STUDIES ON THE BIOSYNTHESIS OF NUCLEIC ACIDS AND PROTEINS

by

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Thesis submitted for the Degree of

Doctor of Philosophy of the

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April, 1964.

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ACKNOWLEDGEMENTS.

This research was carried out with the permission of Professor J.N.Davidson, F.R.S. and was actively helped and accelerated by Dr. H.N.Munro, to whom I am very grateful.

I also thank Dr. A.Fleck, Miss S.Waddington and Mr. T. Duffy for enthusiastically collaborating in several aspects of the work and the Commonwealth Scholarships Commission for providing my entire financial support.

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Abbreviations.

The following abbreviations are used in this thesis:-

AMP	adenosine-2'-3' monophosphate
ATP	adenosine-5' triphosphate
CMP	cytosine-2'-3' monophosphate
DNA	deoxyribonucleic acid
GMP	guanosine-2'-3' monophosphate
GTP	guanosine-5' triphosphate
NADH2	reduced nicotinamide-adenine dinucleotide
PCA	perchloric acid
P-lipid	phospholipid
RNA	ribonucleic acid
TCA	trichloracetic acid
Tris	tri (hydroxymethyl) aminomethane
UMP	uridine-2'-3' monophosphate

GENERAL INTRODUCTION.

A) Aspects of the Ultrastructure and Composition of hepatocytes

Rat liver is composed of a number of different types of cells but the predominant type present is the hepatocyte or parenchymal cell. Though hepatocytes make up only about 60% of the cells in liver on a cell count it has been estimated that they constitute about 90% of the cell volume (Daoust, 1958) and hence they probably account for about 90% of the wet weight of cells in liver also. It is assumed that the compositional and metabolic results obtained in this investigation refer largely to hepatocytes and that no other single cell type contributes significantly to them.

Examination of hepatocytes with the light microscope readily allows them to be differentiated into a cytoplasmic and a nuclear phase and further examination with the light and electron microscope indicates that each of these phases contain a variety of structures.

1) The plasma membrane

The plasma membrane of hepatocytes appears to consist of a single unit membrane about 75 Angstrom units thick (Robertson, 1957), which may be continuous in places with elements of other membranous structures. (Fig. 1). Investigations on other mammalian cells suggest that the plasma membrane contains antigen-like substances which allow like cells to recognize one another and also substances responsible for intercellular adhesion (see review by Weiss, 1963). The plasma membrane also seems to function in the transport of material into and out of cells by pinocytosis and secretion (see review by Woodin, 1963). It seems probable that the

plasma membrane of hepatocytes also participates in these functions of recognition, adhesion and transport.

2) The endoplasmic reticulum.

The endoplasmic reticulum is a ramifying system of unit membranes organized into tubules, vesicles and cisternae, found throughout the cytoplasm in hepatocytes (Fig. 1). The cisternae, which are the most widespread elements of the endoplasmic reticulum, consist of membrane bounded channels generally about 400 - 500 Angstrom units across, the interior of which is termed the intracisternal space. Much of the endoplasmic reticulum is studded with small electron-opaque particles about 100 - 200 Angstrom units in diameter called ribosomes. Reticulum with adherent ribosomes is termed granular or rough-surfaced reticulum (Palade, 1956) or alpha-cytomembrane (Sjostrand, 1956). Continuous in places with the rough-surfaced reticulum is agranular or smooth-surfaced reticulum (Palade, 1956) or gamma-cytomembrane (Sjostrand, 1956), which possesses few or no adherent ribosomes. A high concentration of this smooth-surfaced reticulum is found in the region of the Golgi apparatus (Dalton and Felix, 1956; Rothschild, 1963). It has been claimed that the endoplasmic reticulum is continuous with the plasma membrane so that the intracisternal space connects directly with the extracellular environment (Bennett, 1956; Hoffman and Grigg, 1958); however, though temporary continuities undoubtedly exist it is not generally accepted that the intracisternal space is continuously connected with the extracellular environment (Porter, 1961). /

In addition to those bound to the surface of the endoplasmic reticulum, some ribosomes are also observed unassociated with membranes in normal hepatocytes, these are termed free ribosomes (Palade and Siekevitz, 1956a). The proportion of free ribosomes in hepatocytes is increased in a number of experimental and pathological conditions including prolonged fasting (Fawcett, 1955; Henshaw et al, 1963) maintenance on a protein free diet (Bernhard and Rouiller, 1956), feeding certain carcinogens (Emmelot and Benedetti, 1960), administration of carbon tetrachloride (Recknagel and Lombardi, 1961), treatment with actinomycin (Munro and Korner, 1964), in liver regeneration and in embryonic liver (Howatson and Ham, 1956). It is not known whether there is any common aetiology for the increase in free ribosomes in these diverse conditions.

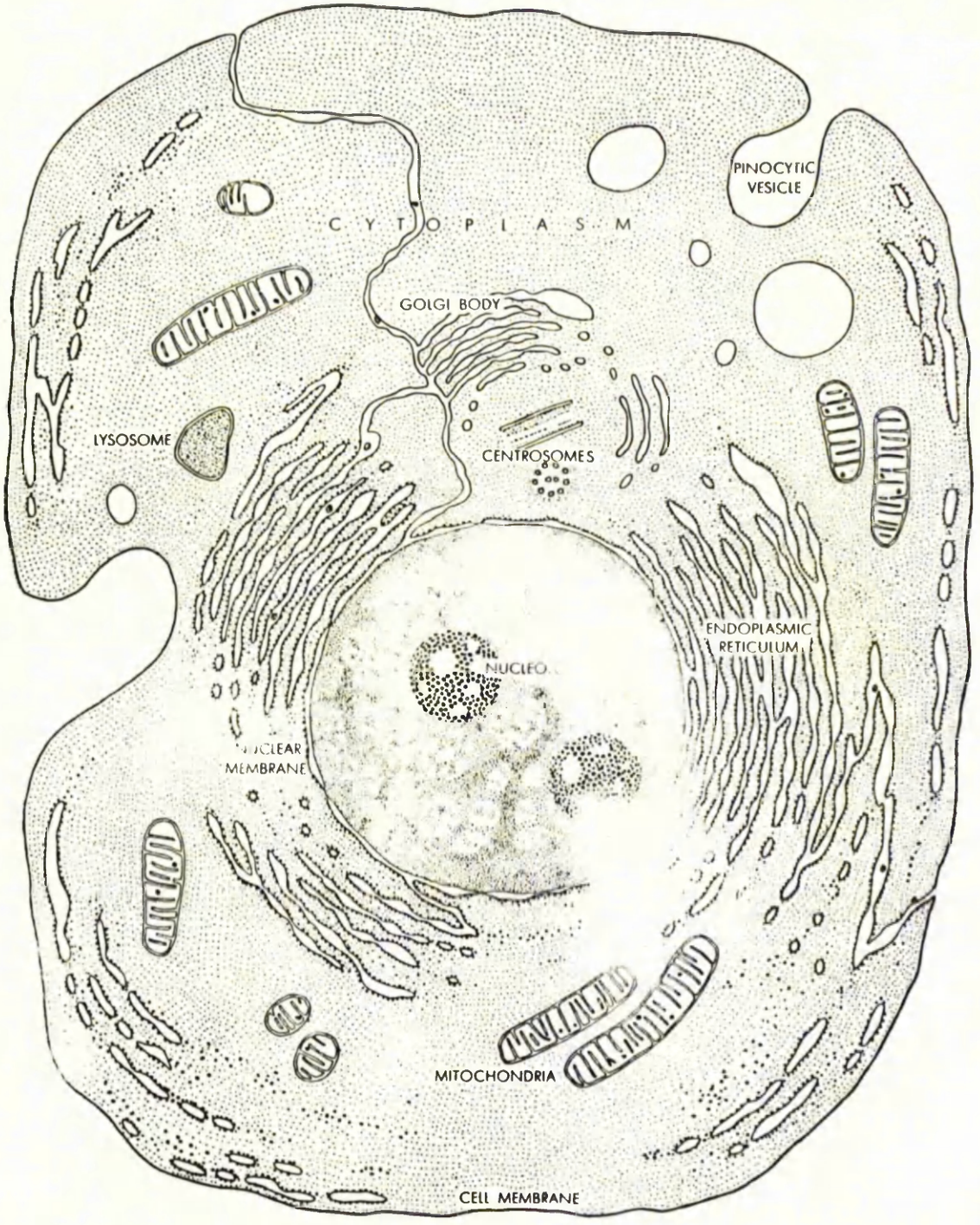
3) The nucleus.

The nuclei of hepatocytes are their largest intracellular organelles. They are divided off from the cytoplasm by a two layered membrane punctuated by large apertures or pores (Watson, 1959) (Fig.1) These pores do not appear to allow free transfer of even small molecules from the nucleus to the cytoplasm as indicated by the high electrical resistance across the nuclear membrane (Lowerstein and Kanno, 1962) and the ability of the nucleus to accumulate sodium ions (Mirsky and Osawa, 1961). The demonstration by Watts and Harris, (1959) that small molecular weight RNA breakdown products are retained within the nucleus and reutilized for nuclear RNA synthesis, under some conditions,

without reaching equilibrium with cytoplasmic RNA precursors, also points this way. The pores in the nuclear membrane may be blocked off with a thin membrane (Merriam, 1961). The outer layer of the nuclear membrane is distinct from the inner one in that it is rough-surfaced, possessing some adherent ribosomes (Buck, 1961). There is evidence of continuity between the nuclear membrane and the endoplasmic reticulum in fixed sections of hepatocytes (Watson, 1955) as well as other cells (Whaley et al, 1960; Porter, 1961), but there are no data on the frequency or duration of these interconnections in living cells. It has been suggested by Whaley et al, (1960) that these interconnections, like the blebs or membranous lamellae seen near the nucleus and interpreted as emanating from it (Watson, 1955; Gay, 1955; Rebhun, 1961), may be much more abundant in undifferentiated cells than in fully differentiated ones like hepatocytes (see also Porter, 1961).

Structures visible within the interphase nucleus of hepatocytes include a fine chromatin network and one or several electron-opaque regions, termed nucleoli. Histochemical staining and observations of nucleoli with the ultraviolet microscope coupled with specific nuclease treatment show that they contain a much higher concentration of ribonucleic acid than the rest of the nucleus (see review by Brachet, 1950). Recent studies of nuclei, with the electron microscope in fixed and sectioned tissues, have shown that they too contain ribosomes, similar to those in the cytoplasm, except that nuclear ribosomes

Fig1. Schematic diagram of a mammalian cell. From Brachet, J., (1961), "The Living Cell". Scientific American 205, 51.



are found exclusively free and unattached to membranes (Callan, 1956; Mirsky and Osawa, 1961).

Other organelles found in hepatocytes include mitochondria, lysosomes, pinocytosis vesicles and the centrosome (Fig. 1) (Brachet, 1961). These all have very important functions; however they are not considered specifically in the following investigation or discussion.

b) Subcellular Localization of DNA, RNA and Phospholipid in Hepatocytes.

There is abundant evidence from histochemical staining, observations with the ultraviolet microscope coupled with specific nuclease treatment, and analysis of isolated nuclei that these contain most if not all of the DNA from liver. However there have been recurrent reports that some DNA may also be associated with mitochondria (Zollinger, 1950; Chevremont et al, 1957), which may not be wholly explained in terms of contamination.

In the case of RNA, it can be calculated that about 90% is associated with the cytoplasmic phase and about 10% with nuclei in hepatocytes (Hogeboom and Schneider, 1955). About 80-85% of the cytoplasmic RNA is located in the microsome fraction. Of the remainder not more than 10% seems to be transfer RNA while perhaps 5% may be associated with mitochondria. At least 90% of the microsomal RNA can be shown by sucrose gradient centrifugation to consist of two species of ribosomal structural RNA (Sibatani, 1963), which sediment at about 29s and 18s respectively (Hiatt, 1962; Hallinan, Munro and Fleck, /

1963; Munro and Korner, 1964). The remaining 10% or so is mainly of lower sedimentation constant. The highest concentration of RNA in nuclei is found in the nucleoli (Brachet, 1950) but nuclear ribosomes (Frenster et al, 1960) and transfer-RNA (Hopkins, 1959; Mirsky and Osawa, 1961), must also account for part of the RNA in nuclei.

Liver phospholipids are located almost exclusively in membranous organelles such as the endoplasmic reticulum, mitochondria and nuclei and very little phospholipid is found in the cell-sap fraction provided this is prepared in such a way that small membranous components are adequately sedimented from it (Spiro and McKibbin, 1956; Biezenski and Spaet, 1961; Bloch, 1960). In fact Spiro and McKibbin's figures show that the cell sap fraction contains only about 2% of the total liver phospholipid. The fraction richest in phospholipids seems to be the microsomes (Spiro and McKibbin, 1956), while purified nuclei are quite poor in phospholipids (Chauveau et al, 1956; Gurr et al, 1963).

c) Structure and Composition of Rat Liver Microsomes.

Any consideration of the structure and composition of microsomes must take into account the conditions used to isolate this fraction, since these can exert considerable influence upon the components found in it. Impulses varying from 17,000g for 2-3 hours (Claude, 1937) up to 150,000g for 2-4 hours (Bernhard et al, 1954) have been used to /

sediment microsomes from homogenates of rat liver, made in a variety of media. The impulse used to sediment the fraction, the viscosity of the homogenate, the types of ions present and the impulse used to sediment premicrosomal particles, which may also bring down a substantial proportion of the heavier microsomes (e.g. Siekevitz and Palade, 1958a) all influence the types of components found in the microsome fraction and hence its composition. There is a tendency now to use an impulse of 105,000g for 1 hour to sediment microsomes from homogenates of liver in 0.25 - 0.44M sucrose, containing buffer, potassium chloride and magnesium chloride and these conditions were used in this study: however an impulse of 105,000g for 3 hours had to be used to sediment microsomes from a 0.88M sucrose homogenate of liver, as shown by Moule et al (1960).

In addition to fragments of the rough and smooth-surfaced endoplasmic reticulum and free ribosomes (Palade and Siekevitz,1956), rat liver microsomes generally contain in addition some small lysosomes (Novikoff, 1957), glycogen particles (Hall and Doty, 1958) plus some enzymes absorbed to them (Luck, 1961), ferritin (Moule et al, 1960) and possibly some fragments of ruptured cell membranes and of membranous structures from the vicinity of the bile capillaries (Palade and Siekevitz, 1956a). Consequently they must be considered to be a very heterogenous fraction indeed. Table I lists some representative figures for the amounts of protein, RNA and phospholipid in rat liver microsomes, /

TABLE 1. Amounts of protein, RNA and phospholipid in rat liver microsomes expressed as a percentage of the amounts of these components in whole liver.

Component	Percentage	Reference
Protein	12	Palade and Siekevitz (1956a)
	18	Greenstein (1956)
	20 - 25	Bernhard et al (1954)
*RNA	33	Palade and Siekevitz (1956a)
	45	Greenstein (1956)
	44	Kuff and Zeigel (1960)
P-lipid	22	Palade and Siekevitz (1956a)
	47	Kuff and Zeigel (1960)
	36	Collins and Shotlander (1961)

* The percentage of whole liver RNA in isolated microsomes is rarely if ever as high as the calculated amount of RNA in microsomal components (see above). Incomplete cell breakage and failure to recover microsomal components contaminating other subcellular fractions probably accounts for this low recovery in most cases, though degradative losses may also occur during subcellular fractionation.

expressed as a percentage of the amounts of these three components in whole rat liver. This gives some idea of the typical range of composition, produced by different conditions for isolating microsomes; none of the figures listed are extreme values.

Extensive studies of protein synthesis (e.g. Roberts, 1958; Harris, 1961; Chantrenne, 1961), drug detoxification (reviewed by Brodie et al, 1958), electron transport (reviewed by Dallner, 1963) and RNA synthesis and turnover (e.g. Strauss and Goldwasser, 1961; Hiatt, 1962; Harris et al, 1963; Hallinan et al, 1963, a,b, 1964; Munro and Korner, 1964) have been carried out on whole microsomes from liver and other mammalian cells, while this fraction has also been used to study phospholipid synthesis (Paulus and Kennedy, 1960) and many other metabolic processes (reviewed by Rothschild, 1963). However, despite the disadvantages of working with so heterogeneous a fraction, comparatively few attempts have been made to study the composition or metabolic behaviour of the fragments of rough and smooth-surfaced endoplasmic reticulum and the free ribosomes, which make up the bulk of liver microsomes. The only one of the above metabolic processes which has been studied at all fully in these sub-fractions, is protein synthesis and this mostly during the last few years while the studies reported here were in progress. This is undoubtedly because no method was available to isolate these three components from rat liver when these studies were begun in 1961 and because the method since developed by Moule et al (1960) and Chauveau et al (1962), though giving /

a good separation, requires at least 24 hours of continuous, high speed centrifugation, with concomitant risks of degenerative changes taking place during this prolonged separation, and it does not allow the free ribosome fraction to be recovered in full (Chauveau et al, 1962). Despite these shortcomings however this method gives an estimate, at least, of the relative amounts of the three sub-microsomal components in liver and their composition as shown in Table 2.

This shows that the rough-surfaced vesicle fraction, composed predominantly of fragments of the rough-surfaced endoplasmic reticulum of hepatocytes, contains about 65% of the total protein, RNA and phospholipid of the microsomes while the smooth surfaced vesicles, derived from the smooth surfaced endoplasmic reticulum and possibly from other agranular membranes, contain about 20% of the protein and phospholipid and only a very small amount of RNA. The free ribosomes contain about 20% of the RNA, 10% of the protein and negligible phospholipid. These figures agree very well with the composition of these components separated by a rapid and convenient method devised by us and described below, except that the free ribosomes and to a lesser extent the smooth surfaced vesicles are incompletely recovered by the above method of Chauveau et al, (1962). The sub-microsomal fractions, separated by the new procedure have been used to study the incorporation of amino acid into protein in vitro and in vivo and RNA and phospholipid turnover in vivo.

~~a) Protein~~ /

TABLE 2. Amounts of protein, RNA and phospholipid in submicrosomal fractions isolated from rat liver.

These figures are computed from published values for total nitrogen, RNA-P and phospholipid-P, assuming that protein contains 16% N, that RNA contains 15.5% N and 9.6%P and that phospholipid contains 1.8% N and 4% P. The smooth-surfaced vesicles and free ribosomes were separated by centrifugation for 20 hours in sucrose of density 1.21. (from data of Chauveau et al, 1962).

Fraction	Percentage of whole microsome content recovered in fraction			RNA / Protein ratio	P-lipid / protein ratio
	Protein	RNA	P-lipid		
rough-surfaced vesicles	65	62	66	0.13	0.56
smooth-surfaced vesicles	17	5.5	26	0.04	0.76
free ribosomes	8	20	0.7	0.31	0.05

d) Protein Synthesis in Liver.

Hepatocytes in adult rat liver turn over so slowly that they can be considered as non-multiplying cells for all practical purposes. However liver synthesizes protein at a substantial rate, so most of this protein must either be for export by secretion as in the case of plasma proteins (Peters, 1962b) or components of enzymes and sub-cellular-organelles, which turn over within the life time of the hepatocyte (Fletcher and Senadi, 1961).

1) Site of protein synthesis.

It is now well established as a result of studies in vivo, by injecting radioactive amino acids into animals and determining the specific activities of total protein or of individual pure proteins in subcellular fractions, that the microsomes are the most active site of protein synthesis in liver and many other tissues (e.g. Keller et al, 1954; Hultin, 1955; Rabinovitz and Olson, 1959; Kern et al, 1959; Peters, 1962a). This has been completely confirmed by comparing the ability of isolated subcellular fractions to incorporate amino acids into proteins in vitro (e.g. Simkin and Work, 1957; Cohn and Butler, 1958, Siekevitz and Palade, 1958).

Further studies have shown that the ribonucleoprotein (ribosome) particles of the microsomes are the immediate sites of synthesis of proteins and not the lipo-protein membranes, which may constitute about 80% of the weight of the fraction in liver (e.g. Littlefield and Keller,/

1957; Simkin and Work, 1957; Korner, 1960; Takanami, 1960). It has now been shown that the most active ribonucleoprotein particles in mammalian microsomes are those with sedimentation constants exceeding 120s, which seem to consist of a number of 80s ribosomes attached to a strand of messenger-RNA (see below) and are termed polysomes or ergosomes (Gierer, 1963; Penman et al, 1963; Warner et al, 1963: Wettstein et al, 1963). It has been shown independently that the most active ribonucleoprotein particles synthesizing protein in liver are also associated with fragments of the rough-surfaced endoplasmic reticulum (Henshaw et al, 1963; Hallinan and Munro, 1963, 1964) so it is quite likely, though not yet definitely proven, that the bulk of the polysomes in liver are bound rather tightly to the rough-surfaced endoplasmic reticulum. Very recent work seems to suggest that the bulk of the polysomes may be associated in bacteria also with a lipoprotein membrane, the protoplast membrane (Schlessinger, 1963), though they are definitely not associated with membranes in the reticulocyte (Warner et al, 1963; Hallinan, unpublished observations). There is good evidence that the polysome structure is not an obligatory requirement for protein synthesis, but that single ribosomes attached to a strand of messenger RNA can synthesize complete peptide chains of haemoglobin (Lamfrom and Knoff, 1963).

2) Mechanism of protein synthesis.

2.1 Initial stages. Proteins are synthesized from carboxyl-activated amino acids which are initially formed as enzyme-bound amino acyl adenylates by the reaction of an amino acid with ATP, a reaction

catalysed by specific amino acid activating enzymes as shown in Equation 1. (Hoagland, 1955; Hoagland et al, 1956).

$$R\text{-}CHNH_2COO^{-1} + ATP \xrightarrow[\text{enzyme}]{\text{activating}} P - P + ROHNH_2CO \quad AMP \quad \text{(Eqn. 1)}$$

Activating enzymes have been found for all of the 18 common amino acids in animal proteins as well as for the amides glutamine and asparagine (Chantrenne 1962) but no enzyme has yet been demonstrated for hydroxyproline; it seems as if hydroxyproline is formed from proline after this is incorporated into proteins (Chantrenne, 1961). Free amino acyl adenylates are extremely reactive and enzyme binding may prevent them from acylating compounds within the cell in a random fashion (Chantrenne, 1961).

However amino acyl adenylates are not the immediate activated precursors which are incorporated into polypeptide linkages. It is now knownthat the amino acid activating enzymes catalyse the conversion of their bound amino acyl adenylate into a complex of the amino acid with an homologous transfer RNA (reviewed by Hoagland,1960), releasing adenosine monophosphate in the process as shown in Equation 2.

$$t\text{-}RNA + Enz - AA \quad AMP \xrightarrow[\text{enzyme}]{\text{activating}} AA\text{-}t\text{-}RNA + AMP + Enz. \quad \text{(Eqn. 2.)}$$

The amino acid is linked in an ester linkage to the 2' or 3' hydroxyl of the ribose of the terminal adenosine of transfer RNA (Zacau et al, 1958) and is carried thus to the site of protein biosynthesis. The transfer RNA molecule is postulated also to contain a trinucleotide sequence (codon), which serves to identify the amino acid by interacting with a complementary sequence at the site of protein biosynthesis /

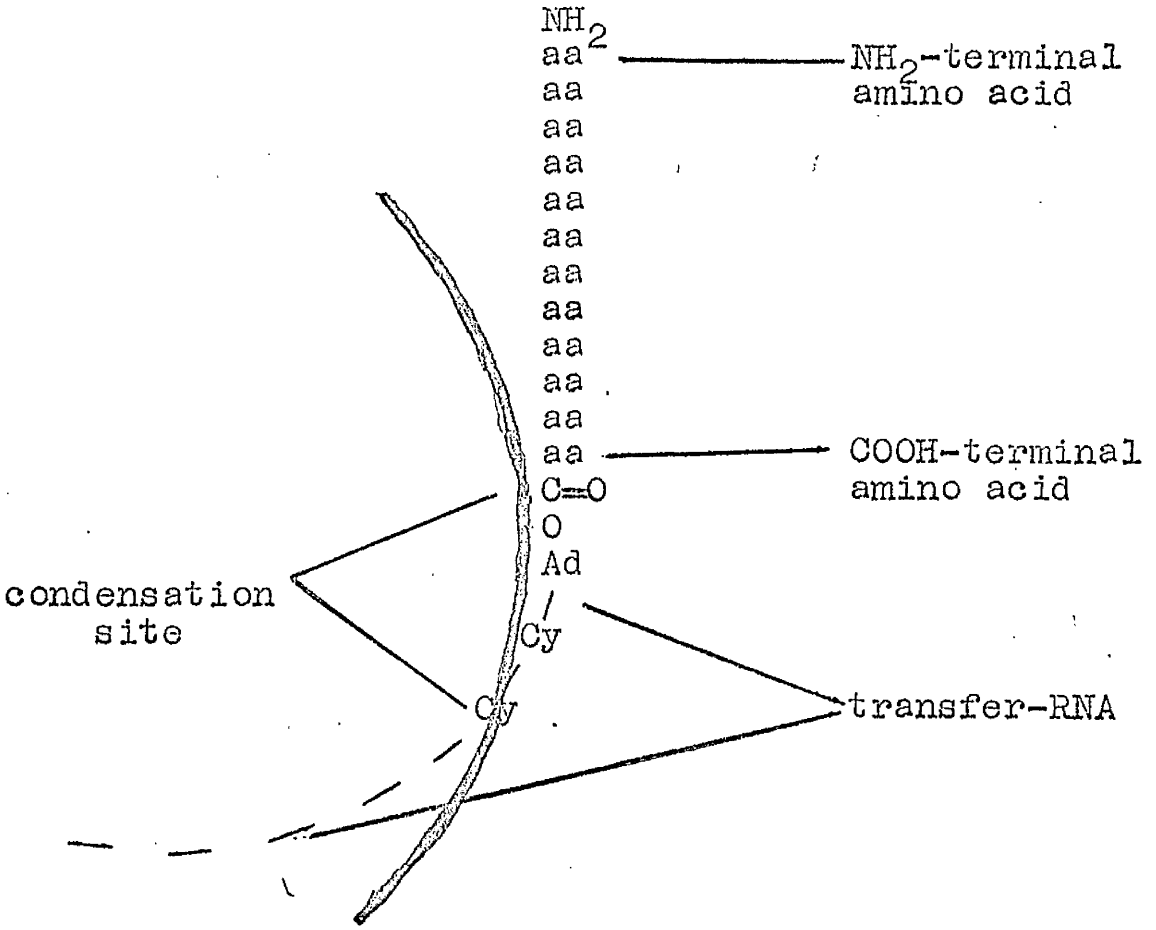
(the messenger's codon), ensuring that amino acids are assembled into polypeptides in the correct order (Nathans et al, 1963).

2,2 The sequential condensation of amino acids into polypeptides.

The initial reactions in protein synthesis described above all occur in the so called cell sap or soluble fraction and have been reconstruct in fairly simple and well characterized systems. They do not require the participation of ribonucleoprotein particles. However, the reactions described below, which accompany the sequential condensation of activated amino acids onto the C-terminal end of a growing peptide chain are much less well understood and have only been convincingly demonstrated so far in complex systems containing ribonucleoprotein particles, GTP, ions and two transfer-enzymes (Nathan and Lipmann, 1961; Noll et al, 1963). The most comprehensive model put forward to account for the condensation process so far is that of Noll et al, (1963).

This model deals with the synthesis of polypeptides by aggregates of '73s' ribosomes from rat liver, strung together on a strand of messenger RNA (polysomes or ergosomes). These ribosomes consist of a '50s' subunit and a '30s' subunit and contain a condensing site, where, in the presence of GTP and two transfer enzymes, t-RNA bound amino acids can be condensed on to the C-terminal end of a partially formed peptide chain. This partially formed chain is probably bound near the condensing site by its carboxyl group to the ribosome surface, t-RNA serving as a ligand at one stage of /

Fig. 2. Bonding of a partially completed peptide chain near the condensation site with a t-RNA molecule as ligand.



the polymerization process (Fig. 2) and the first transfer enzyme at another stage (see below).

