. However, much work is required before this can be said with any degree of certainty.

SUMMARY.

SUMMARY.

1. We have isolated a fraction from a rat liver homogenate which is sedimentable from the supernatant of a 1 hour centrifugation at 105,000g by centrifugation for 3 hours at 105,000g.

2. This fraction, which we have called post-microsomal pellet has an RNA/protein ratio of about 0.2 and a very low content of phospholipid. It represents about 6 mg. material per 5 grams of liver.

3. Both ultracentrifugation and electrophoresis reveal the presence of at least four components in post-microsomal pellet ranging in size from 5 S to 57 S.

4. Post-microsomal pellet contains activating enzymes for a variety of amino acids and is able to replace cell sap pH 5 enzyme in a ribosome-incorporating system. It is also able to inactivate sRNA.

5. Another property of post-microsomal pellet which is quite independent of the activating enzyme activity is the capacity of post-microsomal pellet to incorporate amino acids into a form which is stable to hot PCA. The incorporation of some amino acids is dependent on the presence of ATP but others can be incorporated in the absence of ATP. Neither reaction can be equated to true protein synthesis, however. The relationship of this incorporating system to some mechanisms (other than the classical system) which are proposed in the literature is discussed.

6. The possibility of post-microsomal pellet being a breakdown product of some larger sub-cellular fraction has been explored. Treatment of various sub-cellular fractions with EDTA, sonication, deoxycholate and pyrophosphate did not yield any material with similar properties to post-microsomal pellet. Microsomes did produce some material sedimentable under the same conditions as post-microsomal pellet which could incorporate amino acids. However, the characteristics of the incorporation reaction were different from those of post-microsomal pellet.

7. The possible biological significance of post-microsomal pellet is also reviewed.

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