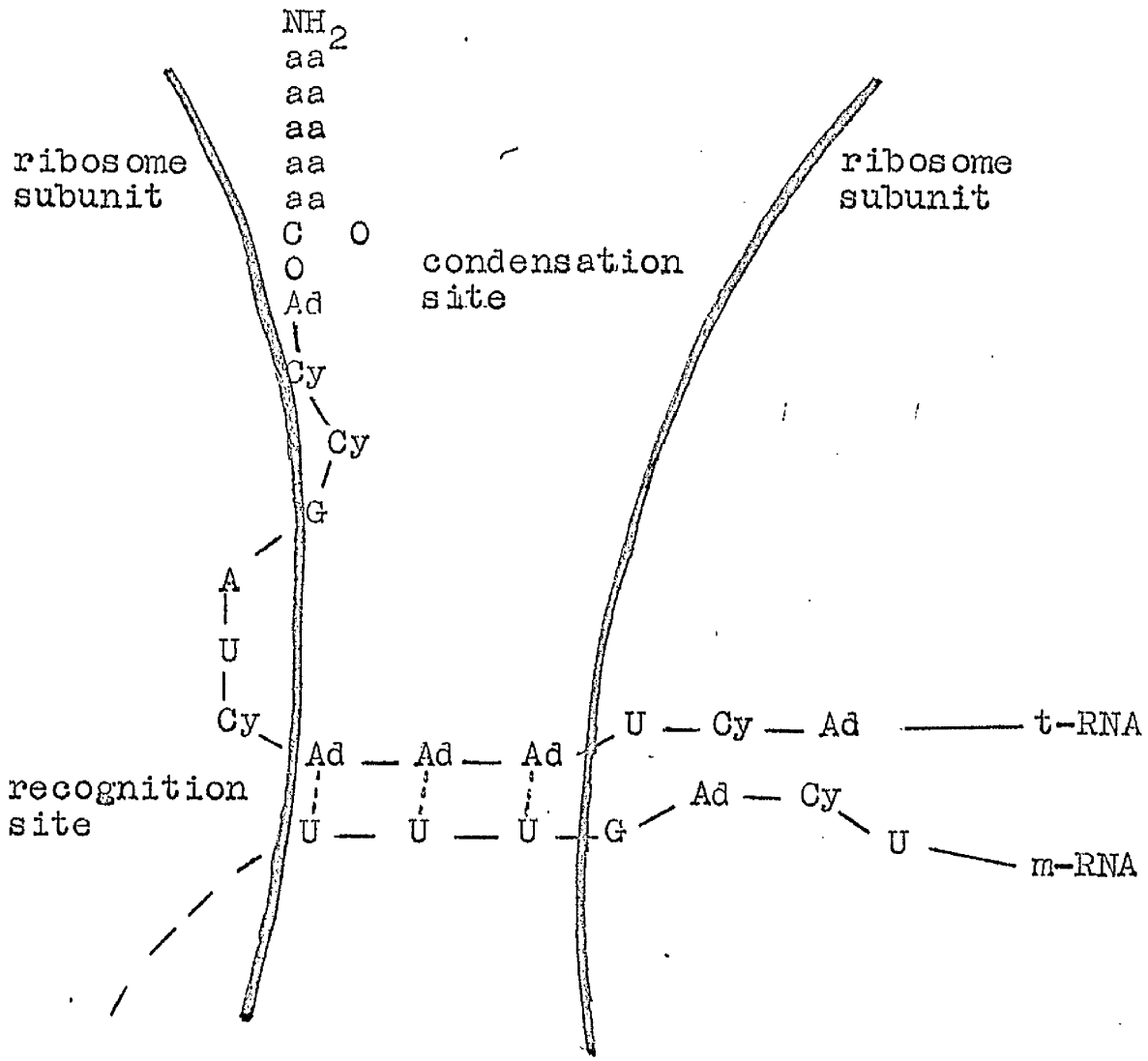
The messenger RNA (m-RNA) consists of a linear sequence of ribonucleotides, specific triplets of which (codons) code sequentially for one amino acid after another in the polypeptide chain *. The synthesis of this m-RNA is directed by the DNA of its corresponding structural gene, which encodes the primary structure (i.e. amino acid sequence) that the polypeptide should have when completed.

The ribosomes also contain a specific recognition site, possibly in or near the groove between its '50s' and '30s' subunits, to which m-RNA is attached (see Fig.3). Only one codon or nucleotide triplet of m-RNA can fit into this recognition site at any one time and when the complementary t-RNA-amino acid complex collides with the site (Ts O and Lubell, 1960), its codon forms a very weak association with the m-RNA codon by hydrogen bonding between their complementarity bases, adenine bonding with uracil and guanine with cytosine. This then allows the t-RNA amino acid complex to bond itself more strongly to the ribosome surface, or one of the transfer enzymes, possibly using its terminal - CCA sequence to form the bond.

However before the new, incoming t-RNA amino acid can condense with the partially completed peptide chain, or even before /

* The consequences of degeneracy in the genetic code are omitted here for simplicity (Crick, 1963).

Fig. 3. Complementation between an m-RNA codon and a t-RNA codon at the recognition site.



its codon can begin to interact with that of the m-RNA as described above, the terminal t- RNA, which coded for the terminal amino acid now on the peptide chain, and which is still attached both to the end of the peptide chain and to the recognition site on the ribosome, must be removed. This is done by replacing it by the terminal phosphate of GTP, so that the peptide chain now ends in an energy-rich acyl phosphate group and the ribosome's recognition site becomes vacant. This reaction is catalysed by the first transfer enzyme (Eqn.3).

$$NH_2aa_1aa_2aa_3aa_4COO\text{-}t\text{-}RNA_4 + GTP \quad GDP + t\text{-}RNA_4 + NH_2aa_1aa_2aa_3aa_4COO\text{-}P \quad \text{(Eqn.3)}$$

Simultaneously, part of the energy released in splitting off the terminal phosphate of GTP powers a translational movement of the m-RNA relative to the vacant recognition site on the ribosome surface so that the next codon on the t-RNA-amino acid then interacts with the new m-RNA codon binding its attached amino acid to the ribosome or the transfer enzyme near the condensation site as described above.

The final step in the process then consists in the interaction of the acyl phosphate group of the partially complete peptide chain with the amino group of the new, incoming t-RNA-amino acid at the condensation site. This reaction, which is probably catalysed by the second transfer enzyme, results in the introduction of the new amino acid-t-RNA at the end of the partially completed peptide chain while phosphate is split off. The cycle can then repeat with the next amino acid in the sequence and so on until the complete peptide chain is synthesized.

There is no general agreement as to how the protein synthesizing system recognizes when a complete peptide chain is synthesized. In polysomes from reticulocytes synthesizing haemoglobin peptides it seems as if individual strands of messenger RNA code for single peptide chains so that when a ribosome traverses the entire length of the messenger and reaches the end, a complete chain is made (Warner et al, 1963; Geirer, 1963). However, it has been speculated that in some cases a single messenger strand may code for more than one polypeptide chain, as say in the biosynthesis of integrated multi-enzyme systems (Ohtaka and Spiegelman, 1963; Horibata and Kern, 1964). In such a case the messenger strand would presumably have to contain some form of 'end-message' to indicate when each peptide chain is complete. This could be some special triplet or one or more unusual bases, such as pseudouridylic acid (Crick, 1963).

2.3 The terminal stages in protein synthesis.

After a complete peptide chain is synthesized by rat liver polysomes, released artificially from the rough-surfaced endoplasmic reticulum membrane by deoxycholate treatment (Wettstein et al, 1963; Noll et al, 1963) the individual '73s' ribosomes become detached from the messenger RNA strand and enter the free ribosome pool (monomer pool) in the cell sap. These '73s' free ribosomes still have the completed polypeptide chain bound to them by their terminal t-RNA molecules /

(Noll et al, 1963) and the completed polypeptides are only released from the free ribosomes very slowly. It is not clear whether the release process normally involves splitting the linkage between t-RNA and the ribosome followed by the linkage between t-RNA and the peptide chain, allowing both the t-RNA and the finished peptide to be released, or whether the t-RNA to peptide linkage is split, releasing the peptide and leaving the terminal t-RNA still bound to the ribosome.

However it is clear that release of the finished polypeptide from the ribosome occurs much more rapidly with intact microsomes than with deoxycholate polysome systems. In liver most of the newly synthesized polypeptides appear very rapidly in the lipoprotein membrane portion of the microsomes, shortly after these are incubated with labelled amino acids and they are presumably passed on to the membrane from the ribosomes (e.g.Peters, 1959; Campbell, 1961; Peters, 1962, a,b.). One would imagine that this would mean that the finished polypeptides are normally released from the ribosomes and passed on to the membranous fraction, before or at the same time as the ribosomes are released from the messenger-RNA strand and leave the rough-surfaced reticulum to enter the free ribosome pool in the cell sap. Otherwise some mechanism would have to exist whereby the endoplasmic reticulum membrane could recognize and re-accumulate newly-synthesized polypeptides from the cell sap. A proteinaceous factor from the cell sap, termed /

'release factor' has been implicated in the process of freeing completed polypeptides from rat liver ribosomes (Hultin, 1962).

Proteins possess a complex secondary and tertiary structure, formed by hydrogen bonds, non-polar (hydrophobic) bonds and in some cases disulphide linkages between different regions of their peptide chains. It is only when this secondary and tertiary structure is established that the protein possesses many of its physiological characteristics such as enzymic activity, serological specificity and ability to bind its prosthetic group. However there is little or no information available yet, on how secondary and tertiary structure is formed or how its formation is controlled (Chantrenne, 1961). It seems to be generally assumed at present that once the primary structure of a protein is established, folding into the secondary and tertiary structure occurs spontaneously (Perutz et al, 1960); however there is plainly a need for further investigation of this aspect of protein synthesis.

Certain proteins (e.g. haemoglobin) are made of several polypeptide chains, either different or identical in primary structure. It is generally believed that association between these separate chains, to make up the complete, physiologically active protein occurs spontaneously (Chantrenne, 1961). However, it has been suggested very recently that two polypeptide components of a protein may be made on a single ribosome, presumably coded by a single strand of messenger RNA /

and that association between them may occur on the synthetic site (Horibata and Kern, 1964).

It should be mentioned here that Peters (1962,a,b,) has shown that serum albumin which is quantitatively the most important protein synthesized by rat liver microsomes, is transferred very rapidly from its site of synthesis on the ribosomes into the rough surfaced endoplasmic reticulum, and thence is transported to the smooth-surfaced endoplasmic reticulum. It seems likely that the newly synthesized serum albumin is transferred from the ribosomes across the endoplasmic reticulum membrane into the intracisternal space of the rough surfaced endoplasmic reticulum and is thence translocated, presumably via a continuous cisternal connection to the smooth surfaced reticulum, where it may accumulate for a short time before being secreted from the liver cells.

e) RNA Synthesis in Rat Liver

As described above, about 90% of the RNA in rat liver seems to be localized in the cytoplasmic compartment and only about 10% in the nuclear compartment. At least 75-80% of the cytoplasmic RNA consists of two different species of ribosomal structural RNA, sedimenting at about 29s and 18s respectively (Hogeboom and Schneider, 1955; Hiatt, 1962; Hallinan and Munro, 1963; Munro and Korner, 1964). The bulk of the remaining cytoplasmic RNA sediments from 4-5s and probably consists mainly of transfer RNA (Sibatani, 1963). A small fraction of the cytoplasmic

RNA of liver turns over rapidly and shows great susceptibility to breakdown by trace amounts of ribonuclease in intact ribonucleoprotein preparations (Staehelin et al, 1964; Noll et al, 1964). Similar rapidly-labelled cytoplasmic RNA from rat liver has been shown by ^{32}P dilution to have a nucleotide base ratio mid-way between DNA and ribosomal structural RNA (Munro and Korner, 1964) and this has been interpreted as indicating that it consists of a mixture of newly synthesised ribosomal RNA and of messenger RNA, with a DNA-like base composition. Noll et al, (1963) have calculated that messenger RNA comprises about 1.5% of the total RNA of a mixed polysome preparation from rat liver cytoplasm. This would mean that it made up about 1% of the cytoplasmic RNA from rat liver (see however Hiatt, 1962).

However, despite the small amounts present, messenger RNA is ascribed a very important function in protein synthesis (see above). It is postulated that messenger-RNA is a replica of the structural gene which encodes information specifying the primary and perhaps also secondary and tertiary structure of proteins (except that uracil replaces thymine in nucleotides),(Jacob and Monod, 1961). It is made under the direction of the structural genes and hence presumably in the nucleus in higher organisms (see however Harris, 1963).

The situation at present is very confused as to the location or the mechanism of synthesis of the different species of cytoplasmic RNA in mammalian cells. RNA-primed RNA polymerase and DNA-primed RNA-polymerase, capable of incorporating all four ribonucleoside /

triphosphates into RNA, have been found both in the nucleus (Burdon and Smellie, 1961; Weiss and Nakamoto, 1961; Abrams et al, 1962; Smellie, 1962) and in cytoplasmic fractions (Strauss and Goldwasser, 1961; Smellie, 1962; see review by Smellie, 1963). On the basis of autoradiographic studies (e.g. Singh et al, 1963) studies with actinomycin, an inhibitor of DNA-primed RNA polymerase, (e.g. Perry, 1962; Merits, 1963), and experiments with anucleate cell fragments (e.g. Prescott, 1959; Goldstein et al, 1960), various groups have concluded that the bulk of cytoplasmic RNA is synthesized in the nucleus. However others have concluded on the basis of similar experiments using auto-radiography (see review by Harris, 1963), actinomycin (Paul and Struthers, 1963) and anucleate cell fragments (Schweiger and Bremer, 1960), that a substantial amount of RNA synthesis may occur in the cytoplasm and that very little of the RNA of the cytoplasm can be derived from the nucleus (Harris, 1963). Hence the question of the origin of cytoplasmic RNA is still very much an open one.

However, since the nucleus contains all of the requisite enzymes, precursors and primers for RNA synthesis, it seems reasonable to assume that nuclear RNA is synthesized by the organelle itself. It also seems fairly well established that the immediate precursors incorporated into RNA are nucleoside triphosphates (see review by Smellie, 1963). Synthesis of a specific RNA species, with a determined nucleotide sequence, seems to involve one of the RNA polymerases,/

the nucleotide sequence of the RNA probably being directed by the RNA or DNA primer used. Polynucleotide phosphorylase does not seem to be able to produce a specific polynucleotide, containing a determined sequence of nucleotide bases (Smellie, 1963).

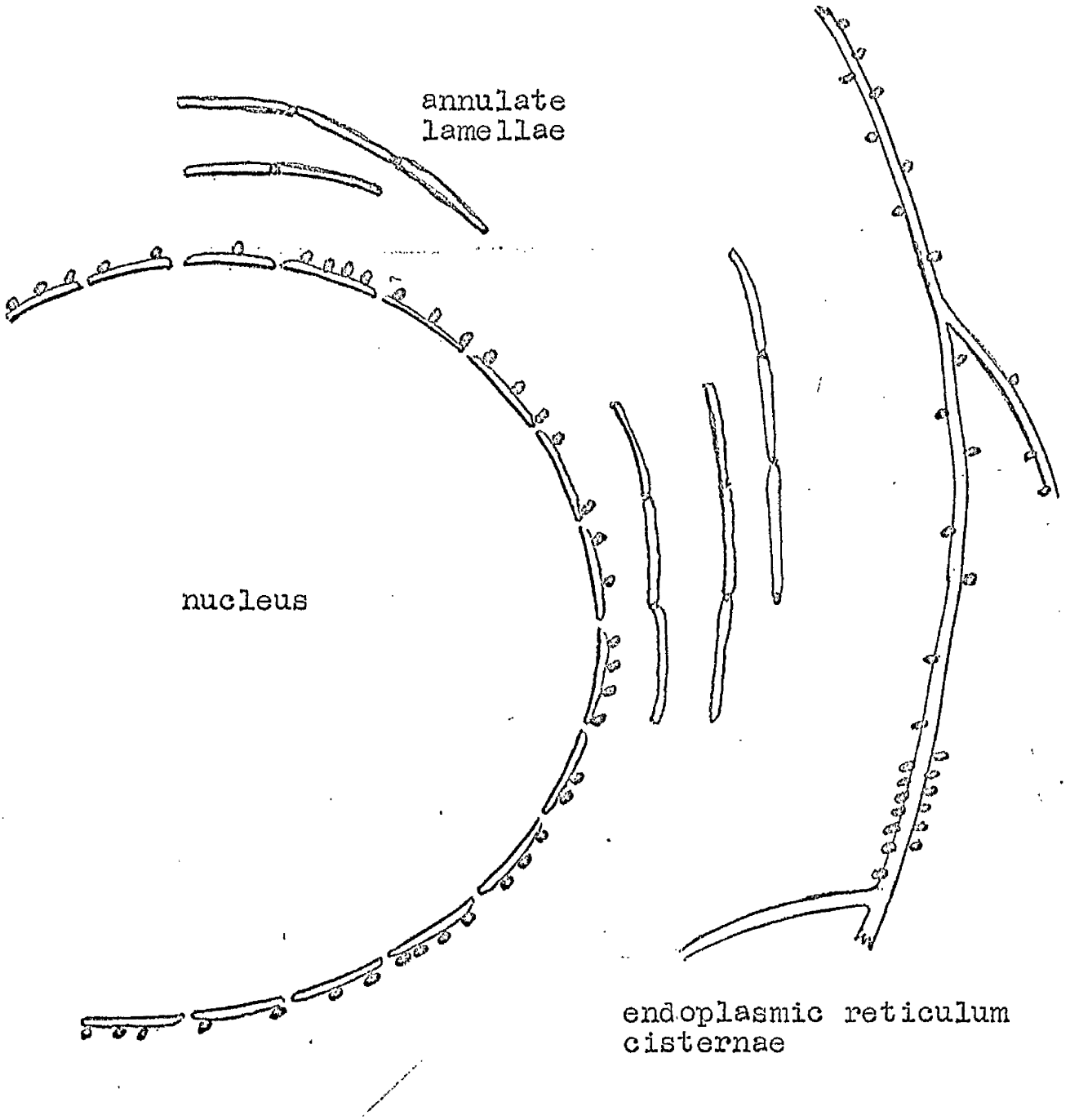
f) Origin of the Endoplasmic Reticulum in animal cells.

There is no definite information available about the origin and synthesis of the endoplasmic reticulum in animal cells, so one can only discuss the several possible mechanisms put forward to account for this process. It has been suggested that the endoplasmic reticulum may be derived from the plasma membrane (Watson, 1955), the nuclear membrane (e.g. Gay, 1955; Swift, 1956; Merriam, 1959; Gay, 1960; Rebhun, 1961; Kessel, 1963), the Golgi apparatus (Manton, 1960), pinocytosis vesicles (Brandt and Pappas, 1962) or by auto-synthesis (Fawcett, 1955; Merriam, 1962). It should also be added that more than one of the above mechanisms may contribute to the formation of the reticulum.

Virtually all of the above theories are based upon observations of fixed sections of cells with the electron microscope, which have revealed structures apparently originating from the various organelles and fusing with the endoplasmic reticulum. Only one preliminary attempt has been made to test any of these theories using tracer techniques (Merriam, 1962), and this yielded inconclusive results.

The problem of the origin of the endoplasmic reticulum is a difficult one to study. Sections used for electron microscopy (150-500 Å thick) sample only about 0.001 of the thickness of a hepatocyte, hence even persistent structural features may only be observed in /

Fig.4. Schematic portrayal of the formation of annulate lamellae from the nuclear membrane.



occasional sections, unless these features occupy a fairly large proportion of the cell volume. It is difficult therefore to determine whether an observed structural arrangement occurs persistently or only rarely during the incessant movement and rearrangement which cellular organelles undergo. Semi-quantitative electron microscopy, which involves examination of sufficient sections to allow statistical deductions to be made (e.g. Burge, et al, 1960) , can go some of the way towards simplifying the problems involved here, but this approach has not yet been applied to the origin of the endoplasmic reticulum. Indeed this type of problem, involving a dynamic interchange between structures within cells, is very difficult in principle to approach by electron microscopy. For example Fig. 4 schematically portrays the theory that the nuclear membrane produces annulate lamellae which serve as precursors of the endoplasmic reticulum (e.g. Rebhum, 1961). These annulate lamellae possess pores similar to those in the nuclear membrane and they are observed in the cytoplasm near the nuclear membrane. This arrangement has been interpreted to mean that the annulate lamellae are being produced by the nuclear membrane, moving into the cytoplasm and becoming endoplasmic reticulum. However electron micrographs of fixed, sectioned tissue only indicate the configuration of these membranes at the instant of fixation and no electron microscopic method has yet been developed to allow living tissue to be examined over a period of time. Hence it is doubtful if this interpretation of the dynamic state of these membranes is justified. It seems equally possible that they might be lying relatively stationary near the nuclear membrane /

or in fact be moving from the cytoplasm towards the nuclear membrane.

Tracer techniques of course permit an approach to the dynamic interrelations between cellular organelles to be made, providing the organelles can be isolated in a suitable state of homogeneity and a chemical component common to them can be labelled (Schoenheimer, 1942; Zilversmit et al, 1943). However application of this approach to the problem of the origin of the endoplasmic reticulum is not easy either, since most of the structures e.g. plasma membrane, nuclear membrane which have been suggested as precursors of it, either have not yet been isolated, or else are extremely difficult to isolate in reasonable yield and with a suitable degree of homogeneity. Because of this, little is known also of the chemical composition of these proposed precursor structures and so it is difficult to choose a chemical component, common to them and the endoplasmic reticulum, which is suitable for labelling.

However, both nuclei and the endoplasmic reticulum membrane contain phospholipids and it has recently been demonstrated that all or most of the lipids of nuclei are contained in their outer membranes (Gurr, Finean and Hawthorne, 1963). With the possibility of preparing nuclei, containing few contaminants, in a reasonably high yield, (Chauveau, Moule and Rouiller, 1956) it seemed feasible to label several of the phospholipids of the nuclear membrane and the rough and smooth surfaced endoplasmic reticulum membranes and follow their turnover, to determine whether this approach showed any precursor-product /

relationships between the various structures.

g) Scope of Present Investigations.

The investigations described below have been focussed on the following problems.

1) Comparison of various methods of sub-fractionating microsomes, culminating in the development of a rapid and efficient method of isolating the three major components of rat liver microsomes, rough - surfaced vesicles (fragments of rough-surfaced endoplasmic reticulum), free ribosomes and smooth-surfaced vesicles (fragments of smooth surfaced cytoplasmic membranes).

2) Comparison of the rates of incorporation of amino acids into protein by the three submicrosomal components, under controlled conditions in vitro and comparison of amino acid incorporation by rough-surfaced vesicles and free ribosomes in vivo.

3) The turnover of RNA in the various sub-microsomal fractions from liver as measured by the incorporation of radioactive RNA precursors in vivo. Studies were made on the total RNA of fractions and preliminary investigations were made of the turnover of ribosomal structural RNA in the rough surfaced vesicle and free ribosome fractions.

4) A comparative study was made of the turnover of total choline-containing phospholipids and purified lecithin and sphingomyelin from rough-surfaced vesicles, smooth surfaced vesicles and nuclei of rat liver, as a preliminary test of the theory that the nuclear membrane is the precursor of the endoplasmic reticulum membrane: ^{14}C choline was used as a tracer to selectively label the choline containing phospholipids in this study.

SECTION 1.

MATERIALS and METHODS.

MATERIALS AND METHODS.

a) Estimations of common chemical constituents.

Phosphorus. Phosphorus was estimated by the method of Allen (1940), used without modification. Organic phosphorus was converted to inorganic orthophosphate by digesting 2-4 hours at 180 - 220°C with 72% perchloric acid. Inorganic phosphorus was estimated in the presence of perchloric acid without digestion. Absorbancy was read at 725 mμ, the absorption maximum of the phosphomolybdic acid.

Nitrogen. Nitrogen was estimated by the microkjeldahl method of Ma and Zuazaga (1942) in samples digested by the method of Miller, Plazin and Van Slyke (1948), with a drop of mercury as a catalyst. Prior to addition of alkali and distillation of the ammonia, the mercuric sulphate was reduced to metallic mercury with approx. 0.2 gm. of zinc dust. Residual zinc and mercury was dissolved at the end of a series of estimations with a few ml. of conc. HNO_3 and the apparatus was thoroughly washed and steamed out. 4-5 drops of 100 vol. H_2O_2 were added to samples containing sucrose when the contents of the digest were thoroughly charred. This accelerates the digestion of the sucrose considerably and has no deleterious effects on the recovery of N from ammonium sulphate, serum albumin or whole liver homogenate as shown below:

Effect of varying digestion conditions on N recovery

Sample	Digestion and conditions	Corrected titre	Mean
2 ml. BSA	Hg catalist	5.14,5.06,5.01	5.07
2 ml. BSA	Hg + H_2O_2+12 mg. sucrose	5.13,5.14	5.14
2 ml. BSA	$CuSO_4$+Se+K_2SO_4 (Ma etal)	4.97,5.01,5.02,4.99	5.00
2 ml. BSA	$CuSO_4$+Se+K_2SO_4+H_2O_2	5.07,5.01,4.91	5.00
1 ml. liver homog.	$CuSO_4$+Se+K_2SO_4	5.95,5.76	5.86
1 ml. " "	$CuSO_4$+Se+K_2SO_4+H_2O_2	5.97,5.61	5.79
0.5 mg.$(NH_4)_2SO_4$-N	$CuSO_4$+Se+K_2SO_4	3.47,3.46,3.47	3.47
0.5 mg.$(NH_4)_2SO_4$-N	$CuSO_4$+Se+K_2SO_4+H_2O_2	3.43,3.45,3.41	3.43

Protein. Readily soluble and sparingly soluble proteins were estimated by the appropriate methods of Lowry, Rosebrough, Farr and Randall (1951). Bovine serum albumin (Armour Pharmaceutical Coy.) calibrated by micro-kjeldahl - N estimation, assuming Adair and Robinson's (1930) figure of 15.6% for its nitrogen content, was used as a standard. These methods allowed estimation of protein in whole tissue homogenates or subcellular fractions with or without acid precipitation. The biuret method of Gornall et al (1951) was found to overestimate the protein of non-acid precipitated rat liver homogenate by about 20%.

Recoveries of liver homogenate protein by the Lowry and biuret methods both with and without acid precipitation.

Acid conc. (P.C.A.)	Lowry O.D.	Biuret O.D.
whole homogenate	0.350	0.425
0.1 N	0.349	0.316
0.2 N	0.361	0.340
0.3 N	0.370	0.352
0.4 N	0.328	0.347
0.5 N	0.321	0.347
0.7 N	0.340	0.349

Comparison of the methods for readily and sparingly soluble proteins on several protein mixtures and pure proteins showed that they gave similar results with most proteins but trypsin and liver homogenate gave significantly lower colour yields in the insoluble method.

Recoveries of proteins estimated by the Lowry methods for readily soluble and sparingly soluble protein.

Protein	Readily Soluble (µg/tube)	Sparingly Soluble (µg/tube)	Mean and % deviation from mean
Liver homogenate	72	60	66 ± 9%
Liver microsomes	59	54	57 ± 4.4%
	55	50	53 ± 4.7%
Casein	56	56	56 ± 0 %
trypsin	74	56	65 ± 13.8%

Lowry et al found that trypsin gave a much higher colour yield than the other proteins they tested by the soluble method but do not seem to have studied its behaviour in the method for sparingly soluble proteins. Apparently 30 mins. treatment with 0.3N NaOH at 100°C, used in the sparingly soluble method, destroys a greater proportion of the chromogenic groupings of trypsin and liver homogenate than in other common proteins.

Estimation of D.N.A.

D.N.A.was estimated by the method of Ceriotti (1952) used without modification. D.N.A, standardized by phosphorus estimation was used as a standard, after partial hydrolysis by heating at 70°C for 20 mins. in 0.5 N PCA. The indole used in the estimation had to be purified by distillation. The method was found applicable both to D.N.A.seperated from acid soluble nucleotides and R.N.A.by the Schmidt-Thannhauser procedure, and to D.N.A.in whole rat liver homogenate containing acid soluble nucleotides,R.N.A.and sucrose. Hence the method seems to be remarkably resistant to interference by liver constituents.

Ribose. Ribose was estimated by the method of Kerr and Seraidarian (1945) in samples of R.N.A.partially hydrolysed with 0.3 N KOH for 1 hr at 37°C and made 0.1 N to PCA. A 30 min. heating period was used as recommended by Fleck and Munro (1962). Ribose figures were multiplied by 4.12, a factor calculated from the base ratios of liver

R.N.A. reported by Davidson and Smellie (1952), to convert them to R.N.A. This method gave a 98% recovery of R.N.A. from a sample of Nutritional Biochemical Corporation yeast R.N.A. standardized by U.V. absorption measurement. The ribose estimation also gave values 97% of the mean of those obtained by combined U.V. absorption, ribose and phosphorus analyses of 6 samples of liver microsomal R.N.A. prepared by the method of Fleck and Munro (1962). T.C.A. and ether (used to remove T.C.A. from R.N.A. digests before U.V. absorption measurements were made) were both found to interfere in this method, producing anomalously high optical densities.

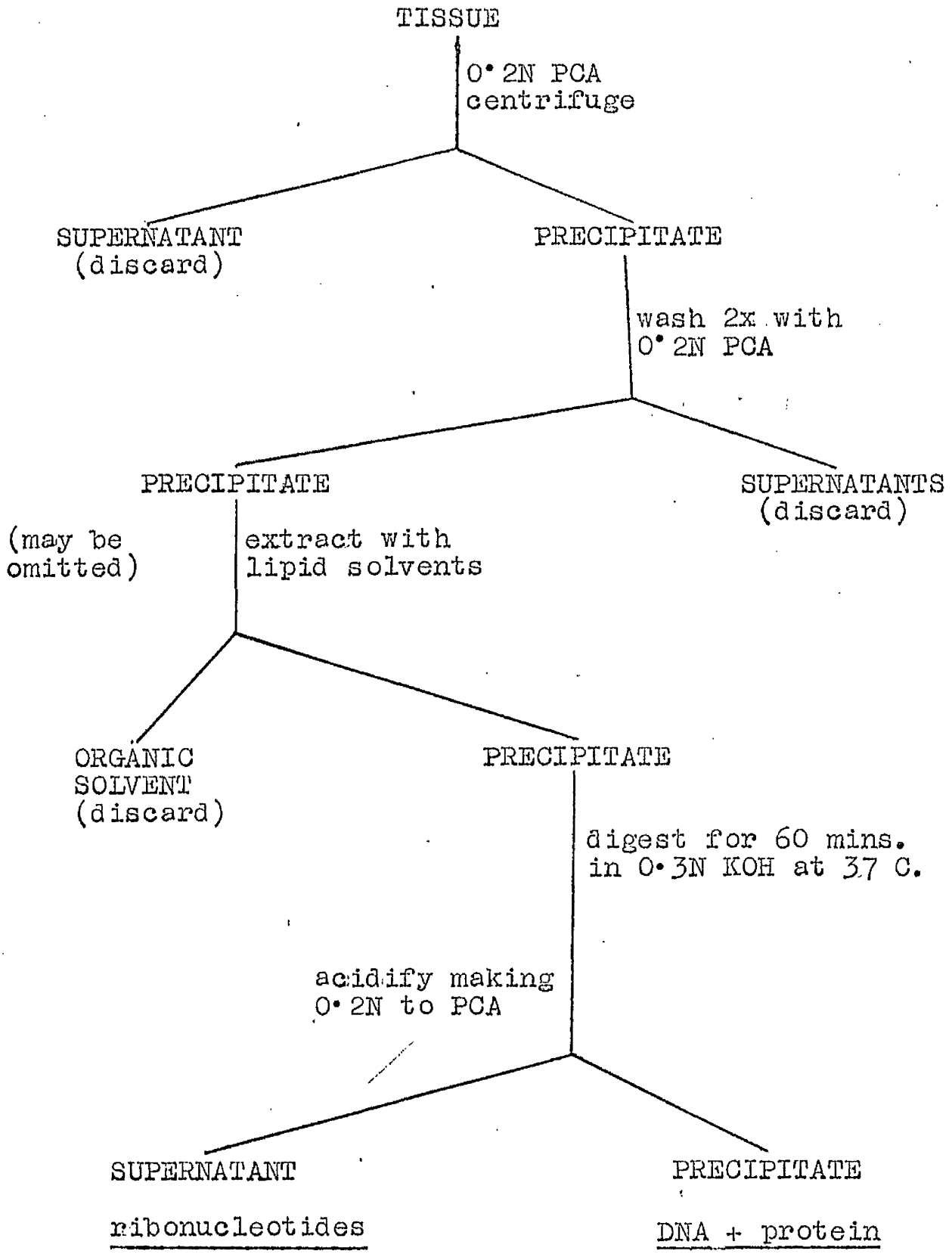
Estimation of R.N.A. by U.V. absorption measurements.

R.N.A. was estimated by measuring the absorbancy of purine and pyrimidine bases at 260 millimicrons in tissue samples precipitated and washed with P.C.A. and then hydrolysed in 0.3 N KOH. This was made 0.1 N with respect to P.C.A. as described by Fleck and Munro (1962) and read in a Unicam S.P. 500 spectrophotometer, using the $E^{1\%}_{1\ c.m.}$ of 300 for rat liver R.N.A. found by them. This method gave 101.5% of the mean R.N.A. figures obtained by the combined ribose, phosphate and U.V. absorption measurements on R.N.A. of 6 samples of rat liver microsomes. This method can also be used to estimate R.N.A. from T.C.A. precipitated samples also, if the T.C.A. is extracted by washing 3 X with 0.25 volumes of ether and a correction is made for the increase in volume due to dissolved ether.

(b) Isolation and Estimation of R.N.A.

R.N.A. was separated from other U.V. absorbing materials by the modified Schmidt-Thannhauser method of Fleck and Munro (1962) (Fig 5)

Fig. 5. Schmidt-Thannhauser method of Fleck and Munro.



and was generally estimated by U.V. absorption as described above. The following aspects of this procedure were investigated for their effect on the recovery of R.N.A.

1) The optimal concentration of P.C.A. and T.C.A. for tissue precipitation and removal of acid soluble nucleotides.

2) The effect of extracting lipids from the tissue.

3) The efficiency of a 1 hr. digestion in alkali for rendering R.N.A. soluble.

4) The optimum concentration of P.C.A. for precipitating D.N.A.

5) The applicability of the ribose, R.N.A. - phosphorus and U.V. absorption methods described above for quantitating the R.N.A. digestion products obtained in the Fleck and Munro method.

(i) Determination of the optimal concentration of acid to precipitate R.N.A. and to wash out acid-soluble components.

The optimal concentration of acid for precipitating rat liver samples and washing them free of acid soluble nucleotides has been found to be 0.2 N for P.C.A; T.C.A. on the other hand shows no marked optimum and any concentration from 2.5 - 15% seems to suffice (Figs. 6,7). If P.C.A. concentrations less than 0.2 N were used, precipitation was not always complete, while if concentrations higher than 0.2 N were used, R.N.A. tended to be extracted into the acid-soluble nucleotide fraction. This could be predicted from the findings of Ogur and Rosen (1950). Normally tissues are precipitated with acid and washed twice more to remove acid soluble nucleotides. This procedure is adequate for

Fig.6. Effect of different concentrations of PCA on the recovery of RNA both with and without lipid extraction.

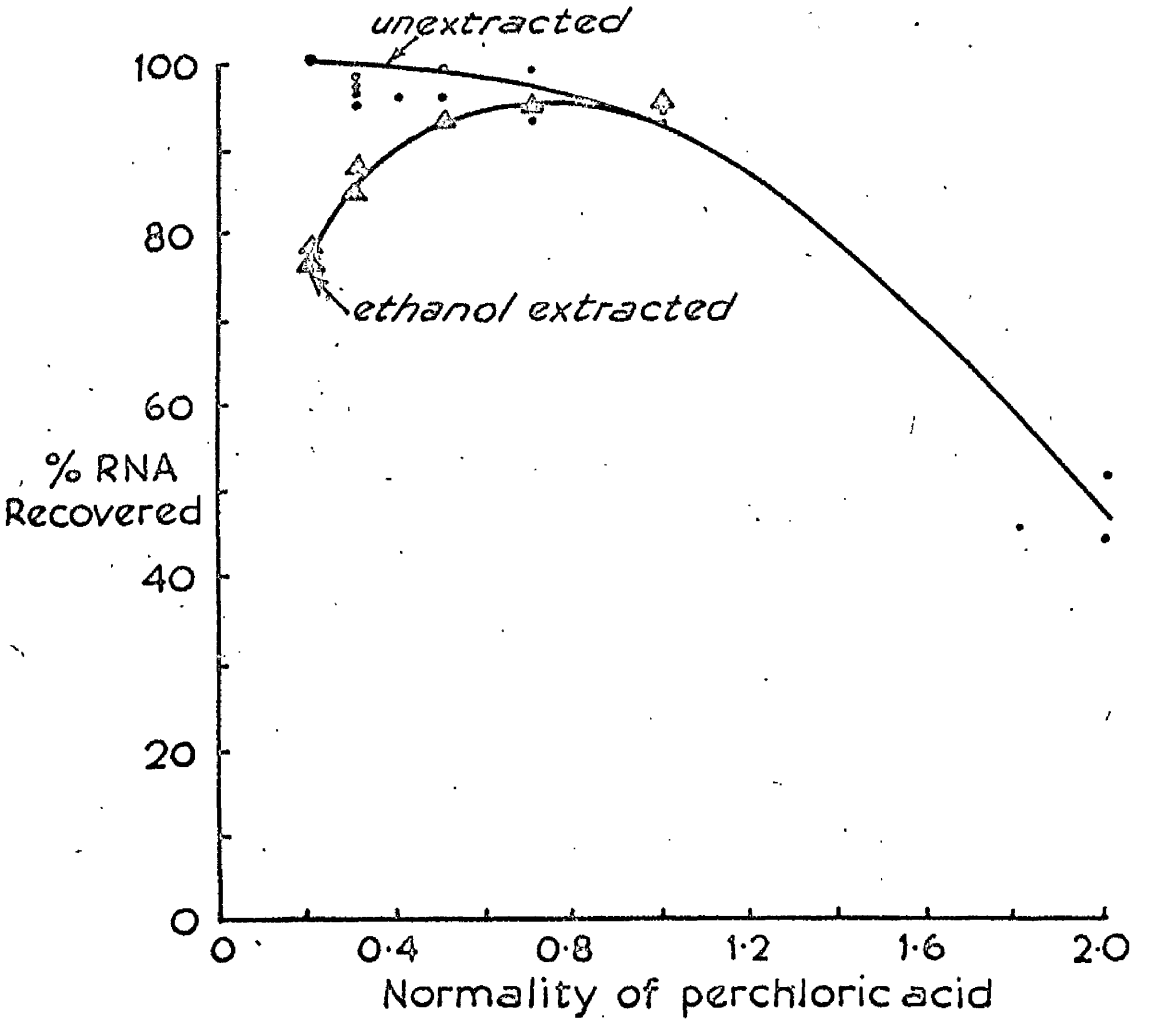
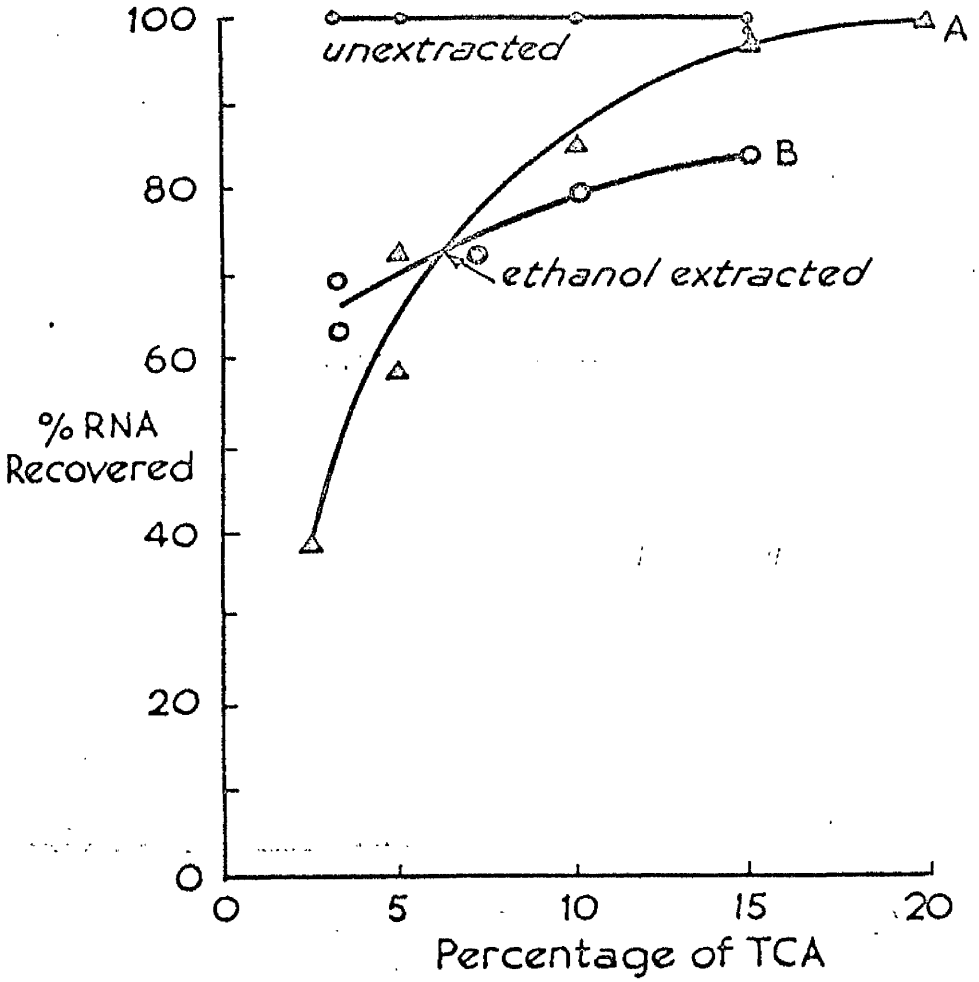


Fig. 7. Effect of different concentrations of TCA on the recovery of RNA both with and without lipid extraction.



removing acid soluble nucleotides, since 5 or 10 more washes with 0.2N P.C.A. did not effect the amount of 'R.N.A.' recovered. This also shows that rat liver R.N.A. is quite resistent to repeated washing with 0.2N P.C.A. since recovery remains constant.

Effect of repeated washes with 0.2 N P.C.A. on the removal of acid-soluble nucleotides and the recovery of R.N.A. from rat liver homogenate.

Replicate samples of rat liver homogenate, corresponding to approx. 200 mg. wet weight of liver were precipitated and washed with 0.2 N P.C.A. and their 'R.N.A.' content was then determined. Each figure is the mean for duplicate samples of homogenate.

Washes	O.D. 260
2	574
5	569
10	584

(2) The effect of lipid extraction on R.N.A. recovery.

In many nucleic acid estimation procedures the acid wet tissue is extracted with lipid solvents. If rat liver homogenate is precipitated and washed with low concentrations of P.C.A., as is necessary if loss of R.N.A. into the acid soluble fraction by the Ogur-Rosen effect is to be avoided, and then extracted with lipid solvents, a significant proportion of the R.N.A. is lost into the lipid solvents. The amount of R.N.A. lost depends upon the amount of acid present in the pellet when it is brought in contact with the organic solvent. This depends upon the concentration of acid used to precipitate and wash the tissue as shown in Figs.6 and 7,

(Hallinan et al, 1962). It is also likely to depend upon factors such as the gravitational field at which the pellet is sedimented, the amount of tissue precipitated and the time and method of drainage used to remove excess acid. R.N.A. is also solubilized by lipid solvents after T.C.A. precipitation, but in this case the solubilisation can be eliminated by raising the T.C.A. concentration to 20%, without encountering difficulties with R.N.A. solubilization as in the Ogur-Rosen effect (Fig. 7), though subsequent estimation of R.N.A. by U.V. absorption or ribose estimation is complicated by T.C.A. interference. All of the lipid solvents tested solubilized R.N.A. from homogenates precipitated with 0.2 N P.C.A. Consequently the procedure finally chosen was to precipitate the samples with 0.2N P.C.A. and then submit them to alkaline digestion without intermediate treatment with lipid solvents.

Solubilization of R.N.A. by different lipid solvents.

R.N.A. recovered from 2 ml. samples of 10% rat liver homogenate, precipitated and washed twice with 0.2N P.C.A. and extracted twice with organic solvents, is calculated as a percentage of the R.N.A. in duplicate samples of the same homogenate precipitated and washed with 0.2N P.C.A. without lipid extraction.

Solvent	% Recovery
ethanol	78
methanol	71
chloroform-methanol	69
acetone	25

3) The efficiency of 1 hr. digestion in 0.3N KOH for rendering liver RNA acid soluble.

The efficiency of a 1 hr. digestion period in 0.3N KOH for rendering soluble the RNA of acid precipitated and washed rat liver microsomes acid soluble was tested by digesting samples (with or without lipid extraction) for 1 hr. at 37°C and isolating the acid soluble RNA, followed by re-digesting the residues for 20 hrs. with 0.3N KOH at 37°C. RNA was estimated by U.V. measurements at 260 and 275 mu. as described by Flock and Munro (1962). Re-digestion for 20 hrs. was found to release only a very small amount of U.V. absorbing material in all cases and this may not have been RNA but protein. This shows that the Flock and Munro method of digesting acid precipitated tissue for 1 hr. with 0.3N KOH at 37°C renders substantially all of the RNA acid-soluble for analysis.

Release of U.V.-absorbing material by re-digesting 1 hr. RNA digestion residues for 20 hrs. with 0.3N KOH at 37°C.

Microsomes from about 1 gm. wet weight of rat liver were digested for 1 hr. in 0.3N KOH at 37°C and the acid soluble RNA isolated and estimated by 260-275 mμ measurements. The acid insoluble residues were then re-incubated for 20 hrs. with 0.3N KOH at 37°C and the amount of U.V. absorbing material 'RNA' (260-275 measurements) was determined and compared with the amount of RNA in the 1 hr. digest.

Treatment	mg. RNA in 1 hr. digest (A)	mg. 'RNA' in 20 hr. digest (B)	B as a % of A.
no-lipid solvents	3.05	0.091	3
	2.94	0.059	2
mixed lipid solvents*	0.96	0.027	2.8
	0.77	0.055	7.1
2:1 chloroform-methanol	2.80	0.084	3
1:1 chloroform-methanol	2.64	0.042	1.6

* acetone; ethanol, ethanol-ether, chloroform-ethanol and ether (Fleck and Munro 1962).

4. The Optimum concentration of PCA for precipitating DNA.

The optimum concentration of PCA for precipitating DNA was found by digesting replicate samples of rat liver homogenate, freed of acid soluble nucleotides, in 0.3N KOH (Fleck and Munro 1962) and precipitating the DNA and protein with concentrations of PCA ranging from 0.1N - 2.0N. The DNA in these samples was then compared with that in duplicate samples of homogenate dissolved directly in alkali without removal of acid-soluble nucleotides or RNA. DNA was estimated by the Ceriotti method.

Effect of PCA concentration on the recovery of DNA.

DNA and protein were precipitated from alkaline digests of rat liver homogenate with different concentrations of PCA and the DNA recovered compared with that in samples of homogenate not freed of acid soluble nucleotides or RNA. DNA was estimated by the Ceriotti method. Each figure is the mean for two samples.

Acid Concentration (N)	O.D. 490
whole homogenate	0.381
0.1	0.383
0.3	0.393
0.5	0.397
0.7	0.391
2.0	0.360

As can be seen, there is no marked dependence of DNA recovery upon the concentration of acid with which it is precipitated, nor is removal of acid soluble nucleotides or RNA necessary for its estimation by the Ceriotti method. Apparently DNA is much less susceptible to direct conversion to an acid soluble form by higher concentrations of PCA than is RNA.

5) The applicability of ribose, phosphorus and U.V. absorption measurements for quantitating RNA digestion products.

The applicability of U.V. absorption measurements, ribose estimations and phosphorus estimations for quantitating the amount of RNA in tissue, after its isolation by the method of Fleck and Munro (1962), was tested on 6 samples of microsomes, some without lipid extraction and others extracted with different lipid solvents after acid precipitation and washing.

Comparison of different methods for estimating RNA from rat liver microsomes.

Results are expressed as mg. of microsomal RNA per gram wet weight of liver.

Treatment of Sample	Method of estimation			
	260-275 mμ absorption	260 mμ absorption	ribose	phosphorus
Unextracted	2.83	2.78	2.74	-
mixed solvents	0.82	0.86	0.80	0.83
2:1 $CHCl_3$-MeOH	2.65	2.69	2.57	2.76
1:1 $CHCl_3$-MeOH	2.49	2.62	2.50	2.70

The results of these estimations show that all methods give remarkably good agreement for the RNA content of microsomes treated in these various ways. (N.B. phosphorus estimation cannot be used to measure the amount of RNA in samples which have not been previously lipid-extracted). It seems as if the one-hour alkaline digest contains very few substances which interfere with these various estimations. It was found to be impossible to obtain such precise agreement between them when these several methods were applied to 18 hr. alkaline digests (Hallinan 1961).

(c) Determination of RNA base ratios

RNA was isolated from submicrosomal fractions by the method of Hallinan, Fleck and Munro (1963) or by the phenol method of Kirby (1962) with naphthalene 1:5 disulphonate and 8-hydroxyquinoline present to inhibit ribonucleases. The RNA was then hydrolysed at 37°C for 16-20 hrs to convert it completely to mononucleotides and then carefully adjusted to pH 6-8 with narrow-range indicator paper and then dried. The nucleotides were

made up to a concentration of 10 mg. per ml. in distilled water and 250 µg. spots were applied to 48 x 34 cm. Whatman number 1 chromatography paper 35 cms. from the bottom and 7.5 cms. from the edge. The descending chromatograms were then developed for 40-55 hrs. in 7:3 isopropanol water with an ammonia vapour phase, as described by Markham and Smith (1952) and the nucleotides were located under U.V. illumination. The chromatograms were then trimmed so that they could be developed at right-angles in ammonium isobutyrate buffer pH 3.6 for about 10-12 hrs. after which they were thoroughly dried for 4-6 hrs. in a warm oven. (All care must be taken to avoid ovens used for drying chromatograms developed in benzene or phenol as these adsorb on the paper and can cause a very high background absorption.) The nucleotide spots were then relocated under U.V. illumination and outlined lightly in pencil. Pseudo-uridylic acid was located by its fluorescence under U.V. and the fluorescent material plus the area of paper adjacent to it were marked (Barr 1961). The outlined spots were out out together with a superimposed area of paper from the same chromatogram which contained no visible U.V. absorbing material. This latter was used as a blank. Both spots and blanks were eluted for 4-6 hours with 0.1N PCA at 37°C to isolate the U V -absorbing nucleotides and materials from the paper. The nucleotides were estimated by absorption measurements at 260 mµ and 280 mµ, using the extinction coefficients of Goswami (1962). It was found impossible to correct for the absorption of the substances in the paper by using the formula derived by Barr (1961),

since this sometimes gave impossible answers (e.g. in a case encountered where O.D.260 nuc. = 0.338; O.D.280 nuc. = 0.233; O.D.260 bl. = 0.047; O.D.280 bl. = 0.035; a zero numerator is obtained in the expression.)

An artificial mixture of nucleotide monophosphates in the same proportion as they occur in rat liver RNA (Davidson et al, 1952) was separated by 2-dimensional Chromatography and used to test the reproducibility of the base ratio figures obtained by the above method. The efficiency of elution for 4-6 hrs. at 37°C was compared with elution overnight at 37°C as used by Crosbie et al.(1952).

Reproducibility of base ratios obtained by two dimensional chromatography and recoveries of nucleotides.

	Base Ratios			% Recovery of nucleotides	
	real	1	2	1	2
G	31.8	30.8	31.3	78.0	78.4
U	22.4	23.5	23.6	89.5	89.4
C	27.1	25.7	25.7	80.5	80.1
A	19.0	19.9	20.0	89.8	89.6

(1 is the mean for duplicate samples eluted for 4-5 hrs. at 37°C while 2 is the mean for duplicates eluted 19.5 hrs. at the same temperature It was concluded from the results of this control experiment that the above procedure gives adequately reproducible base ratios. The recovery of nucleotides however was less satisfying, only 78% and 80% of guanylic and cytidylic acid being recovered, while the recoveries of uridylic and

adenylic acids were 89% and 90% respectively. These reduced recoveries may be due to non-localization of all of the nucleotide monophosphate material in the area of paper cut out, owing to excessive diffusion. Failure to elute the mononucleotides seems a less likely cause since overnight elution gives no improvement in yield over 4-6 hrs. elution. It was observed that phenol-RNA had the same base ratios as that isolated by 1 hr. digestion in 0.3N KOH. This confirms the finding of Goswami (1962) on this point.

d) Extraction and estimation of phospholipid.

A number of different methods of extracting phospholipid from pancreas, pancreas microsomes and rat-liver microsomes, including the methods of Fleck and Munro,(1962), Hallinan et al,(1962) and Folch et al,(1957), were tried. The simplest and most satisfactory method found was that of Folch et al,1957. Virtually all of the phospholipid of liver or pancreatic microsomes could be isolated by shaking in stoppered tubes with 20 vols. of 2:1 chloroform-methanol and this phospholipid could be washed virtually free of non-lipid contaminants by a procedure described below (subsection (8) of this Section.

Number of Folch extractions to isolate phospholipid from pancreatic microsomes.

Lipid - P in first two extracts in µg. (A)	lipid-P in third extract in µg. (B)	B as a % of A
310	0.7	0.2%
455	0.0	0%

Indeed its efficiency was conclusively demonstrated by determining the unextracted fatty acid remaining in the tissue residues after two extractions. This was done titrimetrically by the method of Albrink (1959), after

saponifying the tissue residue with 2% ethanolic KOH at 80°C for 60 mins. These residual fatty acids amounted to only 1 - 6% of the fatty acids in the extracted phospholipids alone, (without considering fatty acids in triglycerides, sphingolipids, cholesteryl-esters etc. in the extract) so the extraction procedure allows total tissue phospholipids to be accurately quantitated. It is conceivable that the unextracted lipids belonged to a particular class, which was poorly represented in the lipid extract, i.e. that the extract was not wholly representative of all of the lipid classes in the tissue. However since our aim was only to estimate the amounts of total phospholipid this possibility was not pursued. The Folch method was effective with undenatured tissue or tissue precipitated with 0.7N PCA and was found capable of considerable precision. The average standard deviation from the mean of 5 groups of samples was found to be only ± 1.7%

Efficiency of Folch method of isolating lipids from pancreas and pancreatic microsomes.

Tissue twice extracted by the Folch method was saponified and the residual fatty acids (FA) estimated titrimetrically and compared with the fatty acids in the extracted phospholipid. Fatty acid figures are given in μ -Eq. per gram wet weight of tissue or per fraction in 1 gm. wet weight of tissue.

Material and treatment	μ-Eq F.A. in extracted P-lipid (A)	μ-Eq residual F.A. in residue (B)	B as % of A	
Whole pancreas	76	0.8	1.1	1.3
	70	1.0	1.4	
Microsomes	30	1.3	4.3	3.2
	30	0.6	2.0	
microsomes + PCA	29	1.5	5.2	5.5
	21	1.2	5.7	

(e) Cytochrome oxidase assay.

Relative cytochrome oxidase activities in microsomes and mitochondria from liver and pancreas were assayed in the reaction mixture of Slater (1949), using 2.85 μ-Molar ascorbate as the reducing agent and a final Cyt. C concentration of 6×10^{-5} M. Aliquots of reaction mixture were removed at suitable intervals after the addition of ascorbate and the proteins precipitated with 4 vols. of 10% metaphosphoric acid, the remaining ascorbate being titrated with 0.01% 2:6 dichlorophenolindophenol as described by Gero,(1949). Oxygen uptake was calculated from the rate of disappearance of ascorbate, assuming 2 moles of ascorbate equivalent to 1 mole of oxygen. The rate of ascorbate disappearance in a blank run was determined in a duplicate reaction mixture containing a final conc. of 7×10^{-4} M KCN, added a few seconds before the ascorbate. It is important to add the cyanide just before the rate measurement is begun, as HCN is rapidly evolved at pH 7.3 and the cyanide activity may otherwise become too low to inhibit cytochrome oxidase completely during the measurement, leading to an anomalously high blank rate. All fractions were homogenized in hypotonic (¼ strength) Campbell's medium prior to assay to ensure adequate access of ascorbate to the site of cytochrome oxidase activity (Potter, 1957). Rates were calculated from the linear portions of the kinetic curves. Liver mitochondria and microsomes oxidise ascorbate at a linear rate for at least 60 mins. but the corresponding particles from pancreas maintain a linear rate for less than 30 mins. Typical QO_2 values for liver and pancreas mitochondria and microsomes are given below, together with the calculated percentage

contamination of microsomes with mitochondria to account for the activity of the former. The results show that contamination of microsomes by mitochondria is minimal.

Cytochrome oxidase activities in mitochondria and microsomes.

	Mit.	5' microsomes*	10' microsomes*	% mit. contamination of 10' microsomes
liver	149	16	14	9%
pancreas	251	35.5	-	-
"	116	27	-	-
"	382	-	9	2%
"	170	-	12	7%

* Microsomes prepared from supernatant after removal of mitochondria etc. by spinning for either 5 min. or 10 min. at 12,000g.

(f) Preparation of Subcellular Fractions.

(i) Microsomes.

Rat liver microsomes were normally prepared from animals maintained on a diet of rat cubes and water ad libitum and fasted overnight before killing. Where other dietary regimes were used, these are noted in the text. Pigeon pancreas microsomes were prepared from birds maintained on rabbit pellets and water ad libitum. Two methods were used to prepare microsomes.

(i) Liver or pancreas was homogenized in 2.5 volumes of medium A (Littlefield and Keller, 1957) and particles larger than microsomes were sedimented by centrifuging at 12,000 g. for 10 mins. Five-sixths of the 12,000g supernatant was removed with a Pasteur pipette and

microsomes were isolated by centrifuging this at 105,000g. for 60 mins. This procedure gave a microsome fraction containing less than 10% of the cytochrome oxidase activity of washed mitochondria prepared from the same homogenate and hence minimally contaminated with mitochondria.

(ii) Liver was homogenized in 4 volumes of 30% (0.88M) sucrose and unbroken cells and large particles were removed by centrifuging at 18,600 g. for 20 mins. The microsomes were prepared from $\frac{5}{6}$ of the supernatant as above by centrifugation at 105,000g. for 180 mins. Moule, Rouiller and Chauveau (1960) showed by electron microscopy that this method yields microsomes minimally contaminated with mitochondria. Microsomes prepared by either of the above methods were washed by resuspension in medium A and resedimentation at 105,000g. for 60 mins.

(2) Mitochondria.

Liver or pancreas was homogenized in 2.5 volumes of medium A and particles larger than mitochondria were centrifuged down at 2,000g. for 10 mins.(Hokin 1955). Crude mitochondria were sedimented from the supernatant by centrifuging at 12,000g. for 5 mins. These were resuspended carefully in medium A and re-centrifuged at 2,000g. for 5 mins. and washed mitochondria were isolated by centrifuging the supernatant from this spin at 8,500g. for 5 mins. (Hokin 1955).

(3) Pyrophosphate 'membrane' and 'ribosomes'.

Washed microsomes from rat liver or pigeon pancreas were resuspended in 0.25M sucrose and mixed with 0.11M sodium pyrophosphate (pH 7.4) in 0.25M sucrose, to give a protein concentration of 3.5-4 mg. per ml. in 0.1M pyrophosphate. This was stood 10 mins. in ice and centrifuged at 105,000g. for 45 mins. (Sachs 1958). Centrifugation yielded a pellet rich in protein

and phospholipid and poor in RNA (pyrophosphate membrane) and a supernatant rich in RNA (pyrophosphate ribosomes) (Goswami 1962).

(4) Isooctane 'membrane' and 'ribosomes'.

Rat liver microsomes prepared by medium A were resuspended in medium A to give protein concentrations of 15-20 mg. per ml. They were then homogenized in a Potter-Elvehjem homogenizer with an equal volume of isooctane for 3-4 strokes at full speed, until emulsified (Hawtrey and Schirren 1962). After standing 20 mins. in ice the emulsion was centrifuged at 105,000g. for 60 mins. This separated the system into membrane fraction, which sedimented centripetally to form a pink pellicle at the interface of the isooctane and aqueous phases (isooctane 'membrane') and a ribosome fraction which sedimented as a tightly-packed, light-tan pellet to the bottom of the tube (isooctane 'ribosomes'). Some reddish unsedimented material remained in the aqueous phase above the ribosomes and below the membrane. No phosphorus or protein could be detected in the isooctane phase.

(5) Deoxycholate ribosomes.

Deoxycholate ribosomes were prepared from microsomes isolated in medium A or from submicrosomal fraction by the method of Kirsch (1962). The particles were resuspended at a protein concentration of about 30mg. per ml. in a solution containing sucrose, 0.1M tris, 0.1M KCl and 0.02M Mg Cl2 pH 7.6 and an equal volume of 2% sodium deoxycholate pH 7.6 was added. The system was then centrifuged for 120 mins. at 105,000g. or 87 mins. at 150,000g. to sediment the DOC ribosomes. Excess DOC was

removed by rinsing the surface of the pellet three times with medium A. The DOC ribosomes had an RNA: protein ratio of about 0.7 - 0.8 compared with Kirsh's figure of 0.5.

(6) The submicrosomal components, rough-surfaced vesicles, smooth surfaced vesicles and 'natural' free ribosomes.

This was performed by a new procedure, the evidence for which will be presented in the Results section. Here, the final procedure adopted will be briefly described.

Rat liver was homogenized in 4 volumes of 30% sucrose and centrifuged at 18,600g for 20 mins. to remove particles larger than microsomes. Rough surfaced vesicles (RSV) were then centrifuged down at 78,000g for 60 mins. Smooth surfaced vesicles (SSV) failed to sediment under these conditions because of their low density while free ribosomes (R) remained in the supernatant because of their relatively small particle size (Fig.14). Smooth surfaced vesicles and free ribosomes were isolated by homogenizing the supernatant from the 78,000g pellet with 0.5 volumes of isooctane. Homogenization was performed in a Potter-Elvehjem homogenizer for 3-4 strokes until an emulsion was formed, and the system was then centrifuged at 150,000g. for 120 mins. This caused the smooth surfaced vesicles to sediment centripetally and form a pellicle at the interface of the isooctane and aqueous phases, while the free ribosomes sedimented in a tightly packed pellet to the bottom of the tube. All fractions were washed by resuspension in medium A and resedimentation at 105,000g for 60 mins. A small amount of centrifugally sedimenting material was released from the smooth-surfaced vesicle pellicle on washing, which

formed a pellet at the bottom of the centrifuge tube. This was not recombined with the washed smooth-surfaced vesicles.

7) Nuclei.

Nuclei were prepared by Miss S. Waddington, using a slight modification of the method of Chauveau et al. (1956). Chopped rat liver was homogenized in 10 volumes of 0.25M sucrose containing 0.002 M calcium chloride and 0.001M magnesium chloride and the homogenate was centrifuged for 5 mins. at 100g to sediment fibrous tissue, unbroken cells and cell debris etc. The 100g-supernatant was layered atop 0.34M sucrose, containing the same concentrations of calcium and magnesium chlorides, and centrifuged for 10 mins. at 600g, which sedimented a crude nuclear pellet, contaminated with erythrocytes, cell membranes and other cytoplasmic debris. This crude nuclear pellet was resuspended in a minimum volume of the above 0.25M sucrose medium and mixed with 14 volumes of 2.13M sucrose-$CaCl_2$-$MgCl_2$, so that the final sucrose concentration equalled 2.2M, with a relative density of 1.273. The suspension was then centrifuged for 60 mins. at 30,000g, which allowed the nuclei, owing to their size and density to sediment to the bottom of the tube, while their less dense and small contaminants either remained in the supernatant or sedimented to the top of the tube. The nuclear pellet was rinsed *in situ* several times with ice cold distilled water, to remove the excess, viscous sucrose and any surface contamination by supernatant. They were then resuspended gently in 0.25M sucrose and sedimented at 2,000g for 10 mins. Nuclei prepared by this method were shown by electron microscopy and chemical analysis to be minimally /

contaminated with cytoplasmic material and to be bounded by an intact 2-layered outer membrane (Waddington, 1964). They were obtained in about 45% yield as shown by DNA-recovery from whole homogenates.

g) Incorporation experiments with radioactive precursors.

1) Incorporation of (^{14}C) - amino acids into protein in vitro.

Particular fractions used in the study of amino acid incorporation were suspended by gentle homogenization in Medium A (Littlefield and Keller, 1957), containing 0.035M tris buffer pH 7.6 instead of bicarbonate, at protein concentrations of 10-20mg. per ml. Of this suspension 0.1 - 0.3ml. were then added to a tube containing 1 µM ATP; 10 µM creatine phosphate; 40 µg creatine phosphokinase; 0.25µM GTP; 10 µM Mg Cl2; 25µM KCl; 350 µM sucrose; 35 µM tris HCl pH 7.6 and an amount of neutralized pH5-fraction protein (from the cell sap) equal to that of particle protein. This mixture was in a volume of 0.9 ml. and 0.1 ml. of isotopic amino acid (normally containing 0.7 µC) was added in medium A and the fractions were incubated for 20 min. at 37°C. The reaction was stopped by the addition of 1 ml. of cold 0.4N PCA containing 4 mg/ml of a (^{12}C)-amino acid quencher. The precipitated protein was then freed of acid-soluble components by three washes with 0.2N PCA, followed by removal of RNA by digestion in 0.3N KOH and precipitation and then three further washes with 0.2N PCA as described by Hallinan, Fleck and Munro (1963). This also removed amino acids bound to activating enzymes, since this linkage is acid labile (Kingdon, Webster and Davie, 1958). /

The protein-lipid residue was then dissolved in 0.3N NaOH and a volume containing less than 1.5 mg. protein was plated out on a lense paper disc on a stainless steel planchette and dried. It has been observed that the self-absorption of amounts of protein less than 2mg. plated in this way is constant (McLean, 1962). The planchettes were counted on a windowless, automatic Nuclear-Chicago gas-flow counter. Controls were always run to determine the incorporation by the system in the absence of an energy source and to determine incorporation by the pH5 fraction, while zero time controls to determine incorporation by particles in the absence of pH5 fraction were frequently run. The figures finally reported are energy-dependent counts/min. into particle protein corrected for energy dependent incorporation by the pH5 fraction i.e. Particle CPM - Zero E blank - (pH5 blank - zero E pH5 blank).

No attempt was made to remove lipids from the protein before counting because all lipid extraction methods tried in this laboratory have been shown to extract substantial amounts of protein which may be more highly labelled than that not extracted (Downie, 1962). It is believed that incorporation into lipids should not interfere with incorporation studies carred out like those above, as lipid incorporation is normally independent of a supply of ATP (Haining et al,1960) and in our studies zero energy controls were always run.

Since experiments were frequently performed to compare the relative rates at which different submicrosomal particles incorporate amino acids into protein, it was necessary to ensure that all of those

Fig. 9. Effect of different concentrations of magnesium ions on amino acid incorporation by the submicrosomal fractions in vitro.

Magnesium concentrations are in mM per litre of incubation medium. (o---o) rough-surfaced vesicles; (x--x) smooth-surfaced vesicles; (□---□) free ribosomes.

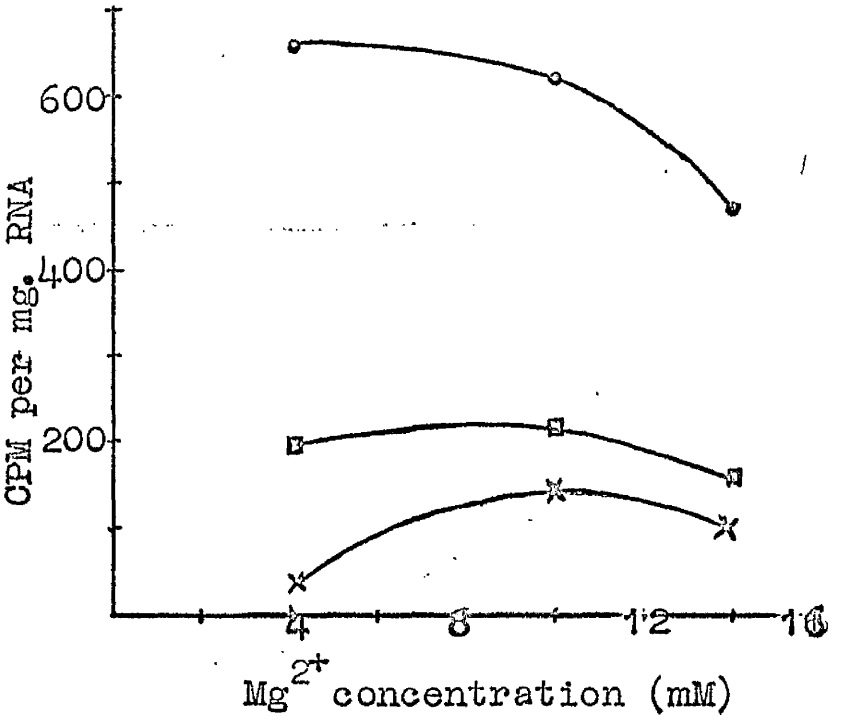
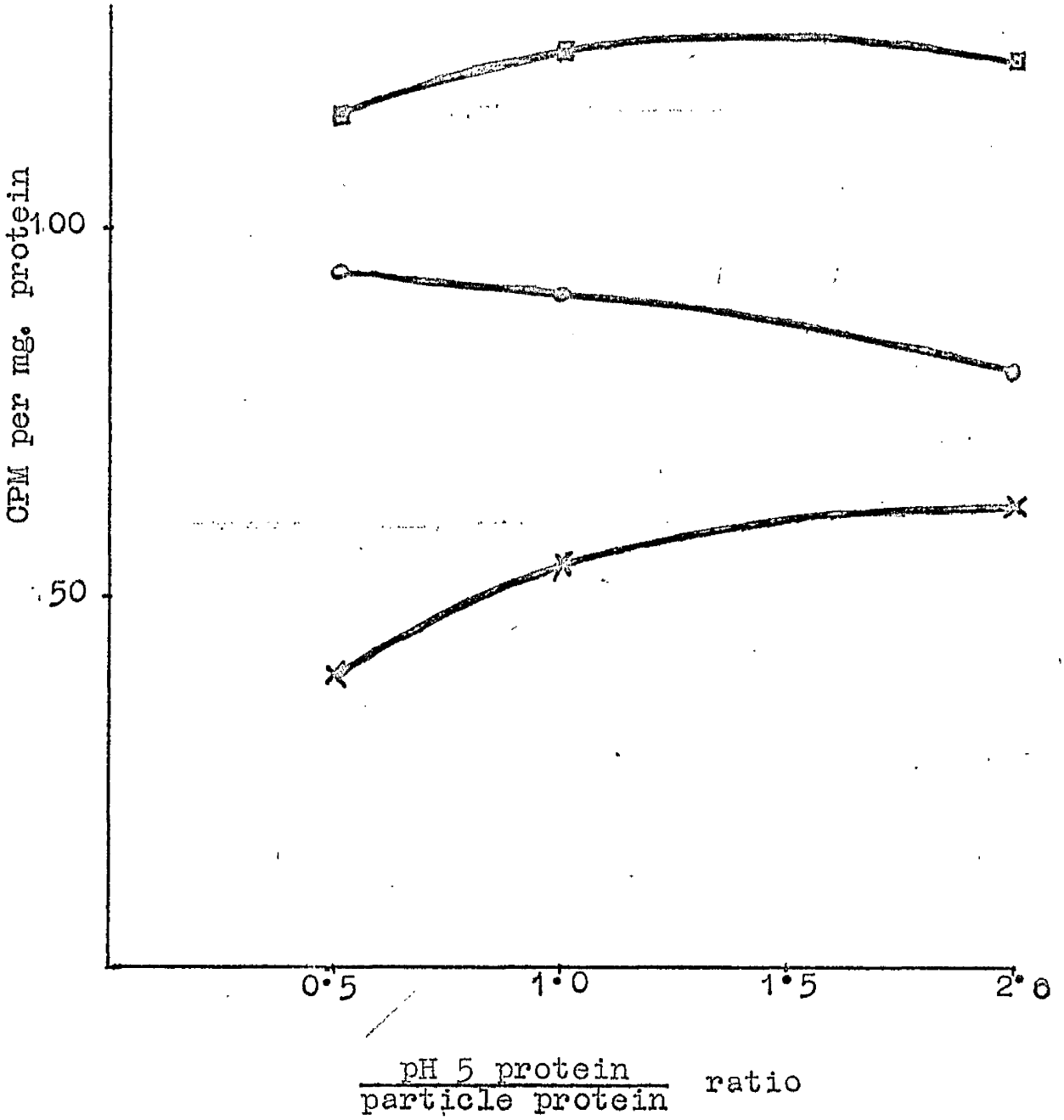


Fig.10. Effect of varying the pH 5 protein/particle protein ratio on amino acid incorporation by the sub-microsomal fractions.

(o--o) rough-surfaced vesicles;(x--x) smooth-surfaced vesicles;(□--□) free ribosomes.



fractions were incorporating close to their optimal conditions. Hence these conditions were approximately determined. A magnesium chloride concentration of 10 μM per ml. and a ratio of pH5 + fraction protein to particle protein of 1.0, was found to cause no significant inhibition of incorporation in any of the fractions (Figs. 9 and 10).

Smooth surfaced vesicles (SSV) isolated by the isooctane method, contain so much lipid and isooctane that after precipitating them with acid they float to the top of the tube when centrifuged and thus cannot be washed free of acid soluble components in the normal way. Hence in experiments in which their incorporation was compared with that of other particles, the reaction was stopped in all fractions by the addition of 3 ml. of 0.3N KOH containing 4 mg/ml of a ^{12}C - amino acid quencher. The tubes were then incubated for 1 hr at 37°C and the protein was precipitated at 0.3N PCA and washed 5 times more with 0.2N PCA. The residue was then dissolved in 0.3N NaOH, reprecipitated at 0.2N PCA and finally dissolved in 0.3 N NaOH and plated and counted as above. This method allows SSV to be counted without any difficulty. When it was applied to ordinary microsomes, this method gave zero energy and zero time blanks as low as the normal method of preparing protein for counting.

In experiments to test the incorporation of amino acids into protein by the submicrosomal fractions <u>in vivo</u>, the radioactive precursor was injected intraperitoneally 15 mins. before killing the

animals and isolating the fractions. The isolated fractions were then freed of acid soluble components and RNA exactly as outlined above for studies *in vitro*, with the exception of the smooth-surfaced vesicles. These were extracted by precipitation with hot 80% (v/v) ethanol (final concentration) and boiled gently for 5 mins. to remove isooctane before treatment with PCA, to avoid flotation and losses when centrifuged. This preliminary extraction with ethanol, which was also used in the *in vivo* studies with RNA precursors described below, was shown experimentally not to lead to losses of RNA.

2) Incorporation of (8 - ^{14}C) - adenine and ^{32}P into RNA *in vivo*

Studies of the incorporation of labelled precursors by the RNA of subcellular fractions of liver were carried out by injecting the precursor intraperitoneally into animals maintained on a selected dietary regime. After suitable time intervals (20 mins. upwards) the animals were sacrificed and the liver subcellular fractions immediately isolated. In the case of ^{14}C-adenine the RNA was then isolated by alkaline digestion exactly as described above, except that 6 washes with 0.2N PCA were used to remove acid-soluble compounds. Alternatively RNA was isolated undegraded by extracting the resuspended particles with 90% phenol - 1% sodium lauryl sulphate in the cold. To inhibit ribonucleases 8-hydroxy-quinoline and naphthalene 1:5 disulphonate were present also. (Kirby, 1962). This phenol - RNA was freed of glycogen by centrifugation /

at 30,000g. for 20 min. (Hall and Doty, 1958) and precipitated with 2 vols. of ethanol and dialysed overnight against 0.05M NaCl, 0.01 M Na acetate; 0.0001 M Mg Cl2 at pH 5.25 to remove ^{14}C contaminants.

^{32}P labelled RNA was always isolated by the phenol-sodium lauryl sulphate method above, since RNA prepared by alkaline digestion is difficult to free from traces of phosphoprotein - phosphorus and phosphoinositede phosphorus contaminants. (Davidson and Smellie, 1952).

3) Incorporation of ^{14}C choline into phospholipids in vivo.

Animals were injected intraperitoneally with 5 - 10 uC of ^{14}C choline and killed after 10 - 65 mins. Nuclei, rough surfaced vesicles and smooth surfaced vesicles were prepared and resuspended in a minimum volume of distilled water before isolating their total lipids by means of two extractions with 2:1 chloroform-methanol as described above. The method of Folch et al, 1957, which has been shown to remove all chemically significant amounts of non-lipid contaminants, was first tried to wash these lipid extracts free of non-lipid, ^{14}C - choline. However addition of free, ^{14}C - choline to rat liver microsomes in amounts expected to occur experimentally followed by extraction and washing of the total lipids by the Folch method, showed that these did not yet have the desired degree of radiochemical purity (Table 3). Hence a small modification was made in the Folch washing technique, which increased its efficiency for removing free, ^{14}C - choline by a factor of ten, and had no deleterious effects on the recovery of phospholipids (Table 3).

TABLE 3. Efficiency of the original and modified Folch procedures for removing contaminating, free ^{14}C choline from lipid extracts.

One microcurie of ^{14}C-choline was added to liver microsomes containing about 60 mg. of protein before extraction of total lipids and washing the extract. Recovery of counts was calculated assuming a counting efficiency of 20% for a Nuclear Chicago Gas Flow counter fitted with a thin mica end window. Recovered counts were corrected for background.

Treatment	CPM	% of added counts recovered in washed lipid extract	lipid-P per 2 ml of washed extract.
3 x rinsed	478	0.0085	11.08
	462		11.14
3 x washed	34	0.0008	10.76
	55		10.78

The initial step of washing the lipid extract by shaking it with 0.2 vols. of 0.73% sodium chloride was retained, but the several subsequent rinses of the organic phase interface with 'pure solvents upper phase'* were omitted. These were replaced by three further washes of the lower, organic phase by shaking it each time with 0.2 vols. of 'pure solvents upper phase', which greatly reduced contamination with free, ^{14}C - choline.

* The equilibrium mixture formed in the upper phase when 0.2 volumes of 0.73% sodium chloride are mixed with 2:1 chloroform methanol.

The purified lipid extracts could then be used for the determination of radioactivity in phospholipids and for the chemical estimation of phospholipid-phosphorus.

h) Sedimentation analysis.

1) Determination of the sedimentation constants of free ribosomes and of ribosomes prepared with deoxycholate from rough-surfaced vesicles.

Free ribosomes prepared from rat liver by the 30% sucrose-isooctane technique described above, were gently resuspended in a medium containing 0.15M potassium chloride, 0.0011 M potassium phosphate pH 7.4 and 0.0025 magnesium chloride (Hamilton et al, 1962) and preliminary measurements were made of the sedimentation constants of the components present. The concentration of particles was adjusted to give approximately 50 µg. of RNA per ml. and sedimentation constants of the components were determined in the Spinco model E, analytical ultracentrifuge, using UV optics and a rotor speed of 29,500 RPM. This showed the presence of two components, present in about equivalent amounts, with sedimentation constants of 62 and 85s respectively. Because of the low particle concentration and the use of a medium with a viscosity equivalent to that of water, these sedimentation constants were not corrected in any way.

Rough surfaced vesicles, isolated from rat liver in 30% sucrose - 0.005M magnesium chloride were solubilized with deoxycholate as described by Kirsch, (1962). The deoxycholate was made up in 0.01 M potassium chloride, 0.005 M magnesium chloride, 0.01 M tris, pH 7.4 /

(Warner et al, 1963) and a weight ratio of deoxycholate/protein of 2/3 was used (Kirsch, 1962). Samples of the solubilised vesicles containing about 10 mg. of protein were run in the analytical ultracentrifuge using a rotor speed of 17,250 RPM and Schlelren optics and approximate sedimentation constants were determined for the components present. These showed uncorrected values of 39, 53.5-58 and 116s. Comparison with particles of a known sedimentation constant of 80s (free ribosomes), run under the same conditions, showed that these now gave a sedimentation constant of 57s. Hence the above values were all multiplied by an arbitrary factor of 1.415 to correct approximately for the effect of particle concentration and the viscosity effect of 30% sucrose, which wetted the original rough surfaced vesicle pellet, and was carried over into the solubilized system. This gave corrected sedimentation constants of 55, 76-83 and 164s for the components in the solubilised rough surfaced vesicles. The 164s component was present at about 3 times the concentration of the 55s one, which was in turn about 3 times more abundant than the 76-83s component. Though its sedimentation constant can only be regarded as approximate, owing to the inaccuracy of the arbitrary correction factor applied, particularly in such a multicomponent system, it is thought possible that the 164s component may represent polysomes. The main objection to this possibility is the sharpness of the peak, polysomes being expected to yield a much more poly-disperse sedimentation pattern, at least from results obtained by zone centrifugation on sucrose /

gradients. However there have been comparatively few studies yet of the behaviour of polysomes in the analytical ultracentrifuge and Schlossinger, (1963) for one, has shown that undetermined factors can sometimes cause them to show hyper-sharp peaks in this instrument. In any case no conclusions can yet be drawn as to whether the 164s component consists of polysomes or not, until its response to trace amounts of ribonuclease and other treatments is studied.

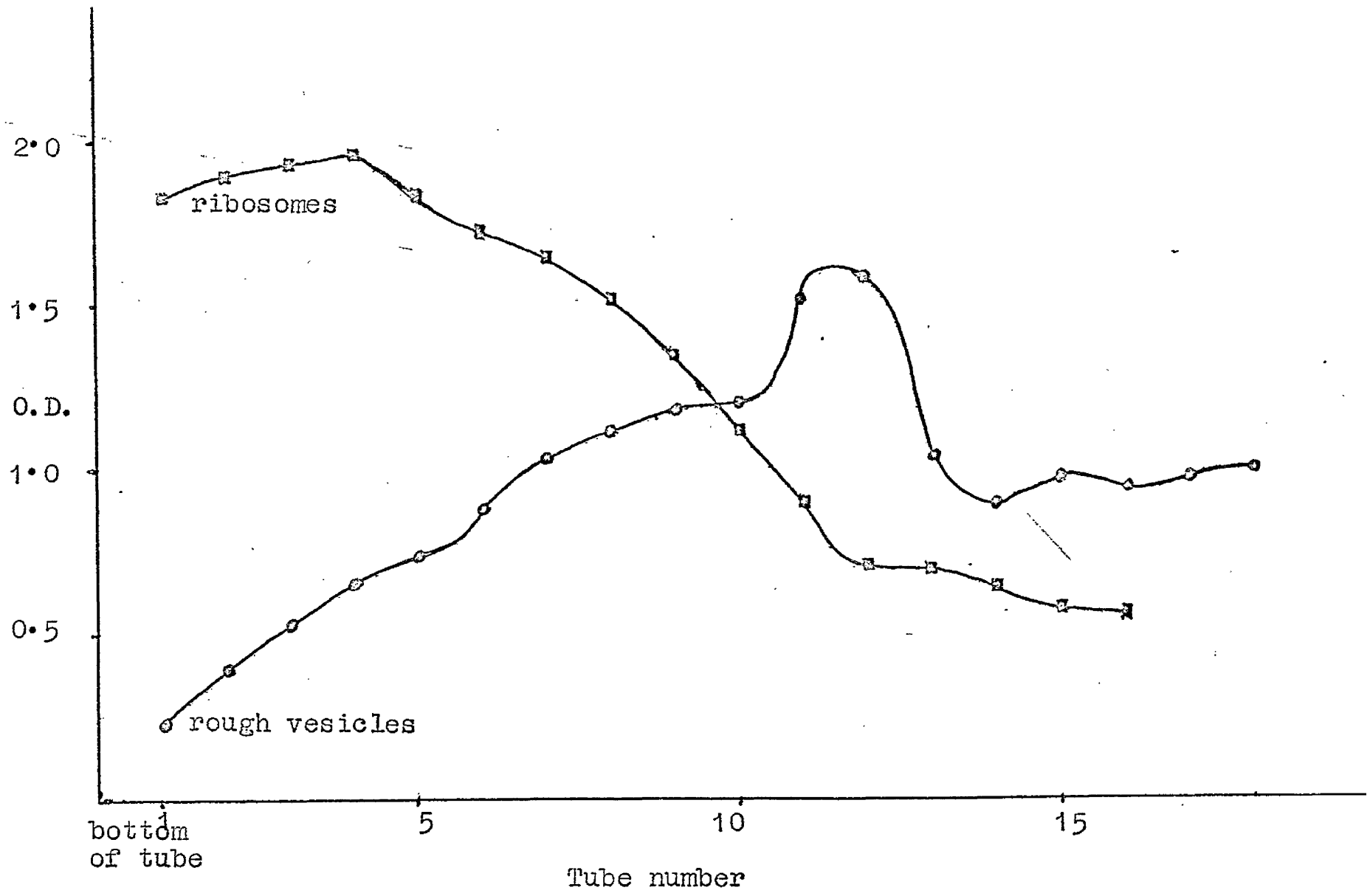
2) Separation of RNA components and ribosomes according to their sedimentation constants and other physical properties on sucrose gradients.

RNA, isolated from submicrosomal fractions by the phenol-sodium lauryl sulphate method and purified as described above, was adjusted to a concentration of 25 - 100 O.D. 260 units per ml. and about 10 O.D. units was applied in a narrow band to the top of a linear or slightly S-shaped sucrose gradient, made by mixing 3% and 30% sucrose in a special mixing chamber (Bolton et al, 1959) The sucrose gradient contained 0.05M sodium chloride, 0.01 M sodium acetate; 0.0001 M magnesium chloride and was adjusted to pH 5.2 - 5.3 with acetic acid. Samples were centrifuged in the gradient for 10 - 14 hrs. at 23,000 RPM in the SW 39 rotor of the Spinco model L preparative ultracentrifuge, distributing their components down the gradient according to their sedimentation constants and partial specific gravity. At the end of the run the tubes were punctured /

through the bottom with a 14 gauge hypodermic needle and the tube contents were separated dropwise as 20 drop fractions. RNA was estimated in each fraction by measuring the absorbtion at 260 m.μ after dilution with 1 ml of distilled water. Radioactivity determinations were carried out on 1 ml. portions of the diluted fraxtions, in some cases after the addition of 1 mg. of bovine serum albumin as a carrier and extraction of acid soluble components with 0.2 PCA.

This procedure of gradient centrifugation was applied also in one experiment to separate the components present in ribosomes prepared with deoxycholate from rough surfaced vesicles (Kirsch,1962) and in free ribosomes, which were also treated with deoxycholate to serve as a control. 13 OD 260 units of these particles were applied in a thin band to the top of a 12% - 30% sucrose gradient, containing 0.025 M potassium chloride, 0.004 M magnesium chloride and 0.035 M tris buffer pH 7.6 (Penman et al, 1963). The samples were centrifuged at 125,000g for 60 mins. in the S.W. 25 rotor of the preparative ultracentrifuge and the gradient was separated into 20 drop fractions and these analysed for RNA by measurement of their absorbtion of 260 mu, which gives an approximate estimate of the amount of ribonucleoprotein in each fraction (Henshaw et al, 1963; Munro and Korner, 1964). The ribosomes from rough surfaced vesicles (Fig.8) were distributed in a single peak around tubes 11 - 13 and in decreasing amounts in lower regions of the gradient, reminiscent /

Fig.8. Separation on sucrose gradients of ribonucleoprotein particles prepared from free ribosomes and rough-surfaced vesicles with DOC.



of the pattern obtained with '80s' ribosomes and polysomes from rat liver (Wettstein et al, 1963). However a substantial proportion of the free ribosomes seemed to aggregate and sediment into the bottom 4 tubes of the gradient (Fig. 8), which should contain components exceeding 300s in sedimentation constant, only (Henshaw et al, 1963). Such heavy components were not detected when free ribosomes were examined in the analytical ultracentrifuge in a medium containing 2.5mM magnesium chloride. Though impossible to come to any definite conclusions on the basis of this one experiment, it is thought possible that the heavy aggregates may have been formed under the influence of 4 mM magnesium chloride, perhaps facilitated by the prior treatment of the free ribosomes with deoxycholate.

1) Isolation of lecithin and sphingomyelin by thin layer chromatography.

Lecithin and sphingomyelin were isolated from purified lipid extracts by Mr. T. Duffy, using a thin layer chromatographic method. The lipid extracts were evaporated to dryness at 37°C in a stream of nitrogen and applied in a minimum volume of chloroform-methanol to silica gel coated thin-layer chromatographic plates, prepared according to the method of Truter, (1963). Using the 'line' technique (Truter, 1963), up to 3 mg. of phospholipid could be applied to a single plate. The chromatograms were developed in a solvent mixture consisting of chloroform-methanol-acetic acid - 0.01 M sodium carbonate (50:25: 8: 4), (Skipski et al, 1962), development taking about 60 mins. Lecithin and sphingomyelin were located by spraying /

the plates with reagent containing bismuth nitrate, silver nitrate and acetic acid, which reacted specifically with the choline-containing phospholipids (Dagendorf, 1953). These areas of silica gel were then promptly scraped off the plates and the lipids eluted with 4 extractions with 2:1 chloroform-methanol. The Dagendorf reagent did not interfere in the subsequent estimation of phospholipid-phosphorus and radioactivity in eluted lipids.

j) Electron microscopic examination of the submicrosomal fractions.

Samples of the submicrosomal fractions were examined by Mr. H. Johnston of the Anatomy Department with the electron microscope. The pellets were fixed *in situ* with buffered osmium tetroxide, dehydrated in increasing concentrations of methanol, cleared in xylol and embedded in araldite (Johnston, *et al*, 1963). The embedded material was sectioned approximately 200 Angstroms thick and stained with lead hydroxide to increase the contrast of ribonucleoprotein particles (Watson, 1958) before examination with a Phillips RCA electron microscope.

SECTION 2.

SUBFRACTIONATION of RAT LIVER MICROSOMES.

INTRODUCTION.

The microsomes from rat liver contain three major components, free ribosomes, granular or rough-surfaced vesicles and agranular or smooth-surfaced vesicles (Palade and Siekevitz 1956a; Moule et al, 1960; Chauveau et al, 1962). These are derived from ribosomes unattached to membranes in the intact hepatocytes and fragments of the granular and agranular endoplasmic reticulum formed during homogenization.

A procedure was sought, which would allow these three major components to be separated from each other and recovered in quantitative yield if possible. Ideally it should be possible to study amino acid incorporation into protein in vitro and in vivo with the isolated components as well as RNA and phospholipid turnover in vivo.

A number of methods were considered for this fractionation.

a) Deoxycholate treatment
b) Pyrophosphate treatment
c) Isooctane treatment
d) Prolonged centrifugation in 30% and higher density sucrose
e) A combination of centrifugation in 30% sucrose and treatment with isooctane.

a) Deoxycholate treatment.

The deoxycholate method was not found very useful for fractionating microsomes because it yields a heterogeneous ribosome fraction and the solubilized membranous components are difficult to separate from one another (Ernster, Siekevitz and Palade, 1962). Deoxycholate ribosomes are a mixture consisting of those initially free of membranes in the microsomes and those bound to fragments of the granular endoplasmic reticulum. Also the detergent is a potent inhibitor of protein synthesis (Littlefield and Keller, 1957) so that the DOC - ribosomes have to be thoroughly rinsed before studying amino-acid incorporation in vitro, and the mixture of solubilized membranes cannot be sufficiently freed of deoxycholate for such studies to be carried out. Finally deoxycholate solubilizes some of the ribosomes as well as the microsomal membranes. The proportion of ribosomes solubilized depends upon the precise conditions under which the microsomes and detergent are mixed (Roth, 1960) and the weight ratio of deoxycholate to microsomal protein (Glen, 1963). In one experiment, washed microsomes, resuspended in medium A were mixed by inversion with an equal volume of freshly made up 1% deoxycholate in medium A, so that the final concentration of detergent was 0.5% and the weight ratio of deoxycholate to microsomal protein was set at 2/3. After centrifugation at 105,000g. for 60 mins. only 63% of the RNA was recovered in the ribosome fraction while 37% was found in the DOC supernatant along with the microsomal membranes (Table 4).

Separation of the RNA of washed microsomes on sucrose gradients shows that at least 90% of it is 29s and 18s ribosomal RNA

TABLE 4.

The distribution of RNA between the ribosome and membrane fractions from deoxycholate treated rat liver microsomes.

Fraction	RNA content (mg)	% of microsomal RNA in fraction
Microsomes	3.0	100
DOC - ribosomes	1.9	63
DOC - membrane	1.1	37

(see below). Hence DOC must solubilize about 25% of the ribosomes under the above conditions. Under different conditions 50 - 60% of the ribosomes may be solubilized (Roth, 1960; Moule et al, 1960). We have found that the RNA recovered in the ribosomes and that solubilized with the membrane both contain pseudo-uridylic acid.

b) Pyrophosphate treatment of rat liver and pigeon pancreas microsomes.

It has been observed (Sachs, 1958) that treatment of rat liver microsomes in the cold with 0.1 M sodium pyrophosphate pH7 in 0.25 M sucrose enabled them to be separated into a fraction sedimenting at 105,000g. in 60 mins and another fraction which did not sediment at that impulse. The fraction which sediments contains most of the protein and phospholipid of the microsomes and about 30% of the RNA and hence contains most of the membranous material; it will be hereinafter referred to as pyrophosphate-membrane. The unsedimented fraction on the other hand contains most of the RNA, little phospholipid and some of the protein of the microsomes and probably consists of ribosomes and some of the more soluble microsomal proteins; it will be referred to as pyrophosphate-ribosomes.

Investigations in this laboratory (Barr, 1961; Goswami, 1962) have shown that the RNA of the pyrophosphate membrane and ribosomes differ significantly from one another in several ways.
(a) after two hours labelling in vivo with 32p the isolated nucleotide

monophosphates AMP, UMP, GMP and CMP of the membrane - RNA possess about twice the specific activity of their counterparts in the ribosome fraction. (b) the membrane RNA has a higher guanylic acid and cytidylic acid content than the ribosomal RNA and contains little or no pseudo-uridylic acid. Since this distribution of bases corresponded fairly closely with several reports of the base ratio of total nuclear RNA, which also contained little or no pseudo-uridylic acid, it seemed possible that the RNA of the membrane fraction obtained by pyrophosphate treatment of liver microsomes might be a unique species containing RNA recently transferred from the nucleus to the cytoplasm (Goswami, 1962). Therefore this fractionation method was investigated further to determine whether it fulfilled the criteria listed above for a desirable fractionation procedure for microsomes.

1) Determination of the sedimentation constant of pyrophosphate membrane RNA from rat liver microsomes.

To determine whether the RNA of the pyrophosphate-membranes survived the preparative procedure without being degraded RNA was isolated from several samples of pyrophosphate-membrane in the presence of different ribonuclease inhibitors and its sedimentation constant was determined in the analytical ultracentrifuge. Naphthalene 1.5 disulphonate (0.015M) + 0.1% 8 - hydroxyquinoline (Kirby, 1962) were used as inhibitors for one sample and 0.2% sodium dodecyl /

sulphate for the other and the RNA was extracted at 5°C to further reduce the chances of degradation during the isolation process. The RNA was freed of glycogen as described in the Methods Section and after precipitation with 2 volumes of ethanol was washed with 3:1 ethanol-water and dissolved in distilled water. It was run on the Spinco model E analytical ultracentrifuge at a concentration of about 50µg per ml. using ultraviolet optics.

The sedimentation pattern obtained was identical, irrespective of which ribonuclease inhibitor was used and it corresponded to a continuously polydisperse population of molecules with sedimentation constants ranging from about 1s to 8s. No peaks were obtained but the average sedimentation constant in both samples was calculated as 4.4s. It was concluded therefore that there was little high molecular weight RNA in the pyrophosphate-membrane fraction and that the RNA present was extensively degraded during the pyrophosphate treatment used to prepare the membrane fraction. This finding was not unexpected as pyrophosphate is a powerful magnesium chelating reagent and it is well known that RNA is extensively degraded in the presence of another magnesium chelator, EDTA (Taka-nami, 1960).

2) Amounts and base composition of pyrophosphate RNA fractions from pigeon liver and pancreas microsomes.

The pyrophosphate fractionation method was further investigated using pigeon pancreas microsomes to determine the /

relative amounts of membrane and ribosome-fraction RNA in this system. Pancreas microsomes were chosen for this study because they contain about twice as many ribosomes per unit weight as liver microsomes. This is evidenced by their high RNA : protein ratio (0.35 - 0.4) compared with that of liver microsomes (0.15 - 0.2) and the abundance of ribosomes visible in electron micro-graphs of pancreatic microsomes (Palade and Siekevitz, 1956). Hence it was expected that they should contain more RNA in their pyrophosphate ribosome fraction and less in their membrane fraction than liver microsomes. The base ratios of the RNA were also determined using two dimensional paper chromatography to see if these differed significantly in the two fractions from pancreas and if pseudo-uridylic acid was absent from the pancreas membrane fraction as it is in liver.

Pancreatic microsomes prepared as described in Methods were treated with 0.1M pyrophosphate and separated into a pyrophosphate soluble-fraction (ribosomes) and a pyrophosphate insoluble fraction (membrane). The amounts of RNA in these two fractions are given below in Table 5 .

The distribution of RNA between the ribosome and membrane fractions obtained from pancreatic microsomes with pyrophosphate is almost the reverse of the distribution observed for rat liver microsomes by Goswami, (1962) which has been confirmed on pigeon liver microsomes in this study. In pancreas 81% of the RNA is found in the membrane fraction and 38% in the ribosome fraction while in liver about 30% /

TABLE 5.

The amounts of RNA in pyrophosphate membrane and ribosomes from pigeon pancreas.

The RNA content of fractions quoted is the amount (mg.) of RNA in the fraction obtained from 1gm. wet weight of pancreas.

Fraction	RNA-content	% of total microsomal RNA
microsomes	9.6	100
P - P ribosome	3.6	38*
P - P membrane	7.8	81*

* Total recovery of RNA in fractions = 119% (See Goswami, 1962 also.)

is found in the membrane fraction (32% with pigeon liver) and 70% in the ribosome fraction (67% in pigeon liver). Hence the pyrophosphate method yields much less RNA in the ribosome fraction from pancreas than from liver despite the greater abundance of ribosomes in the pancreatic microsomes.

The RNA isolated from the pancreatic pyrophosphate fractions by alkaline digestion (see Methods) was separated into its constituent nucleotide monophosphates and its nucleotide composition determined. This is given below in Table 6 .

The RNA from the membrane fraction of pancreas microsomes resembled its liver counterpart in that it contained substantially more CMP than the ribosome fraction RNA. However unlike that from liver the membrane fraction from pancreas contained almost as much pseudo-UMP as the ribosomes.

It was concluded therefore that the pyrophosphate fractionation does not in fact separate the ribosomal RNA from membrane RNA, since in the case of pancreas a substantial proportion of the RNA of the ribosomes was found in the membrane-fraction. Also the absence of pseudo-UMP from membrane-RNA is not a constant feature of this fraction. Furthermore the fractions obtained by pyrophosphate treatment are incapable of amino acid incorporation into protein in vitro (Sachs 1958) and the RNA of the membrane fraction at least was substantially degraded and could not be used to study the turnover of different species of RNA in vivo. Finally the method did not allow the two membraneus components of the microsomes to be

TABLE 6.

Percentage nucleotide composition of RNA from the pyrophosphate membrane and ribosome fractions from pigeon pancreas.

	percentage of recovered nucleotides	
	membrane	ribosomes
GMP	35.8	34.5
UMP	17.4	19.2
pseudo - UMP	1.0	1.4
CMP	25.0	21.5
AMP	20.6	23.4

separated and so it was not investigated further for the fractionation of microsomes.

c) Isooctane treatment.

Hawtrey and Schirren (1962) published a brief account of a novel method of fractionating rat liver microsomes into ribosomes and membranes which was both rapid and convenient. The resuspended microsomes were emulsified with an equal volume of isooctane and centrifuged at 105,000g. for 40 mins. separating the system into two phases. The ribosomes sedimented as a well packed pellet at the bottom of the centrifuge tube while the membranes sedimented centripetally to form a pellicle at the interface of the isooctane and aqueous phases. Some unsedimented material remained in the aqueous phase (Fig. 11).

Both the ribosomes and membranes isolated by this method were able to incorporate amino acids into protein when incubated in a suitable medium with ATP, and therefore the technique was investigated further using whole rat liver microsomes and also rough surfaced vesicles prepared from rat liver.

Washed microsomes and rough surfaced vesicles were homogenized with isooctane and separated into ribosome, membrane and unsedimented fractions and the distribution of RNA, protein and phospholipid among these different fractions was determined. The typical distribution obtained is shown in Table 7.

1) Ribosome fraction.

Fig.11: Separation of microsomes into a 'membrane' and a 'ribosome' fraction by treatment with isooctane (Hawtrey).

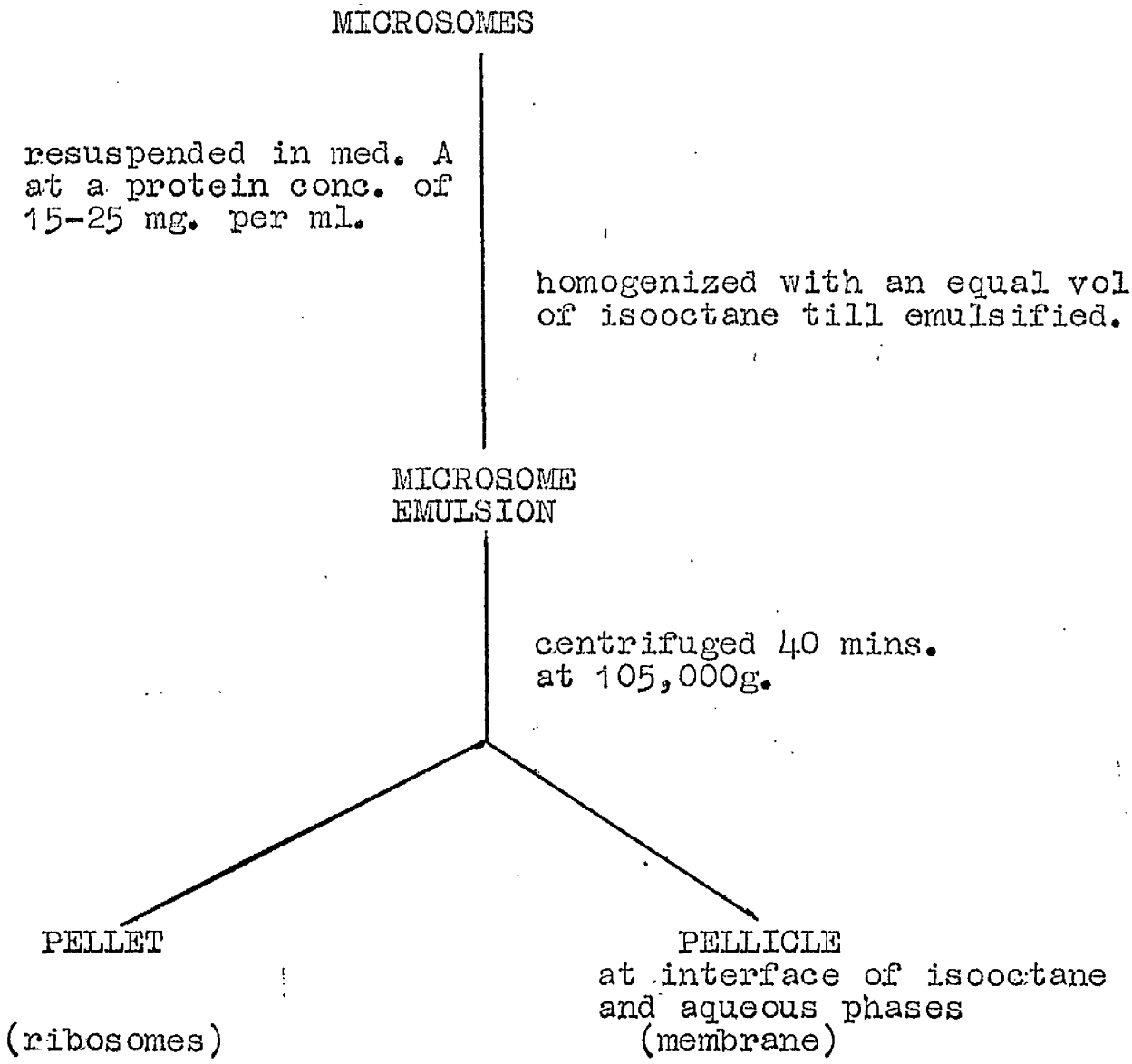


TABLE 7.

Distribution of RNA, protein and phospholipid among fractions from isooctane treated microsomes and of rough surfaced vesicles.

Amounts of chemical components in fractions are expressed as mg. in the fraction obtained from 1 gm. wet weight of liver.

Fraction	RNA (mg)	%	Protein (mg).	%	Phospho-lipid mg.	%	P-lipid protein
microsomes	2.5	100	20.5	100	9.9	100	0.63
membrane fr.	0.64	26	10.6	52	6.7	68	-
ribosome fr.	1.4	58	4.0	20	1.3	13	0.33
unsedimented fr.	0.31	12	2.8	14	1.2	12	-
rough surfaced vesicles	1.4	100	12.2	100	5.8	100	-
membrane fr.	0.77	55	7.4	61	4.5	78	-
ribosomes + unsedimented fr.	0.64	46	4.0	33	0.87	15	-

As expected the ribosome fraction contains large amounts of RNA and had an average RNA / protein ratio of about 0.30. However it also contains considerable amounts of phospholipid, its phospholipid protein ratio being 0.33, or half that of the unfractionated microsomes (0.63). Since it is well known that pure ribosomes are virtually free of phospholipid (Hallinan, Eden and North, 1962) it was considered that the large amounts of phospholipid in the isooctane ribosome fraction must be due to membranous contaminants of the ribosomes.

It is interesting to note here that Hawtrey et al (1962) found NAD H_2 - reductase in high activity in their isooctane ribosomes. This is a typical enzyme of the endoplasmic reticulum membrane (Garfinkel, 1957) also indicating serious contamination of their ribosomes with membrane. Final proof that the isooctane ribosomes were contaminated with membrane was obtained by re-treating the ribosome fraction with isooctane and centrifuging. Each isooctane treatment produced a fresh membrane pellicle from the ribosome fraction and lowered its phospholipid / protein ratio while increasing its RNA / protein ratio (Table 8). After four such treatments with isooctane the ribosomes had an RNA / protein ratio of 0.69, similar to DOC ribosomes from liver or rat reticulocyte ribosomes (Hallinan et al, 1962) and greater than that of free liver ribosomes prepared by prolonged centrifugation (Chauveau et al, 1962). However these purified ribosomes had almost completely lost their ability to incorporate ^{14}C leucine into protein, compared with the crude isooctane ribosome fraction (Table 9).

TABLE 8.

Effect on its composition of re-treating the ribosome fraction with isooctane.

Samples of isooctane ribosomes were treated three and four times with isooctane and analysed.

Number of treatments	RNA / protein	P-lipid / protein
1	0·36	0·32
3	0·62	0·15
4	0·69	0·08

TABLE 9.

Effect on their ability to incorporate amino acids into protein of re-treating ribosomes with isooctane.

Samples of isooctane ribosomes were treated three and four times with isooctane and their ability to incorporate ^{14}C leucine into protein *in vitro* was assayed before and after the additional isooctane treatments.

Number of treatments	Uptake of ^{14}C leucine in vitro
1	490
3	-
4	43

2) Membrane fraction.

The membrane fraction prepared with isooctane from either whole microsomes or rough surfaced vesicles contained most of the protein and phospholipid of the starting material as expected (Table 7). However it also contained a large and variable proportion of the RNA, which led us to suspect that it was still contaminated with incompletely removed ribosomes. The proportion of RNA in the membrane fraction prepared from whole microsomes was less than that in the membrane from rough surfaced vesicles. This is probably because free ribosomes, which are readily separated from the membranous components with isooctane make up about 30% of the RNA in the whole microsomes (see below), while virtually all of the RNA of rough surfaced vesicles is contained in the membrane bound ribosomes, which appear to be only partially detached from the membranes by isooctane.

To test whether isooctane did in fact incompletely remove bound ribosomes from the membrane fraction, a sample of this was subjected to three further treatments with isooctane. Each treatment produced a fresh crop of ribosomes and unsedimented material. Analysis of the original membrane fraction and that thrice treated with isooctane, and also of the further ribosome and unsedimented fractions obtained by repeated isooctane treatment revealed that 27% of the RNA of the original membrane fraction was due to contaminating ribosomes (Table 10). However despite the removal of those ribosomes /

TABLE 10.

Effect of re-treating the membrane fraction with isooctane.

A sample of isooctane membrane was treated three further times with isooctane and analysed. The ribosome and unsedimented fractions obtained with the three additional treatments were pooled and analysed also. Amounts of chemical components are expressed as mg. in the fraction obtained from 1 gm. wet weight of liver.

	RNA (mg)	Protein (mg)	P-lipid (mg)	RNA / protein	P-lipid / protein
membrane (orig.)	0.65	10.6	6.75	0.063	0.64
membrane (4x treat.)	0.28	5.3	5.2	0.053	0.98
ribosomes	0.17	0.6	0.06	0.28	0.01
unsedimented fr.	0.08	2.0	0.20	0.04	-

contaminating it the RNA/protein ratio of the thrice treated membrane was not significantly lower than that of the original isooctane membrane. This is apparently due to the concomitant release of protein into the unsedimented fraction, which accompanies the removal of contaminating ribosomes from the membrane (Table 10). The protein released into the unsedimented fraction seems to be mainly non-lipoprotein, as it is not accompanied by a corresponding amount of phospholipid; hence retreatment of the membrane fraction with isooctane increases its phospholipid/protein ratio from 0·64 to almost 1, (Table 10).

3) Unsedimented Fraction.

As well as the the particulate ribosome and membrane fractions obtained when microsomes are treated with isooctane, part of the microsomal material remains unsedimented at 105,000g for 60 mins. in the aqueous phase (See Methods). This unsedimented material contains RNA and phospholipid as well as protein and hence is more complex than Hewtrey and Schirren,(1962) originally believed it to be.

4) Base composition of the RNA of the fractions obtained from rat liver microsomes by isooctane treatment.

To characterise further the fractions obtained by treating rat liver microsomes with isooctane, the RNA was isolated from each by alkaline digestion (Fleck and Munro,1962) or with phenol (See Methods) ; this isolated RNA was then completely hydrolysed and

TABLE 11.

Percentage nucleotide composition of the RNA from the isooctane microsome subfractions.

The figures for the ribosome and membrane fractions are the mean (± S.E.) for three samples, with the exception of pseudo-UMP which was determined in two samples of membrane and one of ribosomes. Figures for the unsedimented fraction were obtained from one fraction only.

	Percentage of recovered nucleotides		
	Membrane	Ribosomes	Unsedimented
GMP	34.0 ± 0.6	34.7 ± 0.2	37.6
UMP	16.8 ± 0.6	17.7 ± 0.4	16.7
pseudo-UMP	2.3 ± 0.3	1.5	-
CMP	30.2 ± 0.6	29.9 ± 0.8	28.1
AMP	17.6 ± 0.3	16.7 ± 0.2	17.6

separated into its component nucleotide monophosphates by two-dimensional paper chromatography. The percentage base composition of the RNA from each fraction is given below in Table 11.

There is no difference between the base composition of the RNA of the membrane and ribosome fractions, which is not surprising since at least 90% of the RNA in both fractions is probably ribosomal in origin. The unsedimented fraction may contain an RNA species distinct from that in the other two fractions which contains additional GMP. However this possibility is only based upon determinations on a single sample of unsedimented fraction.

5) Conclusions on the applicability of the isooctane technique for fractionating rat liver microsomes.

The ribosome fraction obtained by isooctane treatment is heavily contaminated with membrane and cannot be purified without losing its capacity to incorporate amino acids into protein. Furthermore the membrane fraction is contaminated with ribosomes, removal of which is accompanied by the loss of membrane-protein. It was concluded therefore that isooctane cannot be used to detach bound-ribosomes from membrene or bound membrane from ribosomes. Furthermore the crude ribosomes obtained are a mixture consisting of those originally bound to membranes and those free in the microsome fraction. Finally, the membrane fraction obtained consists of rough surfaced membrane partially freed of its adherent ribosomes and smooth surfaced membrane. Hence the method was not used further for the fractionatinn of microsomes in the form above. /

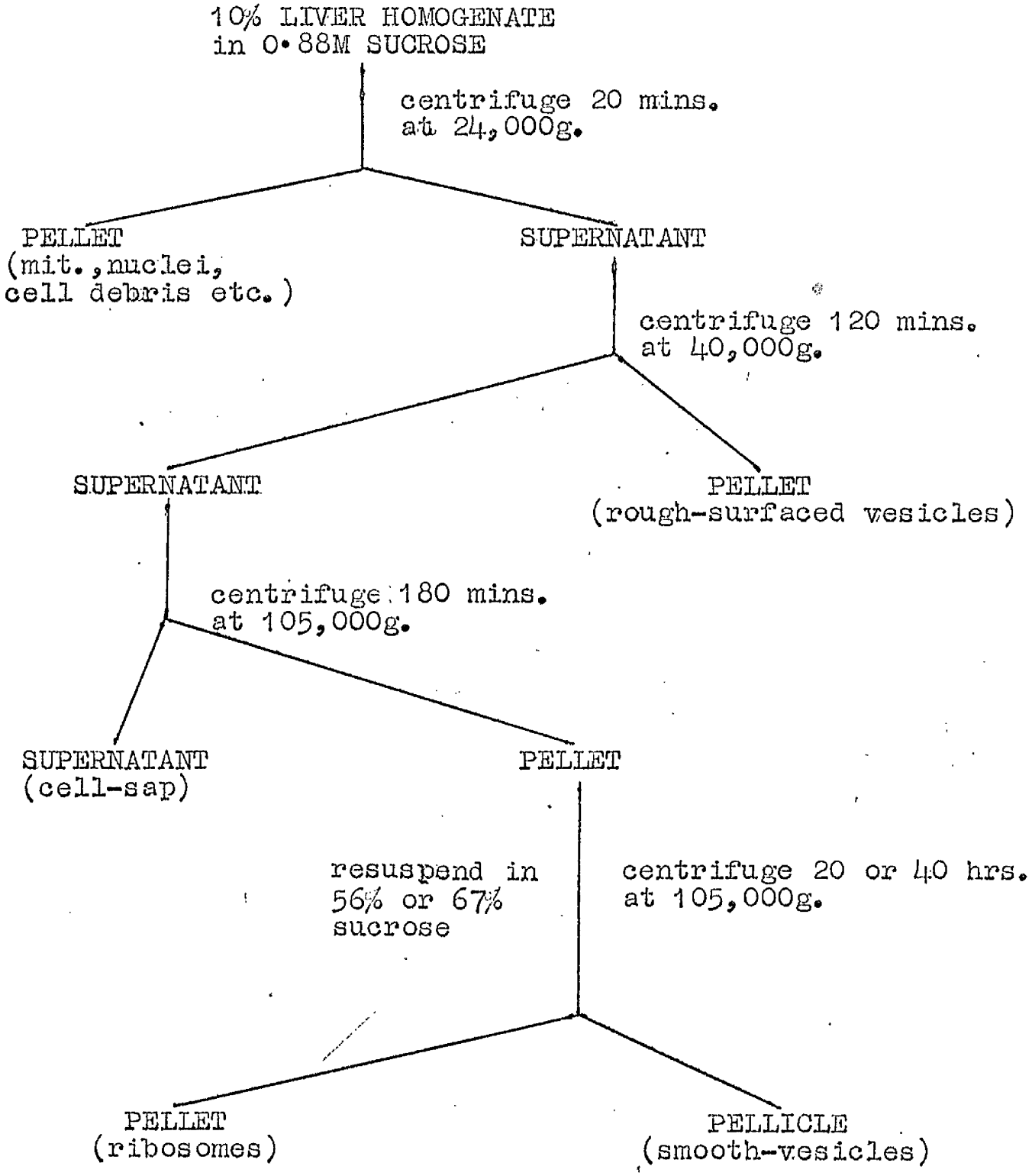
d) Centrifugation in high density sucrose.

Moule et al (1960) developed a relatively rapid method of isolating the rough-surfaced vesicle fraction from rat liver microsomes. This was done by simply centrifuging the post mitochondrial supernatant from a 0.88 M sucrose homogenate at 40,000g for 2 hrs., which was sufficient to sediment the rough surfaced vesicles without bringing down the free ribosomes and smooth surfaced vesicles.

However to separate the free ribosomes and smooth surfaced vesicles it was necessary first to sediment them as a mixed pellet from the rough surfaced vesicles - supernatant. This required 3 hrs centrifugation at 105,000g. The mixed fraction was then resuspended in sucrose of density 1.21 (40%) and centrifuged at 105,000g for 20 hrs. or was resuspended in 1.25 density sucrose (60%) and centrifuged for 40 hrs at 105,000g (Fig 12). The density of these sucrose solutions exceeded that of the smooth-surfaced vesicles so these sedimented centripetally and formed a pellicle at the top of the centrifuge tube, while the free ribosomes, whose density exceeded that of the suspending medium, sedimented to form a pellet at the bottom of the centrifuge tube.

The fractions obtained by this technique were shown by means of electron-micrographs of fixed and sectioned samples to be minimally cross contaminated (Moule et al,1960; Chauveau, et al, 1962); this was also confirmed by determination of the chemical composition of the fractions. Consequently the technique seemed to /

Fig.12. Separation of microsomes into rough-surfaced vesicles, smooth-surfaced vesicles and free ribosomes by prolonged, high-speed centrifugation (Moule and Chauveau).



give a perfectly adequate separation of the liver sub-microsomal components. Therefore further studies of this method were undertaken to check its reproducibility and to see if the fractions obtained could be used for metabolic investigations.

1) Composition and yield of the submicrosomal fractions obtained by the Moule-Chauveau technique.

To check on the reproducibility of the above method for preparing fractions, a sample of liver was taken right through the fractionation scheme, the smooth vesicles and ribosomes being separated by 20 hrs. centrifugation in sucrose of density 1.21. The distribution of microsomal protein, RNA and phospholipid among these fractions was then determined. This is ~~submicrosomal~~ given in Table 12, along with corresponding figures calculated from the data reported by Moule et al (1960) and Chauveau et al (1962) for their fractions.

Comparison of these figures show that the method is easily reproducible and yields fractions in our hands, which closely resembled those of Moule and Chauveau in their chemical composition.

2) Incorporation of amino acids into protein in vitro by the submicrosomal fractions.

Having established that the method gave reproducible results, an experiment was undertaken to see whether the fractions retained their ability to incorporate amino acids into protein in vitro after their prolonged isolation. Fractions were resuspended/

TABLE 12.

Yields of protein RNA and phospholipid in the submicrosomal fractions compared with the yields obtained by Moule and Chauveau.

The yields and composition of the fractions of Moule and Chauveau are calculated from their figures for RNA-P, phospholipid-P and total nitrogen in rough surfaced vesicles and ribosomes and smooth surfaced vesicles isolated by centrifuging for 20 hrs in sucrose of density 1.21. For the purpose of these calculations it was assumed that RNA contains 9.6% P, phospholipid 4% P, protein 16% N, RNA 15.5% N and phospholipid 1.8%N. The figures for microsomes refer to the amounts of chemical components in the microsome fraction from 1 gm. wet weight of liver.

	Protein (mg)	RNA (mg)	P-lipid (mg)
Whole microsomal composition	24	3.1	10.3
Moule-Chauveau microsomes	21.5	2.9	11.65

Distribution of components in fractions as a % of their amounts in whole microsomes.

	Protein	RNA	P-lipid	RNA / Protein	P-lipid / Protein
Our rough vesicles	59	56	68	0.12	0.49
Moule-Chauveau	65	62	66	0.13	0.56
Our smooth vesicles	17	5.4	24	0.04	0.60
Moule-Chauveau	17	5.5	26	0.04	0.76
Our free ribosomes	6	14	0.4	0.31	0.03
Moule-Chauveau	8	20	0.7	0.33	0.05

in medium A and incubated with 0.7 μC of ^{14}C leucine in a suitable medium (See Methods) for 20 mins. at 37°C. The incubated fractions were precipitated at a final concentration of 0.2N PCA and freed of RNA as described above and the leucine incorporated into their protein was determined. Table 13 shows the ATP-dependent incorporation by each fraction in terms of its protein content and its RNA content. Expression of specific activity on an RNA basis allows direct comparison to be made between the activity of the free ribosomes and the membranous fractions, in which the ribosomes, responsible for incorporation are diluted many-fold with membrane protein.

In this experiment the rough surfaced vesicles incorporated amino acid most rapidly on an RNA-basis, showing that this fraction contains the most active ribosomes. However incorporation in all fractions was rather low, only about ½ to 1/10 of that normally observed with whole microsomes incubated under the same conditions. This suggests that the fractions lose a significant proportion of their incorporating activity during their prolonged isolation. Comparison of the activity of the rough surfaced vesicles with rough surfaced vesicles prepared by 3.5 hrs. centrifugation (See below) suggests that this fraction loses proportionately more activity during the prolonged isolation than the smooth vesicles or free ribosomes.

Hence though the Moule-Chauveau method provides a means of obtaining all of the major submicrosomal fractions minimally cross contaminated, it is very time-consuming. Also the fractions obtained /

TABLE 13.

Incorporation of ^{14}C leucine into protein by the isolated submicrosomal fractions in vitro.

Samples of the three fractions containing 1.5 - 2.5 mg. of protein were incubated with 0.7 μC of ^{14}C leucine in 1 ml. of buffered medium. Incubation proceeded for 20 mins. at 37°C and was terminated by the addition of 0.2N PCA. Incorporation is corrected for ATP-independent uptake and uptake by the pH5 fraction.

	Ribosomes	Rough Vesicles	Smooth Vesicles
CPM for mg. protein	95	32	15
CPM per mg. RNA	310	430	350

from it have lost some of their ability to incorporate amino acids into protein *in vitro*. Finally because of the viscosity of the 40% or 60% sucrose used in the separation of the free ribosomes and smooth surfaced vesicles, the free ribosomes are only partially recovered. The fraction of free ribosomes not recovered remains with the unsedimented material between the smooth vesicle pellicle and the free ribosome pellet and it can reach 25-30% with sucrose of density 1.25 (Chauveau *et al*, 1962).

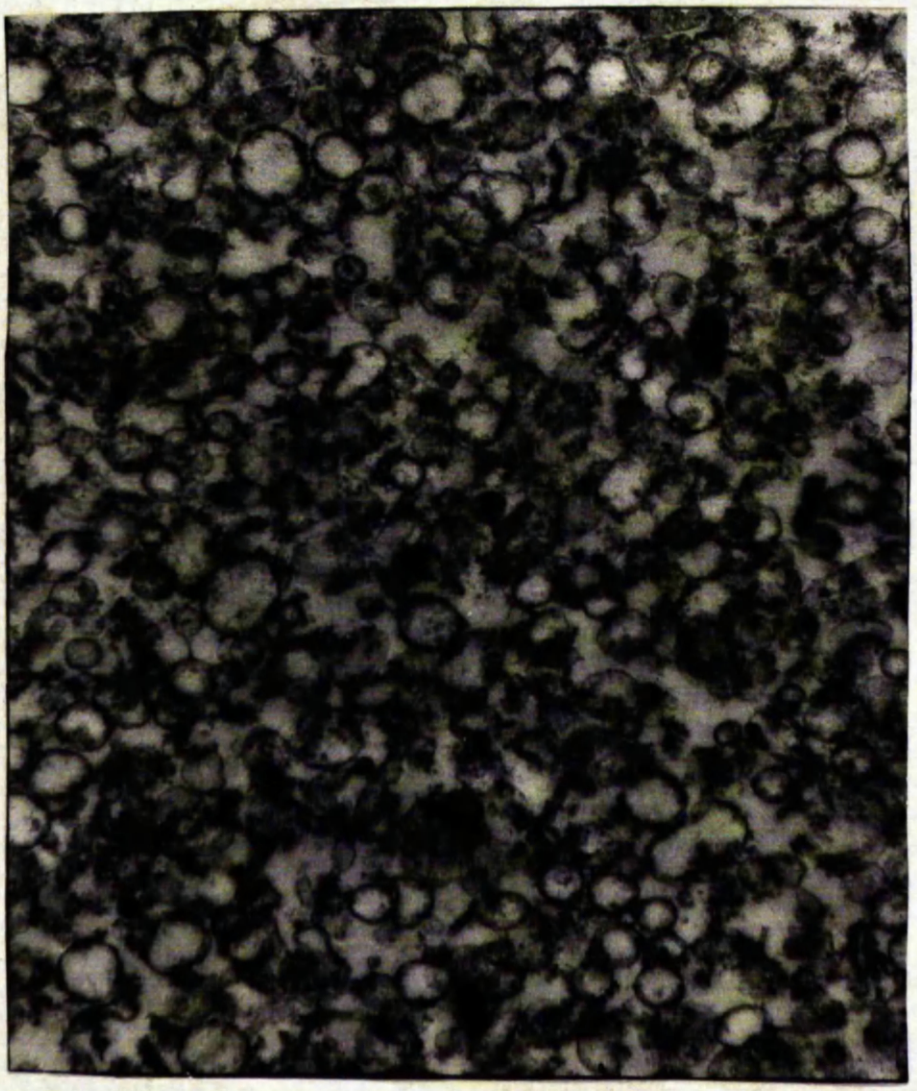
e) *A combination of centrifugation in 30% sucrose and treatment with isooctane*.

The rough surfaced vesicle subfraction was separated from the other components of the microsomes by the method of Moule *et al* (1960) by centrifuging at 78,000g for 1 hr. This yielded a fraction minimally contaminated with other components, as shown in electron micrographs of fixed sections of the fraction (Fig. 13). Moule *et al* also produced electron micrographs which show few other microsomal components contaminating the rough surfaced vesicle fraction. The supernatant after centrifuging down the rough-surfaced vesicles contained the smooth-vesicles and the free ribosomes and experiments were carried out to see if these could be adequately separated using the isooctane technique, with all the advantages of rapidity and convenience it offers.

Introduction

Fig.13. Fixed section of rough-surfaced vesicles (X 29,000)

The fraction consists of rounded vesicles and some fragments of cisternae, which are still flattened. These bear small electron-opaque ribonucleoprotein particles on their surface. In addition ribonucleoprotein particles can be seen in places apparently unattached to membranes: it is likely however that the bulk of these are in fact attached to membranes, underlying the plane of focus. A few small mitochondria are visible towards the bottom of the pellet but these only represent minor contaminants. Few smooth-surfaced membranous structures are present.



Initially the smooth-surfaced vesicles and free ribosomes were cosedimented in a pellet by centrifuging the supernatant from the rough vesicles at 150,000g for 2 hrs (Moule et al, 1960). This pellet was then resuspended in medium A at a protein concentration of 15-25mg. per ml. and homogenized with an equal volume of isooctane and centrifuged at 150,000g for 40 mins. This separated the smooth surfaced vesicles as a pellicle at the interface of the isooctane and aqueous phase from the free ribosomes, which formed a pellet at the bottom of the centrifuge tube. The chemical composition of these two fractions is given in Table 14.

Clearly the bulk of the phospholipid of the system is found in the smooth membrane, while the bulk of the RNA is found in the free ribosome fraction. The RNA: protein ratio of 0.065 for the smooth membrane is about 50% greater than the figure of Chauveau et al (1962) for their fraction prepared by 20 hrs centrifugation in sucrose of density 1.21 (see Table 12) but the P-lipid: protein ratio of the free ribosomes prepared by this method (0.06) is only slightly higher than the value of Chauveau et al. Hence this procedure separates the smooth surfaced vesicles and free ribosomes fairly cleanly in 2 hrs 40 mins. total centrifugation time.

It was found possible however to obtain a still cleaner separation of smooth surfaced vesicles and free ribosomes and at the same time to decrease the period for separating them by eliminating one centrifugation step. This was done simply by treating the post-rough-surfaced vesicle supernatant with isooctane without prior

TABLE 14.

Chemical composition of the smooth surfaced vesicles and free ribosomes obtained by treating a mixed pellet of these components with isooctane.

A mixed pellet of ribosomes and smooth surfaced vesicles sedimented at 150,000*g* for 2 hrs was resuspended and homogenised with an equal volume of isooctane and separated into membrane and ribosome components by centrifuging at 150,000*g* for 40 mins and analysed.

	RNA (mg).	Protein (mg)	P-lipid (mg)	RNA / protein	P-lipid / protein
ribosomes	0.66	2.6	0.15	0.25	0.06
smooth membrane	0.33	5.0	2.1	0.065	0.42

sedimentation of the smooth vesicles and free ribosomes from the cell sap as was done above. The rough vesicle supernatant was homogenized with half its volume of isooctane and centrifuged at 150,000g for 2 hrs. This spun up the smooth surfaced vesicles to form a pellicle at the interface of the isooctane and aquoud phases while the free ribosomes sedimented to the bottom of the tube as a tight-packed pellet and the cell sap remained unsedimented in the aquoud phase. (This cell sap was used later to prepare active pH5 enzyme fraction).

1) Composition of the microsomal subfractions obtained with the final modification of the combined centrifugation - isooctane technique.

The composition of the fractions obtained by this final modification of the subfractionation technique (shown in fig. 14), is given below in Table 15. The figures calculated from the data of Moule et al, (1960) and Chauveau et al, (1962) already given in Table 12 are repeated to allow ready comparison to be made between the fractions obtained by the two methods.

The distribution of RNA, protein and phospholipid among the rough and smooth vesicles is almost the same in subfractions prepared by the two methods and the smooth surfaced vesicles prepared by the isooctane technique have a slightly smaller RNA/ protein ratio than those of Chauveau et al, (1962) indicating that they are minimally contaminated with the other two RNA-rich subfractions. ~~Electron~~ /

Fig.14. Rapid separation of rough-surfaced vesicles, smooth-surfaced vesicles and free ribosomes from rat liver.

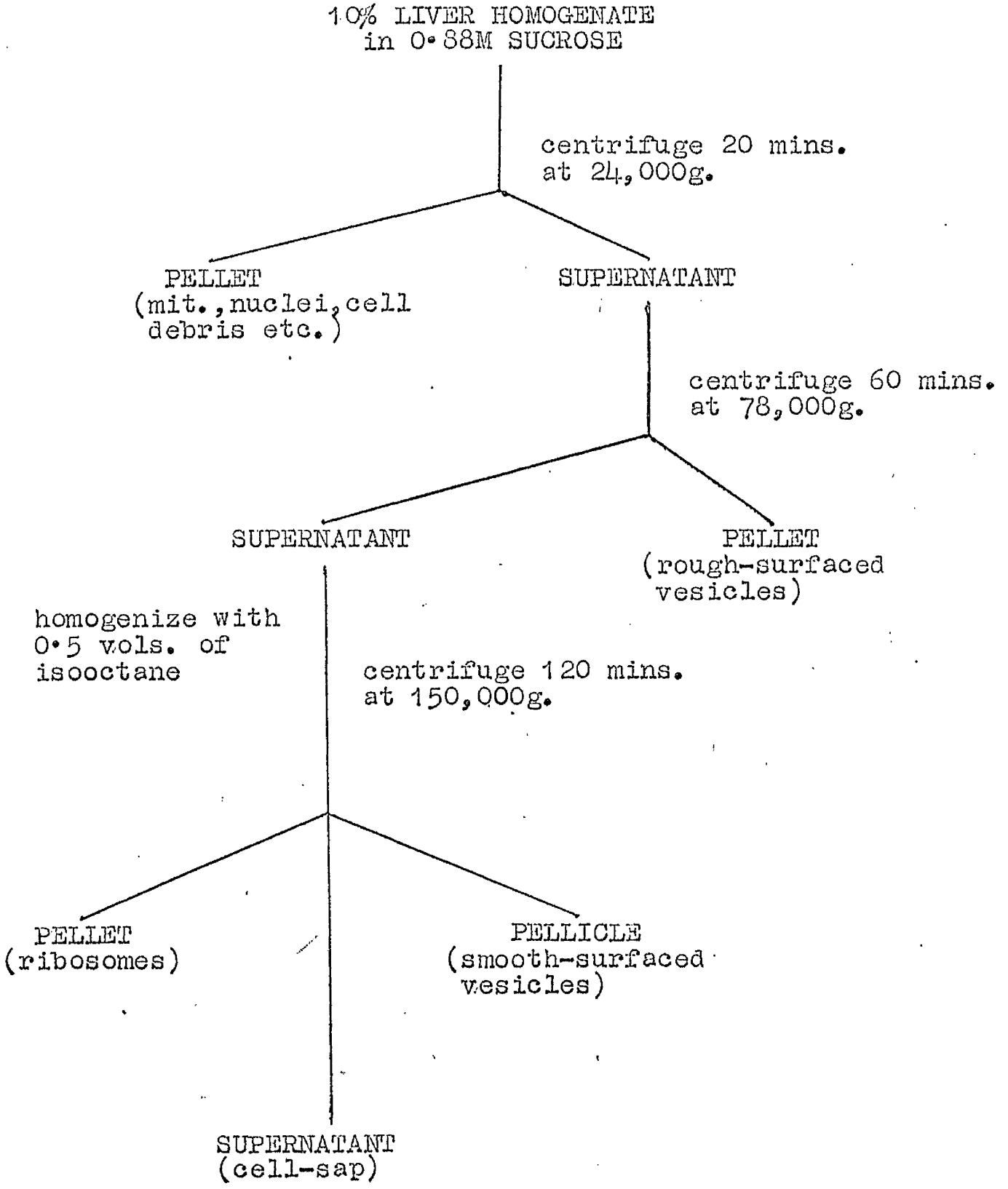


TABLE 15.

The composition of the submicrosomal fractions prepared by the combination of the Moule and isooctane techniques compared with the fractions isolated by Moule and Chauveau.

The rough surfaced vesicle fraction was sedimented from the post-mitochondrial supernatant at 78,000g for 1 hr while the smooth vesicles and free ribosomes were isolated by treating the rough vesicle supernatant with half its volume of isooctane and centrifuging at 150,000g. for 2 hrs. The figures for microsomes refer to the amounts of chemical constituents in the microsome fraction from 1gm. wet weight of liver.

	Protein (mg)	RNA (mg)	P-lipid (mg).
Whole microsome composition	17.0	2.9	7.7
Moule-Chauveau microsomes	21.5	2.9	11.65

Distribution of components in fractions as a % of their amounts in whole microsomes.

	Protein	RNA	P-lipid	RNA / protein	P-lipid / protein
Our rough vesicles	65	63	58	0.16	0.40
Moule-Chauveau	65	62	66	0.13	0.56
Our Smooth vesicles	24	5.0	31	0.035	0.59
Moule-Chauveau	17	5.5	26	0.04	0.76
Our free ribosomes	22	34	2.2	0.27	0.05
Moule-Chauveau	8	20	0.7	0.33	0.05

Electron micrographs of fixed sections of the smooth surfaced vesicle fraction completely bear out the chemical evidence, showing that the fraction consists almost completely of different sized vesicles devoid of adherent ribosomes (Fig. 15). Only very few, small rough surfaced vesicles or free ribosomes could be seen in the fraction.

The only marked difference in composition between the isooctane and Moule-Chauveau membranous subfractions is that the RSV AND SSV prepared by the isooctane method contain less phospholipid as indicated by their lower phospholipid protein ratios. However this is due solely to the fact that the whole microsomes used as starting material for fractionation contained less phospholipid than those used by Moule and Chauveau. This was also true in the experiment in which the reproducibility of the Moule-Chauveau method itself was checked (see Table 12) and is probably because younger rats (130-170 gm.) were used in our experiments than in those of Moule and Chauveau. It has been shown by Dawkins, (1959) that the amount of phospholipid in the liver microsomes increases from immature to old adult rats.

The composition of the free ribosome fractions obtained by the two methods, i.e. their RNA protein and phospholipid protein ratios, are also very similar, indicating that the same general type of particle is isolated by both techniques and that these particles are not significantly contaminated with the membranous subfractions. This is confirmed also by the virtual absence of membranous structures in fixed sections of the free ribosome fraction examined with the

Fig.15. Fixed section of smooth-surfaced vesicles (× 24,000)

The fraction consists of smooth-surfaced vesicles ranging widely in size and appearance. Some seem to contain electron-opaque material. An occassional ribonucleoprotein particleis visible, either attached to small membrane fragments or free. At the bottom of the pellet a few very extensive membranous structures were seen: it is thought that these could have been almost intact plasma membranes.

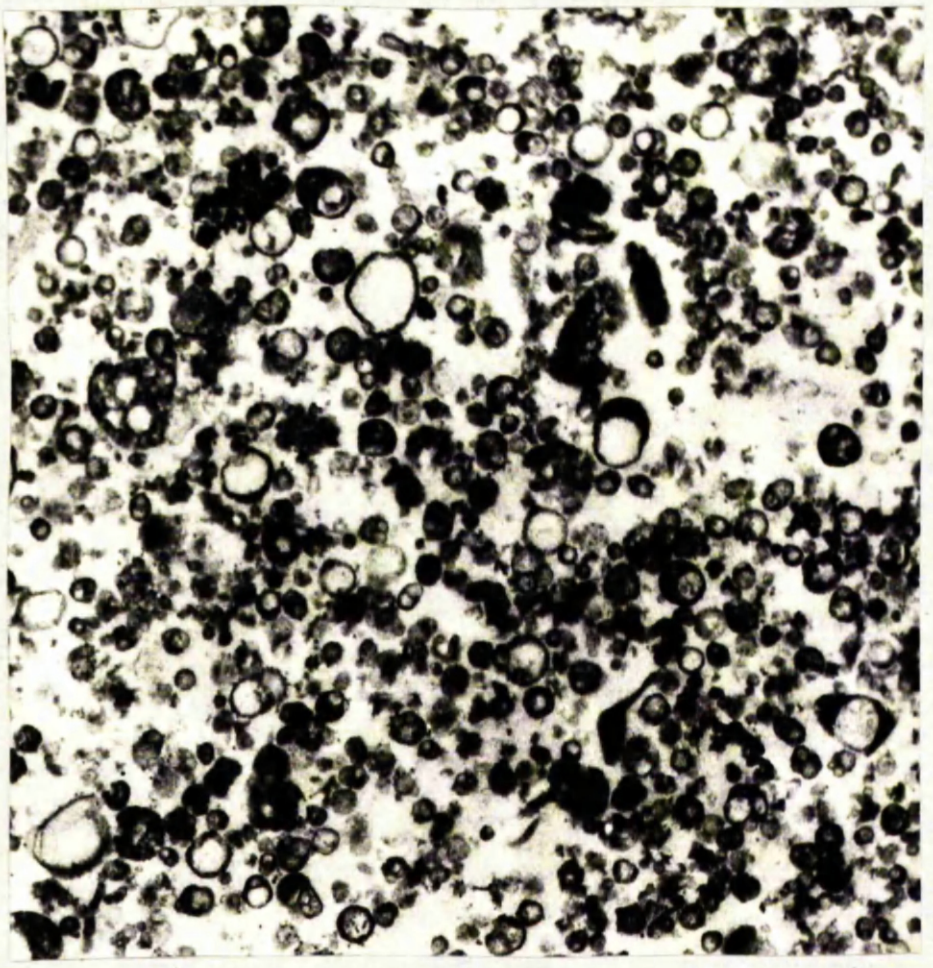


Fig. 16. Fixed section of free ribosomes (× 21,000).

This fraction consists almost exclusively of ribonucleoprotein particles with very occassional rough-surfaced vesicles as contaminants.

electron microscope.

However the yield of free ribosomes obtained by the isooctane technique is about double that obtained by 20 hrs. centrifugation in sucrose of density 1.21, according to Chauveau et al, (1962). The reason for this low yield in the Chauveau method is the viscosity of the 40% sucrose used to separate the SSV and ribosomes as discussed above (see also Chauveau et al, 1962). This effect is not encountered to the same extent with 0.88M sucrose (30%), so it seems that the yield of free ribosomes obtained by the isooctane method gives a more reliable estimate of the quantity of free ribosomes in rat liver microsomes. In five experiments carried out as above, the free ribosomes accounted for 34% ± 5%(S.D) of total RNA of the microsomes.

The RNA/protein ratio of the free ribosomes averaged 0·37± 0·03 (S.D.) in ten preparations. This is almost identical with the figure of 0·38 obtained by Keller, Cohen and Wade, (1963) for free ribosomes from pancreas. This ratio is only about half that of the deoxycholate ribosomes from rat liver microsomes or of the free ribosomes from rat reticulocytes (Hallinan, Eden and North, 1962). Keller et al, (1963) ascribe the low RNA/protein of their free pancreas ribosomes to binding of extraneous basic proteins, so this may well be the case with the free ribosomes from liver also. Certainly the additional protein can be removed by treating the particles with deoxycholate without reducing their capacity to incorporate amino acids into protein.

2) Effect of cations on the yield of different submicrosomal fractions.

Addition of cations, especially the divalent cations magnesium and calcium, to the 0.88M sucrose affects the yields of the various submicrosomal fractions. Comparison of the yields of the various subfractions from liver homogenised in 0.88M sucrose alone and in 0.88M sucrose buffered with 0.035M tris-HCL at pH 7.6 and containing concentrations of magnesium chloride, potassium chloride and calcium chloride, used by others in their preparative media shows that maximal yield of all the subfractions is obtained with 0.88M sucrose alone (Table 16). Higher concentrations of magnesium chloride (10 - 15mM) or 3mM calcium chloride greatly reduce the yield of free ribosomes and substantially reduce the yield of smooth surfaced vesicles, especially 3mM calcium chloride. In the presence of calcium chloride the yield of free ribosomes was reduced to 40% and that of smooth vesicles to 50% of normal.

It is noteworthy that there is a reduction in the yield of protein in the rough surfaced vesicle fraction when the concentration of magnesium chloride in the isolation medium is increased, but that no significant change occurs in the amount of RNA in this fraction over the concentration range studied. This eliminates the possibility that magnesium ions reduce the yield of free ribosomes and smooth vesicles by preventing the artefactual detachment of ribosomes from the rough surfaced vesicles to form components which might sediment like free ribosomes and smooth surfaced vesicles. No sign that magnesium exerts any protective effect on rough surfaced vesicles from liver,

TABLE 16.

Yields of submicrosomal fractions isolated in media containing various concentrations of $MgCl_2$, KCl and $CaCl_2$.

Equal weights of chopped rat liver were homogenized in the various media and the submicrosomal fractions were prepared from the homogenate as described above. RNA and protein were determined in the various subfractions and their amounts are expressed as mg. in the subfraction obtained from 1 gm. wet weight of liver. The abbreviations used for the media are as follows:

None - 0.88M sucrose alone, no added cations or anions.
Mg 4 - K25 -0.88M sucrose buffered at pH 7.6 with 0.035M tris - H Cl and containing 4mM Mg Cl 2 and 25 mM K Cl[1].
Mg 10 - K 25 - buffered 0.88M sucrose + 10mM Mg Cl 2 and 25 mM K Cl[2].
Mg 14 - K 25 - buffered 0.88M sucrose + 14 mM Mg Cl 2 and 25mM K Cl.[3]
Mg 4 - K 80 - buffered 0.88M sucrose + 4mM Mg Cl 2 and 80mM K Cl.[4]
Ca - 3 - buffered 0.88M sucrose + 3mM Ca Cl 2.[5]

Additions to medium	Rough vesicles		Ribosomes		Smooth vesicles	
	RNA	protein	RNA	protein	RNA	protein
None	1.95	17.5	0.790	2.8	0.175	5.1
None	1.98	17.8	-	-	0.205	5.8
Mg4 - K25	2.05	14.0	0.430	1.9	0.120	4.2
Mg4 - K25	-	-	0.425	1.9	-	-
Mg10 - K25	1.85	12.7	-	1.3	0.120	-
Mg14 - K25	1.95	-	0.265	1.2	0.120	4.2
Mg4 - K80	1.75	13.2	0.430	1.6	0.120	4.4
Ca - 3	1.60	12.4	0.295	1.6	-	2.3

1. Littlefield and Keller, (1957)
2. Optimal incubation conditions for amino acid incorporation into protein (see Methods).
3. Kirsch, (1962)
4. Korner, (1961)
5. Henshaw et al, (1963).

as it does on the somewhat analagous membrane-bound polysomes from B.Megaterium (Schlessinger, 1963), was detected.

Rather the particles lost from the free-ribosome and smooth surfaced vesicle fractions in the presence of high magnesium concentrations seem to aggregate with the heavy premicrosomal fraction, spun out at 3.6×10^5g. av.min; which then contains more RNA than the fraction obtained with 0.88M sucrose alone. This is consistent with the recent demonstration by Dallner, (1964), that part of the smooth surfaced vesicle fraction from liver can be aggregated with magnesium ions so as to sediment at relatively low centrifugal forces. The aggregation of isolated free ribosomes in magnesium chloride can also be demonstrated by sucrose density-gradient centrifugation. Figure 8 shows the optical density profile obtained when free ribosomes are centrifuged on a 12 - 30% sucrose gradient, made up in medium A which contains 4 mM $MgCl_2$. Ribosomes isolated from rough surfaced vesicles are run on an identical gradient as a control. The free ribosomes shows a large amount of material towards the bottom of the tube, which, under the conditions of centrifuation (60 mins. at 125,000g), must have had a sedimentation constant $\hat{s}_{20,w}$ of 300 s or greater (Henshaw et al, 1963), while the ribosomes from the rough-surfaced vesicles show much less material in this region of the tube. Free ribosomes, run in the analytical ultracentrifuge at a magnesium concentration of 1.5mM (Peterman and Pavlovec, 1963) contained only 62 and 83s components, so consequently these large particles must be artificial aggregates formed under the influence of the elevated magnesium concentration. /

3. Effect of ions on the recovery of labelled protein of submicrosomal fractions labelled in vivo.

As well as affecting the yield of the various submicrosomal fractions, some ions also alter the amount of labelled protein recovered in submicrosomal fractions labelled *in vivo* with ^{14}C leucine before isolation. Table 17 shows the effect of adding different concentrations of magnesium chloride, potassium chloride and calcium chloride to the homogenization media on the recovery of labelled protein in the submicrosomal fractions.

It can be seen that addition of 80mM potassium chloride or 3mM calcium chloride to the homogenization medium almost halves the specific activity of the free ribosome fractions protein and reduces the specific activity of the rough vesicle fraction's protein also. 14mM magnesium chloride on the other hand seems to enhance the specific activities of these fractions slightly. Potassium chloride at a concentration of 70 mM has been shown to cause the release labelled protein from ribosomes *in vitro*, so this probably accounts for the reduction in the specific activity of the free ribosome fraction isolated in 80mM potassium chloride. A similar effect may account for the reduction in specific activity by calcium chloride also, but obviously if this is so, calcium must be about 25 times more effective in causing the release of labelled protein than potassium. It is /

TABLE 17.

Recovery of labelled protein in submicrosomal fractions isolated in media containing various ions.

Equal weights of chopped rat liver from animals injected with 10 μC of ^{14}C leucine 15 mins. before killing were homogenized in the various media and the submicrosomal fractions were prepared as above. These were freed of acid soluble components and RNA and the amounts of radioactive amino acid incorporated into their proteins was determined as above. Radioactivity was converted to specific activity per mg. RNA, for ready comparison of the activity of the rough vesicles and ribosomes. The abbreviations for the media used are the same as those in Table 16.

Medium	Counts per min. per mg. RNA	
	rough vesicles	ribosomes
zero	2085	149
M4 - K25	2050	145
M10 - K25	1960	-
M14 - K25	2340	183
M4 - K80	1840	91
Ca - 3	1780	69

interesting to note that potassium releases much less labelled protein from rough-surfaced vesicles than from free ribosomes. This may indicate different modes of binding of labelled proteins to these two particles or possibly even differences in the proteins which are bound.

Clearly calcium is the prime candidate for the cation most to be avoided especially in studies of amino acid incorporation in vivo by the submicrosomal fractions. It is three to five times more effective than magnesium in reducing the yield of free ribosomes and smooth surfaced vesicles and about twenty-five times more effective than potassium in reducing the specific activity of the protein from the free ribosomes. Hence the free ribosome fraction, isolated in a medium containing 3mM calcium contains only one sixth as much labelled protein as that isolated in sucrose alone. 3mM calcium chloride was the addition to the isolation medium used by Henshaw et al,(1963).

DISCUSSION

The combination of the method of Moule *et al*, (1960) for isolating the rough-surfaced vesicles and the isooctane technique for separating the free ribosomes from the smooth surfaced vesicles has proved the most useful of the methods tried for fractionating microsomes. The fractions obtained are morphologically identifiable and minimally altered and there is little cross contamination of one fraction with the others, while all are obtained in quantitative or almost quantitative yield. Also, the fractions retain sufficient activity to be used for *in vitro* studies of amino acid incorporation as well as *in vivo* studies of this process and of RNA-turnover (see next Section).

Indeed the method is probably the most versatile developed so far for fractionating submicrosomal components. Early methods used by Palade and Siekevitz (1958b) and Kuff and Zeigel, (1960) which involved differential centrifugation were only partially successful for separating rough surfaced vesicles and free ribosomes from liver furthermore these latter particles were always mixed with smooth surfaced vesicles. These methods seem to have been somewhat more effective for separating free ribosomes from rough surfaced vesicles in Novikoff hepatoma (Kuff and Zeigel, 1960) and in pancreas (Siekevitz and Palade, 1958b). Recently Henshaw *et al* (1963) developed a sucrose gradient centrifugation method which allows rough surfaced vesicles /

and free ribosomes to be isolated in a fairly pure state, as indicated from their chemical composition. However they used 3mM calcium chloride in their centrifugation media which would be expected on the basis of our findings to aggregate about 60% of their free ribosomes and greatly reduce the amount of radioactive protein associated with the remainder of these particles in their in vivo labelling experiments. This in fact seems to have occurred; Henshaw et al recovered only about 30% as much RNA in their free ribosome fraction as was found in the free ribosomes in our study, and their free ribosomes, in some experiments at least, had much lower specific activities relative to the rough-surfaced vesicles than were found in our study (see next section). With the method of Henshaw et al and the several others discussed above, it was not possible to isolate the smooth surfaced vesicle fraction from liver microsomes.

Other methods have been developed recently which allow the smooth surfaced vesicles to be isolated from rat liver but these do not permit the rough surfaced vesicles to be separated from the free ribosomes. (Fouts, 1961; Peters, 1962a; Rothschild, 1963 and Dallner, 1963). These yield a smooth surfaced vesicle fraction with a similar RNA/protein ratio to that of our smooth vesicle fraction. However in all cases the methods require either prolonged centrifugation (8-12 hrs) or a special centrifuge, which allows very high gravitational fields to be generated (Dallner, 1963). Dallner, (1964) has very recently refined his fractionation method to isolate two different classes of smooth surfaced vesicles, which differ in size and morphology and their /

ability to be aggregated under the influence of magnesium chloride. This refinement is an improvement upon our method of dealing with smooth surfaced vesicles and may be able to be incorporated into our technique.

Other methods have been developed which allow a crude smooth surfaced vesicle fraction to be separated from the rest of the submicrosomal fractions. (Barbieri and Di Marco, 1963) and supposedly allow the total microsomal membranes (membranes from rough vesicles as well as smooth surfaced vesicles) to be separated from the total ribosomes (free ribosomes + those attached to rough surfaced vesicles), by treatment with 0.26% deoxycholate (Ernster, Siekevitz and Palade, 1962). However there is some doubt whether all of the smooth membranes are recovered by this method, and the 'ribosome' fraction is definitely heavily contaminated with membrane, as indicated by its phospholipid/protein ratio of 0.47 which is 77% of the ratio for the whole microsomes!

1. Origin of the submicrosomal fractions from cytoplasmic structures in the hepatocyte

Rough surfaced vesicles are believed to be derived from fragments of the granular endoplasmic reticulum, i.e. endoplasmic reticulum bearing adherent ribosomes on its outer surface (Moule et al 1960). The ribosomes seem to be tightly bound to the reticulum membrane since only about 45% of the RNA of the rough surfaced vesicles is removed by homogenization of the resuspended vesicles with isooctane and this proportion is not increased by adding 1 M urea or 1.6mM heparin to the resuspended vesicles prior to isooctane treatment. Furthermore /

the ribosome content of the vesicles is not reduced by preparing them in the absence of magnesium, unlike a somewhat analagous fraction from B. megaterium, consisting of polysomes bound to fragments of the protoplast membrane, where about 95% of the RNA is released in the absence of magnesium (Schlessinger, 1963).

The smooth-surfaced vesicle fraction contains a variety of membranous forms devoid of adherent ribosomes which are probably derived from a number of cytoplasmic structures in the hepatocyte. Some of these are fragments of the agranular (smooth surfaced) endoplasmic reticulum (including the Golgi apparatus) as shown by the preferential accumulation of newly synthesized protein by the smooth surfaced vesicle fraction in vivo (See next section). However, others may be fragments of the plasma membrane or of biliary microvilli or perhaps be small pinocytosis or secretory vesicles, all of which are smooth-membrane structures.

The significance of the small amount of RNA in the smooth-surfaced vesicle fraction, which represents only about 5% of the total RNA of the whole microsomes is difficult to evaluate. Examination of this RNA isolated without degradation and separated on sucrose gradients shows that it contains the same major components, present in about the same proportions, as the free ribosomes and rough surfaced vesicles. These consist of 50-60% of a 29s component about 30% of an 18s one and about 10% of small molecular weight RNA (see below). It is well established now that 29s and 18s RNA corresponds to the structural RNA of the 60s and 40s subunits of mammalian ribosomes (reviewed by Sibatani, 1963). Consequently it can be concluded that 90% or so of the RNA of the smooth surfaced vesicle fraction is ribosomal in origin.

This ribosomal RNA may be part of the smooth vesicles or may be due to trace contamination of the fraction with free ribosomes or rough-surfaced vesicles. Occasional rough-surfaced vesicles and free ribosomes can be seen in electron micrographs of fixed sections of the smooth vesicle fraction but these seem much too rare to account for all of the RNA of the fraction, which is the conclusion also reached by Chauveau et al, (1962). However electron microscopy may not be the most sensitive tool to detect a low degree of contamination such as could occur in this case. It will be seen in the next Section that the total RNA of the smooth-surfaced vesicle fraction exhibits distinctly different metabolic behaviour to that of the rough-surfaced vesicles and free ribosomes, particularly under certain dietary conditions. These differences are very marked in some cases and it is difficult to explain them unless it is assumed that a substantial portion of the RNA of the smooth vesicle fraction is in fact distinctly different from that of the other two sub-fractions.

The free ribosomes probably correspond to those ribosomes which can be seen unassociated with membranes in electron micrographs of sectioned rat hepatocytes (Palade and Siekevitz, 1956a), though these seem fewer in number relative to the membrane bound ribosomes than was found in this study. Our fractionation method yields a free ribosome fraction containing about a third of the total microsomal RNA. The free ribosomes from bovine pancreas isolated by a somewhat similar method to that used by Chauveau et al, (1962) account for some 50% of the total /

microsomal RNA from that tissue (Keller, Cohen and Wade, 1963). The approximate figure of 10% arrived at by Henshaw, Bojarski and Hiatt (1963) for the contribution of free ribosomes to the total RNA of the microsomes is almost certainly several fold in error due to their use of .3mM calcium chloride in their homogenization medium, which seems to aggregate free ribosomes and cause them to sediment with the heavy premicrosomal particles, as shown earlier by us. Magnesium chloride at concentrations greater than 2.5mM seems to cause similar aggregation of free ribosomes, the extent of aggregation depending upon the concentration of magnesium and possibly also upon the pH and ionic strength (Petermann and Hamilton, 1961), as well as the presence of other cellular components. The almost ubiquitous use of magnesium chloride in excess of 2.5mM concentrations in homogenizing media for the preparation of liver microsomes has probably contributed to the general underestimation of the proportion of free ribosomes in rat liver cytoplasm.

The other possibilities, namely that a significant proportion of the particles in our free ribosome fraction are artifactually released from the nuclei or from the granular endoplasmic reticulum under low magnesium concentration conditions during homogenization, were considered and dismissed as not likely. There is about 4% as much RNA in the total nuclear ribosomes as there is in the cytoplasm (Waddington, 1964) while the free ribosomes account for about 25% of the total cytoplasmic RNA; so, in practice, contamination of the free ribosome fraction with nuclear ribosomes cannot be /

significant. The fact that separation of the sub-fractions in 0.88M sucrose alone, without the addition of magnesium leads to a maximal recovery of RNA in all of them, rough surfaced vesicles as well as free ribosomes and smooth vesicles is strong evidence against the possibility that a proportion of the free ribosomes are derived by detachment of bound ribosomes from the rough surfaced vesicles under these conditions. This is also borne out by the lack of effect of different concentrations of magnesium on the RNA-content of the rough surfaced vesicles. Finally, the demonstration in the next Section that the free ribosomes differ very markedly from the rough surfaced vesicles, or ribosomes released from the rough vesicles with deoxycholate, in their rates and kinetics of amino acid incorporation and RNA-turnover, make it difficult to accept the idea that they cross-contaminate one another to a significant extent.

SECTION 3.

TURNOVER STUDIES ON THE PROTEIN, RNA AND PHOSPHOLIPID OF THE SUBMICROSOMAL FRACTIONS.

INTRODUCTION

a) The Role of Submicrosomal Components in Protein Synthesis

The first systematic investigation of the role of submicrosomal components in protein synthesis was carried out with relatively crude fractions of rough-surfaced vesicles and free ribosomes from guinea-pig pancreas. (Siekevitz and Palade, 1958). This showed that the protein of the rough surfaced vesicle fraction became labelled earlier *in vivo* than that of the free ribosomes after ^{14}C leucine was injected into intact guinea pigs. It was shown after treatment of the rough vesicles with deoxycholate that insoluble ribosomes contained the protein of highest specific activity and not the vesicle membranes (or the soluble ribosomes). A somewhat analagous situation was found to exist in liver where the rough surfaced vesicle fraction became labelled earlier than a composite fraction consisting of smooth surfaced vesicles and free ribosomes. Several further investigations with composite fractions from liver have shown that the rough surfaced vesicles + free ribosomes became labelled earlier than the smooth surfaced vesicles *in vivo* (Peters, 1962 a,b) while Campbell and Cooper (1963) have recently shown that a fraction of smooth surfaced vesicles, somewhat contaminated with free ribosomes, incorporate amino acids into protein more slowly than whole microsomes *in vitro*.

This evidence, which suggests that the rough surfaced vesicle fraction synthesizes protein more rapidly than either the smooth vesicles or free ribosomes, has been called into question by Barbieri and Di Marco (1963), who suggested in a recent study that the smooth surfaced vesicles synthesize protein most actively of all the submicrosomal components in normal rat liver but not in hepatoma. Consequently, we undertook an investigation of the rates of amino-acid incorporation into protein by the three submicrosomal fractions from liver under standard conditions in vitro and in vivo (Hallinan and Munro, 1963, a,b). While our investigation was in progress, Henshaw, Bojarski and Hiatt (1963) reported that the rough-surfaced vesicle fraction from liver synthesizes protein much more rapidly than do the free ribosomes.

b) RNA. Turnover in the Submicrosomal Fractions.

The first investigation of RNA-turnover in submicrosomal fractions was also made by Siekevitz and Palade (1959) on the same relatively crude fractions of rough surfaced vesicles and free ribosomes from guinea pig pancreas, which they used in their earlier studies of protein synthesis. They showed that the RNA of the free ribosomes turned over at a similar rate to that of the rough surfaced vesicles, being a little faster than the deoxycholate-insoluble ribosomal RNA of the rough vesicles and a little slower than the deoxycholate soluble RNA. However the RNA of a fraction sedimenting at higher centrifugal forces than the free ribosomes, the 'second postmicrosomal fraction', which had an /

RNA protein ratio of 0.12, turned over two to three times faster than that of either the free ribosomes or the rough surfaced vesicles. Electron micrographs showed that this latter fraction contained some free ribosomes as well as large amounts of protein. No investigations were made of the molecular species of RNA turning over in these sub-fractions.

Chauveau et al (1962), working with liver submicrosomal fractions virtually identical with those used in our studies showed that the total RNA of the free ribosomes had three times the specific activity of RNA from the smooth surfaced vesicles, two hours after injection of ^{32}P into fasting rats. However, no other injection times were tried and no details were given of the methods used to isolate and purify the ^{32}P labelled RNA for counting, which makes evaluation of these results rather difficult. The only other data on RNA turnover in submicrosomal fractions from liver were given in a brief report by Henshaw, Bojarski and Hiatt (1963a), in which they state that a rapidly-labelled RNA with a sedimentation constant of 6 - 14s, (messenger RNA?) 'sediments with bound, but not with free ribosomes' when these particles are separated on sucrose gradients. The bound ribosomes referred to by Henshaw et al correspond approximately with our rough surfaced vesicles, while their free ribosomes correspond to that portion of the free ribosome fraction which can be isolated from an homogenate containing 3mM calcium chloride, (see Section 3) or about 30% of the total free ribosome fraction.

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Further investigations have been undertaken with well characterized submicrosomal fractions to supplement this rather fragmentary picture and to determine the rates of turnover of individual RNA species in the submicrosomal fractions. These include studies of the turnover of the total RNA of sub-fractions and of different RNA species comprising the total RNA, which were separated on sucrose-gradients. Preliminary experiments have also been made to test the effect of several different dietary regimes on RNA turnover in the submicrosomal fractions. These regimes are suspected of altering the proportion of polysomes present and the intensity of protein synthesis in rat liver microsomes.

c) Comparison of Phospholipid Turnover in the Membranous Subfractions of the Microsomes and in nuclei.

According to a very popular and widely disseminated theory, endoplasmic reticulum membranes are formed from the nuclear membrane, delaminating off the surface of the nucleus into the cytoplasm as shown schematically in Fig. 4. This theory is based wholly on observations of fixed sections of cells from a wide variety of tissues with the electron microscope (Gay, 1955; Swift, 1956; Merriam, 1959; Gay, 1960; Rebhun, 1961; Kessel, 1963.) There is some disagreement on the question of whether the membranous precursors of the endoplasmic reticulum are made within the Nucleus (Merriam, 1959; Afzelius, 1963) or on the cytoplasmic side of the nuclear membrane, but the concensus of opinion seems to favour the latter view (Swift, 1956; Rebhun, 1961) /

It is also generally agreed that newly synthesized endoplasmic reticulum is initially smooth surfaced (agranular) and only subsequently acquires ribosomes in the cytoplasm and becomes granular reticulum. (Fawcett, 1955; Rebhun, 1961; Kessel, 1963). Hence the simplest statement of the theory, which incidentally would not be accepted unreservedly by all of the workers quoted above, is as follows:-

Nuclear membrane → Agranular reticulum → Granular reticulum

Clearly, such a theory is open to ready test by normal, biochemical precursor-product techniques, so consequently preliminary experiments have been commenced to test it in this way. This has been done by using the rate of labelling of their total-phospholipid, lecithin and sphingomyelin as a measure of the turnover of the different membranous components. As far as it is known both lecithin and sphingomyelin function solely in a structural role and Fletcher and Sanadi (1961) have shown that lecithin turnover gives an accurate measure of the turnover of rat liver mitochondria. ^{14}C choline was injected into rats and the specific activities of the two choline-containing phospholipids, lecithin and sphingomyelin from liver nuclei, smooth-surfaced vesicles and rough surfaced vesicles were determined at various times after injection of the precursor. Most experiments were done on animals fasting 18 hours after a normal diet but animals regenerating endoplasmic reticulum after being maintained on dietary regimes known to reduce the amount of endoplasmic reticulum in hepatocytes, were also studied.

RESULTS.

a) The Role of the Submicrosomal Components in Protein Synthesis

1) Comparison of the rates of amino acid incorporation by the three submicrosomal fractions in vitro.

When the three submicrosomal fractions were incubated in a suitably buffered medium containing an optimal concentration of magnesium ions and pH5-fraction protein (See Methods Section), incorporation of ^{14}C leucine into protein by the rough-surfaced vesicles and free ribosomes was largely dependent upon externally supplied ATP in the incubation medium. (Table 18) As much as 50% of the uptake by the smooth-surfaced vesicles, on the other hand, was independent of added ATP.

It was assumed that the ATP independent incorporation by the smooth surfaced vesicles was not due to protein-synthesis, since it has been repeatedly demonstrated that this process requires added ATP in microsomes. The possibility that the membranous components of the microsomes, both of which had a higher ATP independent incorporation than the free ribosomes, might be able to generate some endogenous ATP for protein synthesis was considered (Morton and Raison, 1963); however it was thought more likely that the ATP-independent incorporation by the membranous subfractions could be ascribed to some process like transpeptidation (Suttie,1962) or incorporation into the abundant lipids of the fractions (Haining /

TABLE 18. Energy dependent and energy independent incorporation of amino acids by the submicrosomal fractions.

Incorporation is expressed as counts per min. per mg. of particle-RNA and is corrected for incorporation by the pH5-fraction.

	Incorporation - ATP	Incorporation + ATP	ATP dependent incorporation
rough vesicles	100	730	630
ribosomes	16	236	220
smooth vesicles	142	290	148

et al, 1960). Consequently the energy independent incorporation was not investigated further.

The comparative rates of ATP-dependent amino acid incorporation into protein by the three submicrosomal fractions is given in Table 19. RNA is used instead of protein as a basis for comparing the specific activities of the fractions, as this allows direct comparisons to be made between the free ribosomes and the membranous fractions, in which the ribosomal protein is diluted many-fold with membrane protein. It can be seen that incoporation per mg. of RNA is about twice as rapid into the rough surfaced vesicles than the smooth vesicles or free ribosomes, the last two of which exhibit quite similar activities. The difference between the rates of incorporation per mg. of RNA in the rough-surfaced vesicles and free ribosomes is highly significant (P is less than 0.01) (Hallinan and Munro, 1963 a,b.) Approximately the same relative activities are shown by particles prepared by our 30% sucrose-isooctane technique or by 24 hrs. centrifugation in high density sucrose (Moule et al, 1960; Chauveau et al, 1962), and in consequence it can be safely assumed that the differences in activity between fractions are not due to any inhibitory effect of isooctane, used to prepare free ribosomes and smooth-surfaced vesicles but not rough surfaced vesicles. However to establish this important point further, we incubated microsomes in a reaction mixture for amino-acid incorporation containing visible excess isooctane and compared the activity of these microsomes with that of a normal control sample: no differences were found between the two (Hawtry and Schirren (1962) /

TABLE 19. ATP-dependent incorporation of amino acids into protein by the submicrosomal fractions in vitro.

Particles were incubated at a concentration of 1.5 - 2.5mg. of protein per ml with an equal weight of pH5 fraction protein in a medium containing 350m - Molar sucrose 10m-Molar magnesium chloride, 25m-Molar potassium chloride, 35m-Molar tris-hydrochloride pH 7.6, 1m-Molar ATP, m-Molar creatine phosphate and 40 µg. ofcreatine phosphokinase. 0.7 µC of ^{14}C leucine was added to each tube and incubation was carried out at 37°C for 20 mins. Activity figures are corrected for incopporation by the pH5-fraction and incorporation in the absence of added ATP. Specific activities are expressed per mg. of particle RNA or protein.

CPM per mg. of RNA		Rough vesicles	ribosomes	smooth vesicles
Exp.	1	1360	800	-
	2	680	220	370
	3	630	220	148
	4	504	562	-
	5	475	154	93
	6	290	110	-
Moule-Chauveau method		430	310	350

CPM per mg. of protein		Rough vesicles	ribosomes	smooth vesicles
Exp	1	192	255	-
	2	83	136	34
	3	70	114	13
	4	58	178	-
	5	53	80	8
	6	38	32	-
Moule-Chauveau method		32	95	15

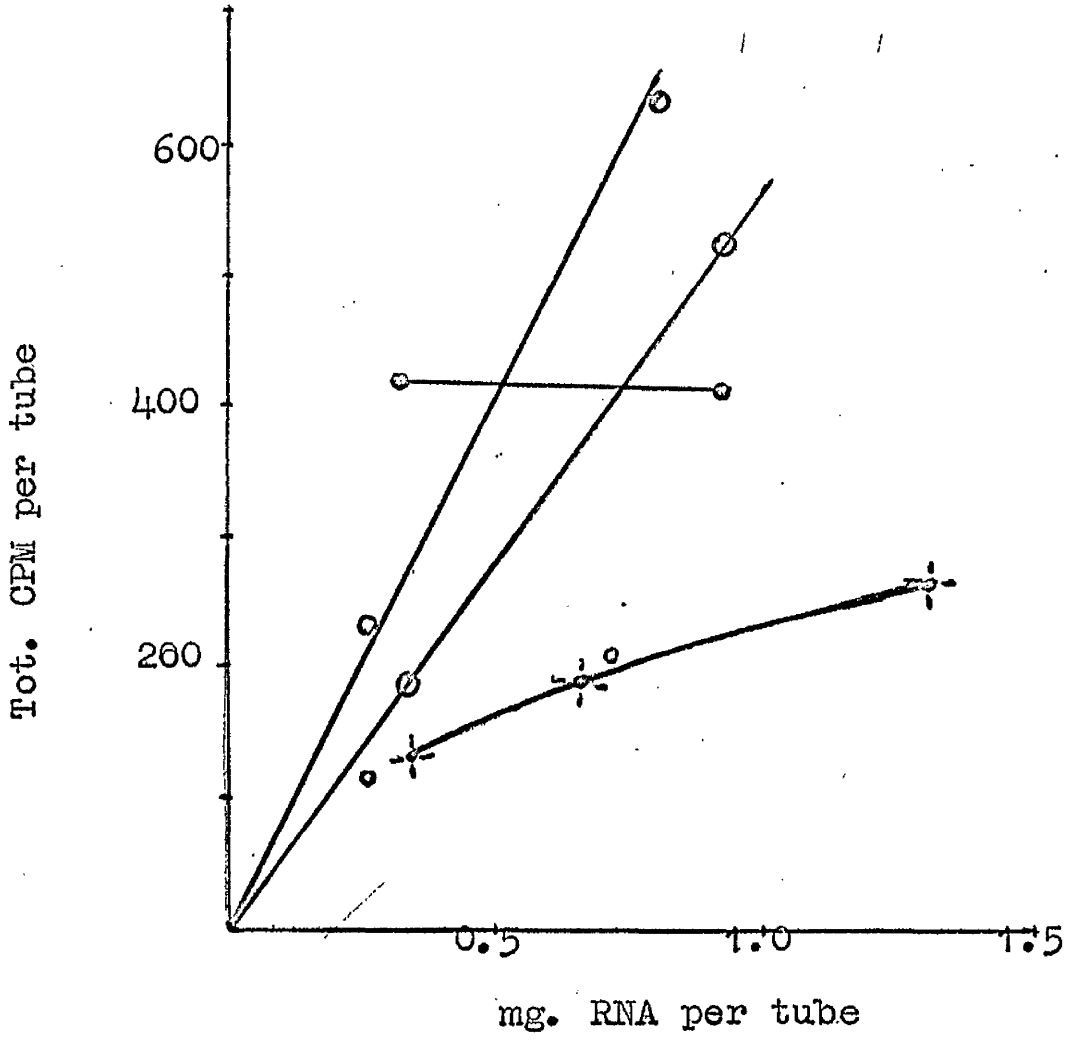
similarly found isooctane non-inhibitory for protein synthesis.

If incorporation is expressed per mg. of particle protein instead of per mg. of RNA, free ribosomes show the greatest activity of all of the fractions, (Table 19). Hence these particles were more active *in vitro* in our system than in that of Henshaw, Bojarski and Hiatt (1963, b), where free ribosomes showed negligible activity in the absence of added polyuridylate. As mentioned above in Section 2 Henshaw *et al* isolated their particles in a medium containing 3 mM calcium chloride, which when added to our isolation medium decreased the yield of free ribosomes by a factor of three and reduced the specific activity of particles labelled *in vivo* by a factor of two. Hence the low activity of the free ribosomes in Henshaw's system may well be due to this calcium effect. Calcium chloride at concentrations around 3 mM has been shown to aggregate deoxycholate ribosomes from rat liver (Takanami, 1960).

As fig. 17 shows, rough surfaced vesicles and free ribosomes do not differ only in their rates of amino-acid incorporation into protein, but also in the effect exerted by the concentration of particles in the assay system on incorporation. Free ribosomes give a constant rate of incorporation per mg. of RNA over a three-fold concentration range (1-3mg. of particle protein per tube), while incorporation by rough-vesicles can be reduced as much as 60% by raising the particle concentration from one to three mg. of protein per tube. This concentration dependence is not due to limiting /

Fig.17. Effect of increasing the particle concentration on amino acid incorporation by free ribosomes and rough-surfaced vesicles.

Varying amounts of particles were incubated *in vitro* with ^{14}C leucine as described in Methods. Free ribosomes incorporated the amino acid at a linear rate over a 3 fold range of concentration, while incorporation by rough-vesicles and ribonucleoprotein paticles prepared from them by means of deoxycholate, was strongly inhibited at higher particle concentrations. (o-----o) free ribosomes; (●-----●) rough-surfaced vesicles; (+-----+) DOC-ribonucleoprotein particles from rough-surfaced vesicles.



magnesium or pH5 fraction in the assay system (See Section) or to isotopic dilution of the ^{14}C precursor with ^{12}C intermediates associated with the bulky rough vesicle fraction. The latter possibility is ruled out by the fact that ribosomes isolated with deoxycholate from the rough surfaced vesicles show identical concentration-dependence to that of the rough vesicles from which they are isolated (Fig. 17), although the deoxycholate washing must remove a large proportion of soluble ^{12}C intermediates from the ribosome particles. This experiment also shows that the concentration dependence is due to the ribonucleoprotein particles of the rough-surfaced vesicle fraction and not to its membranous component.

It is obviously technically important to be aware of this factor of dependence of incorporation on particle concentration and to make allowance for it when working with rough surfaced vesicles or whole rat-liver microsomes, which also show it (Leggate and Fleck, 1964).

2) Labelling of the protein of the submicrosomal fractions with ^{14}C leucine in vivo.

Ribonucleoprotein particles, isolated by treating rough-surfaced vesicles and free ribosomes with deoxycholate 15 mins. after the injection of 20μC of ^{14}C leucine into intact rats, show the same relative rates of labelling as the isolated submicrosomal fractions in vitro (Table 20). However, in vivo, rough-surfaced vesicle ribosomes are about five times more heavily labelled than free /

TABLE 20. Labelling of the submicrosomal fractions in vivo.

Submicrosomal fractions were isolated 15 mins. after injection of 20 μC of ^{14}C leucine into fasted, 150 gm. rats. Some fractions were treated with 0.5% deoxycholate to prepare DOC-ribonucleoprotein particles from them. All fractions were freed of acid-soluble components and RNA before the extent of incorporation of radioactive leucine into their protein was determined.

	CPM. per mg. RNA	CPM per mg. protein
1) Intact rough vesicles	24,000	-
2) DOC-ribonucleoproteins from rough vesicles	8,400	1260
3) Free ribosomes (DOC-treated)	1,780	531
4) Intact smooth vesicles	55,000	1940

ribosomes, compared with only about twice as heavily *in vitro*. This may mean that the rough surfaced vesicles examined *in vitro* have lost a substantial proportion of their activity during isolation.

If the intact rough-surfaced vesicles are compared with free ribosomes after 15 mins. labelling *in vivo* it is found that they have acquired even more label than their ribonucleoprotein particles and are about thirteen times more heavily labelled than free ribosomes. This is most likely to be due to the temporary accumulation within the membranous portion of the rough-surfaced vesicles, of radioactive proteins made earlier in the time course by the ribonucleoprotein particles, a phenomenon which has been previously demonstrated by others (e.g. Peters, 1959; Campbell, 1961). It seems that even more of this labelled protein is translocated to the smooth-surfaced vesicles during the labelling period of 15 mins. (Peters, 1962 a,b.). This translocated protein accounts for the very heavy labelling of our smooth vesicle fraction on an RNA basis, (Table 20) compared with its low activity for amino acid incorporation into protein *in vitro*. Indeed, the smooth vesicle fraction has the highest specific activity even on a protein basis after a period of labelling of 15 mins. and hence contains most radioactive protein by this time. Labelled protein is apparently translocated from the rough-surfaced (granular) endoplasmic reticulum to the smooth-surfaced (agranular) reticulum via cisternae which link portions of these (Peters, 1962 a,b.).

Barbieri and Di Marco (1963) ignored this factor of translocation of proteins from their site of synthesis on the ribonucleoprotein particles, to other locations in the cell, like the smooth-surfaced vesicles, in interpreting their results. Therefore, when they found most of the labelled protein in a crude fraction of smooth-surfaced vesicles after 30 mins. labelling *in vivo*, they wrongly concluded that the smooth-surfaced vesicles were themselves responsible for synthesizing this protein instead of merely serving as temporary receptors for protein synthesized elsewhere. Our studies of amino acid incorporation *in vitro* and *in vivo* together with the results of Peter's experiments (Peters 1962, a,b.), show that the smooth-surfaced vesicles are only capable of very sluggish protein synthesis and are certainly not an important site of synthesis in rat liver.

3) Conclusions on the role of the submicrosomal components in protein synthesis.

The experiments described above on the incorporation of amino acids into proteins, both *in vitro* and *in vivo*, combine to show that the rough surfaced vesicle fraction is the most active of the submicrosomal components in protein synthesis. Just how much more active it is than the other two components is difficult to say. However considering that the rough vesicle fraction contains about 60% of the microsomal RNA, compared with about 34% in the free ribosomes and 5% in the smooth vesicles and taking the activities of the three fractions /

for amino acid incorporation *in vitro* as a measure of their capacity to synthesize protein, it can be calculated that in the intact cell the rough vesicles synthesize 4-5 times as much protein as the free ribosomes and 24-30 times as much as the smooth vesicles. However it is likely that the activity of the rough-surfaced vesicles *in vitro* underestimates their actual capacity for protein synthesis, due to preparative damage during isolation; consequently if the degree of labelling of their ribonucleoprotein particles *in vivo* is taken as a measure of the capacity of the rough vesicles and free ribosomes to synthesize protein, it can be calculated that the rough vesicles synthesize about 8 times more protein than free ribosomes.

Wettstein *et al* (1963) taking another approach to determine the site of protein synthesis in hepatocyte cytoplasm, came to the conclusion that polysomes were the most important protein-synthesizing units as is the case also with reticulocytes (Warner *et al*,1963) and bacteria(Risebrough *et al*, 1962). Wettstein's group isolated their polysomes by treatment of the post-mitochondrial supernatant with deoxycholate, which solubilized all of the membranous elements in the cytoplasm and probably some ribonucleoproteins also. Hence they did not consider the question of whether polysomes existed *per se* free in the cytoplasm or in association with cytoplasmic membranous elements. Henshaw *et al* (1963) sought free polysomes in liver cytoplasm without success and speculated that these could be bound to the rough surfaced endoplasmic reticulum in liver. This would /

imply that the polysomes are located in our rough-surfaced vesicle fraction.

This is consistent with our finding that the rough-surfaced vesicles are the most important site of amino acid incorporation into protein *in vitro* and *in vivo*. Also we have fragmentary evidence, on the basis of zone centrifugation and analytical centrifugation, that rough-surfaced vesicles contain at least 50% of large ribonucleoprotein aggregates with s-values greater than 150s, which fall within the size range of polysomes. Our finding of strong dependence of incorporation-rate upon the concentration of particles in the incubation system for rough-surfaced vesicles and their derived ribonucleoprotein particles, which was not shown by free ribosomes is also consistent with the former containing polysomes absent from the free ribosome fraction since the results of Fessenden, Caincross and Moldave (1963) show that artifical polysomes made by adding poly-uridy late to rat liver ribosomes, exhibit a similar strong dependence of incorporation on concentration. Finally, the yield of RNA in our rough-surfaced vesicle fraction is almost identical with the yield of RNA in the polysome fraction (their'C-ribosomes') separated by density-gradient centrifugation from free-ribosomes by Wettstein *et al* (1963). Very recent work with protoplasts from *B. megaterium* suggests that, in bacteria also, the polysomes are associated with a membranous structure, in this case the protoplast membrane (Schlessinger, 1963).

/

If the polysomes are bound to the membrane of the rough-surfaced endoplasmic reticulum in liver, one is faced with the problem of accounting for the incorporation of amino acids into protein by the other two submicrosomal fractions, free ribosomes and smooth surfaced vesicles. On the basis of our *in vitro* studies it seems as if the smooth-surfaced vesicles could only synthesize 3-4% of the microsome-protein and the significance of this very low level of synthesis is probably bound up with the allied problem of the origin and significance of the small amount of RNA in the smooth vesicles. However the free ribosomes could account for 10-25% of the microsomal protein synthesis and so pose the larger problem.

The free ribosome fraction does not contain any detectible polysomes, when examined in the analytical ultracentrifuge using ultra-violet optics, which should allow detection of components comprising as little as 5-10% of the total ribonucleoproteins. Hence single ribosomes would seem to be responsible for the amino acid incorporation in this fraction. This incorporation could be due to protein synthesis by single ribosomes attached to a strand of messenger RNA as shown by Munro, Jackson and Korner (1963) and by Knopf and Lamfrom (1963), the latter having shown that single ribosomes are able to initiate and complete the synthesis of a whole polypeptide chain of haemoglobin. Alternatively the incorporation by free ribosomes may not represent protein synthesis at all, but some undirected process of amino acid polymerization. A choice between these alternatives will probably require the products of synthesis of the free ribosomes to be isolated and characterized.

b) RNA TURNOVER IN THE SUBMICROSOMAL FRACTIONS.

1) Rates of incorporation of ^{14}C adenine and ^{32}P into the total RNA of the submicrosomal fractions in vivo.

Pairs of 150 gm rats were injected with ^{14}C adenine and killed at various times thereafter. The submicrosomal fractions were washed by resuspension in medium A and resedimentation to reduce adherent s-RNA and their total RNA was extracted by alkaline digestion, after removal of acid-soluble nucleotides (Fleck and Munro, 1962; Hallinan, Fleck and Munro; 1963). The free ribosomes always showed the most rapid initial rate of incorporation of RNA precursors by fasted rats, followed generally by the smooth and then rough-surfaced vesicles, which incorporate at rather similar rates, being about half that of the free ribosomes at early time intervals (Fig. 18). Similar results are obtained using ^{32}P as a precursor for a 40 mins. period of labelling, though here the RNA had to be isolated by the phenol-method and dialysed to free it of labelled impurities (See Section 1) The incorporation curve for the free ribosomes mostly levelled off and sometimes dropped at later time courses while the other fractions were labelling fairly linearly, reminiscent of a precursor plot, however this did not occur in all experiments and sometimes incorporation continued to occur into the free ribosomal RNA at a fairly steady rate at later times.

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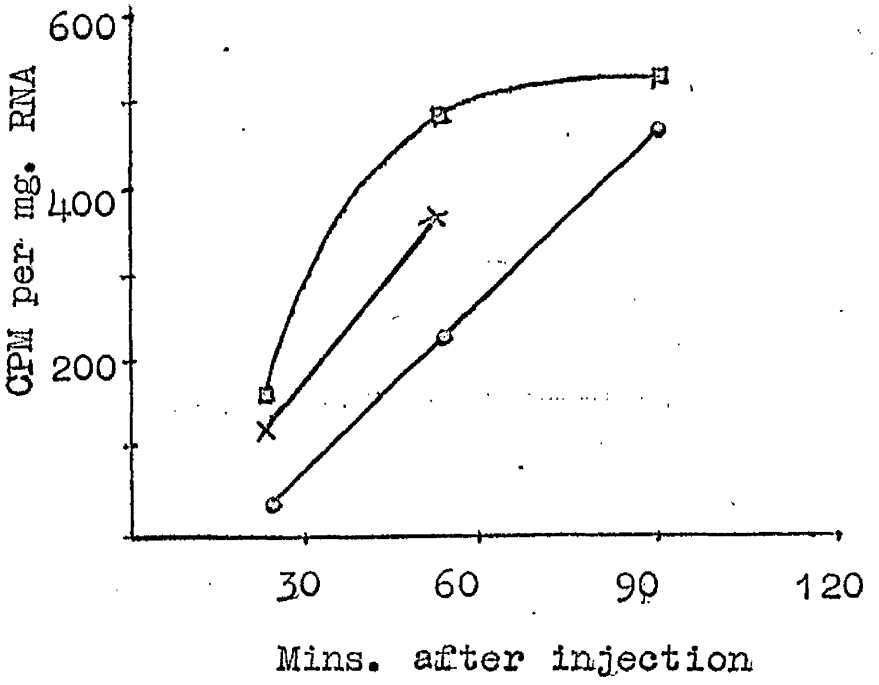
Fig.18. Time course of ^{14}C adenine incorporation into the RNA of the submicrosomal fractions from fasted rats.

Animals were fasted 18 hrs. before injection of the precursor. Total RNA was isolated by alkaline digestion after 6 washes with 0·2N PCA to ensure complete removal of acid soluble components from the fractions. (o--o) rough-surfaced vesicles; (x--x) smooth-surfaced vesicles (□--□) free ribosomes.



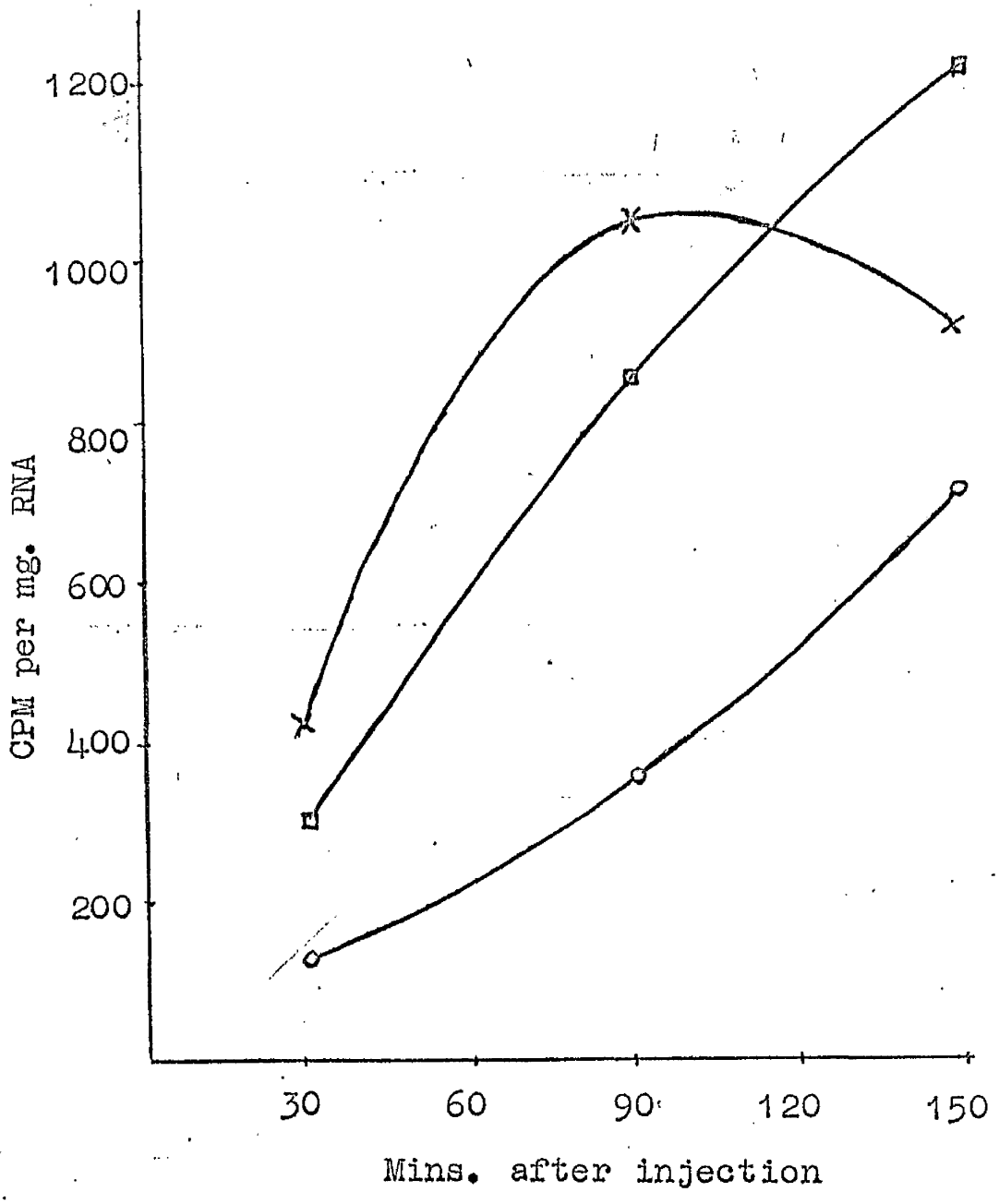
If instead of fasted rat#s, animals are used which have been fed 2 gm. of casein or allowed to feed ad libitum on a protein-rich diet before injection of the radioactive RNA precursor, a very different picture is obtained of the labelling of the RNA in the submicrosomal fraction, (Fig. 19). The smooth surfaced vesicles now show the most rapid initial rate of labelling, about 50% greater than that of the free ribosomes, which in turn exceed the rough-surfaced vesicles by about a factor of two. The labelling curve of the RNA of the smooth-surfaced vesicles either flattens out or actually declines at later time courses in all cases, while the radioactivity in the other fractions increases at a fairly linear rate. There is some evidence that protein feeding, particularly in the case of rats previously maintained on a low protein diet as in the experiment depicted in Fig. 19 ,may produce a small increase in the microsomal polysome population and an increased capacity to incorporate amino acids into protein in vitro. (Fleck and Leggate, 1964), so it is of particular interest that the RNA of the smooth-surfaced vesicles, which comprises only about 5% of the total microsomal RNA, should show such a dramatic increase in labelling under these circumstances.

2) Separation and examination of the individual RNA species from the submicrosomal fractions.

In order to shed further light on the turnover of the submicrosomal fraction RNA in vivo, RNA was isolated unde#graded from these fractions and purified as described in Section 1.

Fig.19. Time course of ^{14}C adenine incorporation into the RNA of the submicrosomal fractions from protein fed rats.

RNA was isolated by alkaline digestion after 6 washes with 0·2N PCA to ensure complete removal of acid soluble contaminants. (o--o) rough-surfaced vesicles; (x--x) smooth-surfaced vesicles; (□--□) free ribosomes.



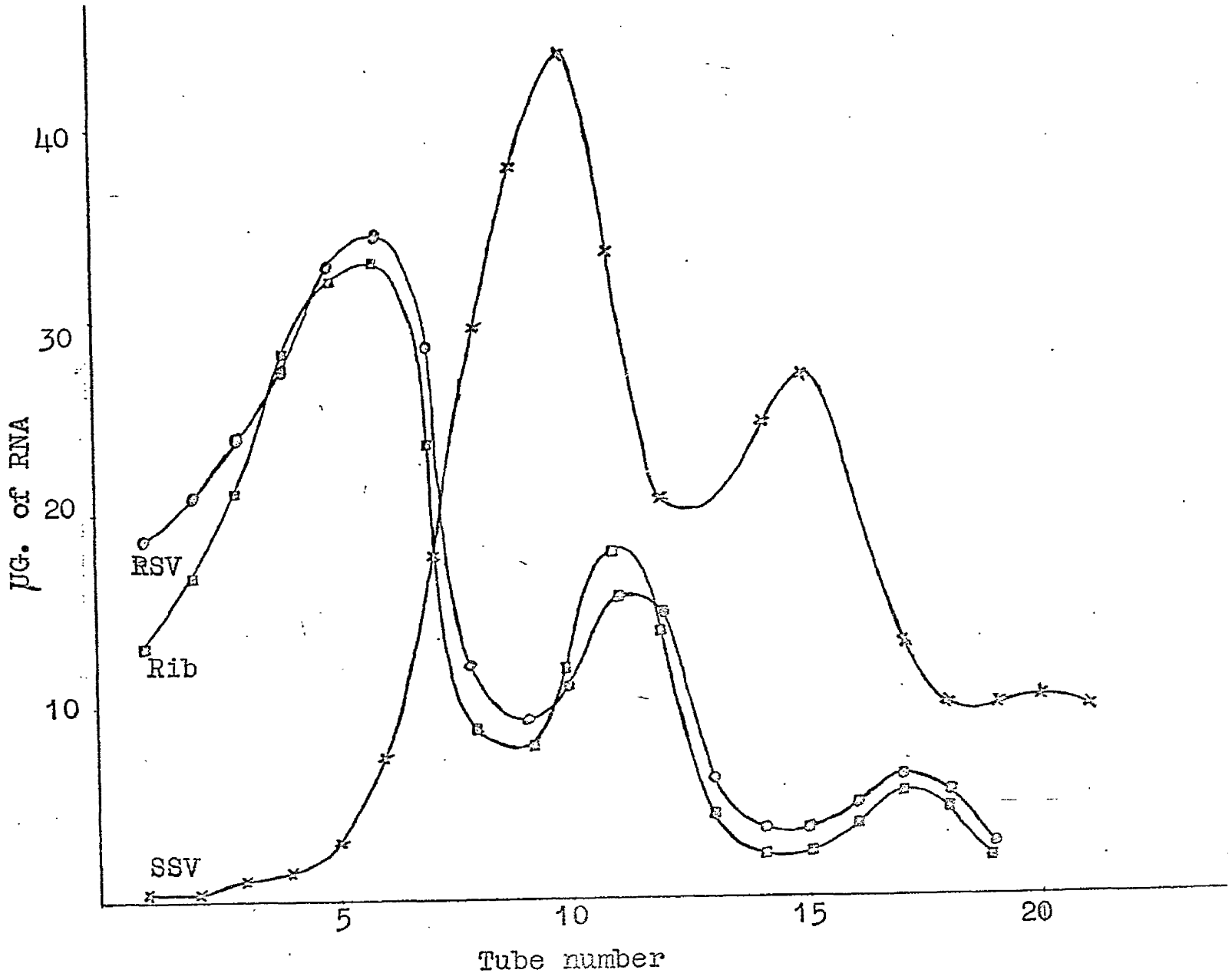
This purified RNA was then separated by sucrose gradient centrifugation into different species according to its sedimentation rate. Typical sedimentation patterns for the RNA from the three fractions are shown in Fig. 20 . These are identical in appearance showing 3 components, one about 29s which comprises 50 - 60% of the RNA of each fraction, another about 18s, comprising about 29% of the total and a heterogeneous fraction sedimenting at less than 18s and comprising about 10% of the total.

The 29s and 18s components are now well recognized as the slowly turning over, so-called 'structural RNA' of mammalian ribosomes, the 29s component being found in the '60s' sub unit and the 18s component in the '40s' sub unit of 80s mammalian ribosomes (see review by Sibatani, 1963). The components sedimenting at less than 10s include messenger RNA, which exhibits a considerable range of s-values in liver (Staehelin et al, 1964), transfer-RNA which seems to have an s-value around 4.5s and probably a variety of degradation products of other RNA species.

It is of considerable interest that the smooth-surfaced vesicle RNA contains exactly the same components, present in the same proportions as in the free ribosomes and rough-surfaced vesicles; indeed the absence of any detectible difference between the RNA of these fractions argues against the existence of 'membrane-RNA', a supposedly unique RNA species associated with the endoplasmic reticulum membrane. (Shigeura and Chargaff, 1958) Chauveau et al, /a

Fig. 20 Sedimentation patterns of the RNA components from rough and smooth-surfaced vesicles and free ribosomes.

RNA from RSV and rib. was centrifuged for 12 hrs. and that from SSV for 8 hrs. at 23,000 RPM in the SW 39 head of the Spinco model L ultracentrifuge.



1962; Schulman and Bonner, 1962).

It is important to note that the different species of RNA could only be isolated without degradation from unwashed submicrosomal fractions. Washing the fractions by resuspension in medium A and recentrifugation, which was initially used to deplete them of trapped transfer-RNA, always led to a considerable loss of the 18s ribosomal RNA component and a corresponding increase in the amount of material sedimenting at less than 18s (Fig. 21). This may be because washing removes the natural ribonuclease inhibitor from the particles, which is found in the soluble fraction of rat liver (Roth, 1958). The selective destruction of the 18s ribosomal-RNA component in the washed particles has also been observed by Staehelin et al (1964) when liver ribonucleoproteins are incubated with small amounts of ribonuclease.

3) Labelling of the individual RNA-species from the submicrosomal fractions.

Purified RNA, isolated from the liver submicrosomal fractions of animals injected with ^{14}C adenine or ^{32}P was separated into its constituent species by gradient centrifugation and the radioactivity of the two ribosomal RNA-species and the RNA sedimenting at less than 18s was determined.

Under optimal conditions, the distribution of radioactivity shown in Figs. 22 and 23 was observed for free ribosomes and rough-surfaced vesicles from fasted rats. The two ribosomal RNA components sedimenting at 29s and 18s contained about 50% of the total counts which agrees well with a value of about 60% calculated from the data /

Fig. 21. Sedimentation pattern of RNA isolated from washed rough-surfaced vesicles.

The rough-surfaced vesicles were washed by resuspension and sedimentation from medium A for 60 mins. at 105,000g. RNA was isolated, purified and separated as in Fig. 19. Note the reduced amount of 18s ribosomal, structural RNA.

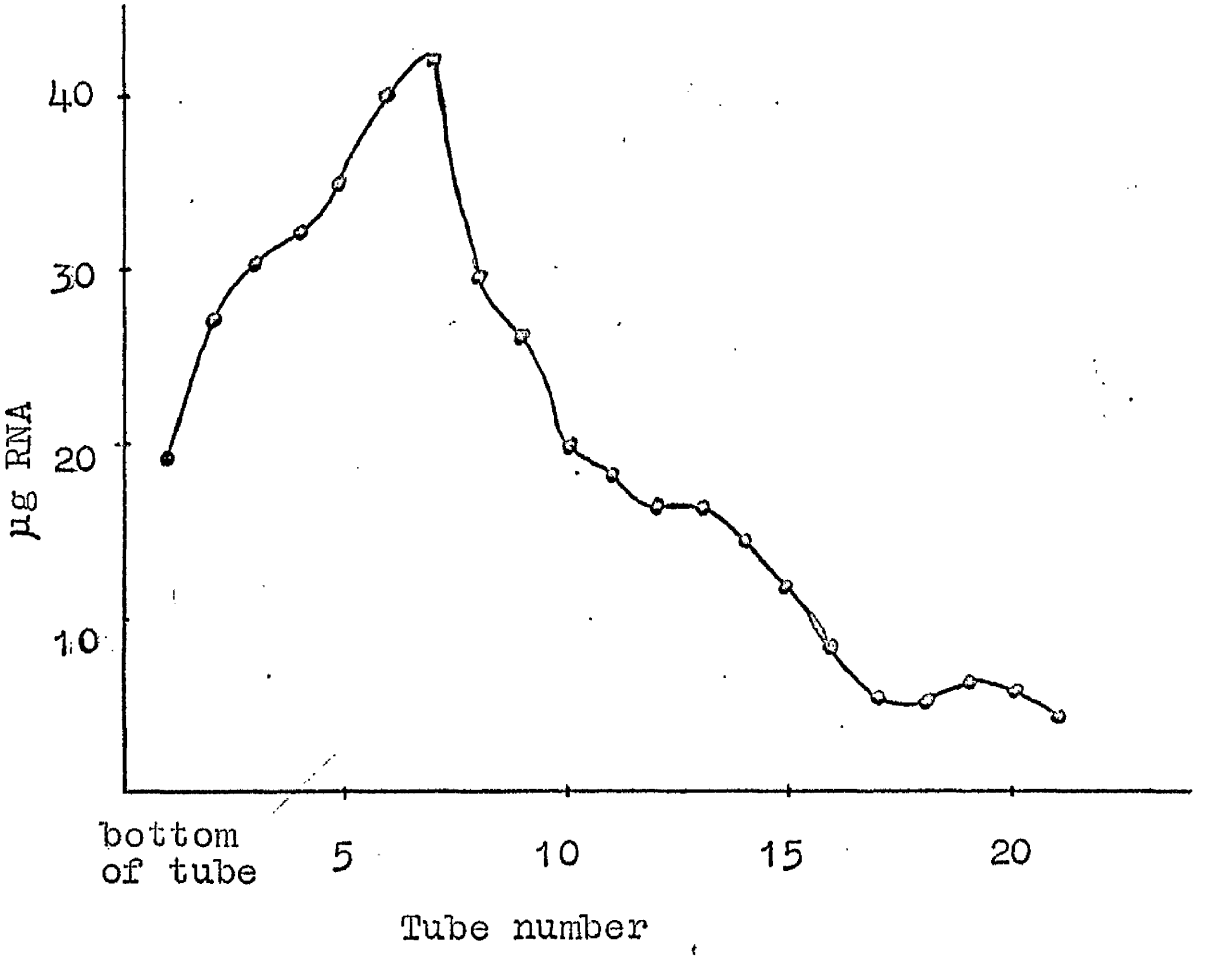


Fig. 22. Distribution of radioactivity between the RNA components from rough-surfaced vesicles and free ribosomes.

RNA was isolated and purified from the submicrosomal fractions of fasted rats, injected with 20 μC of ^{14}C adenine 70 mins. before killing. 280 ug of RNA from each fraction was layered atop a 3-30% sucrose gradient and separated into its components by centrifugation for 12 hrs. at 23,000 RPM as described above.
The dotted lines represent RNA and the solid lines, counts.

Fig. 22.

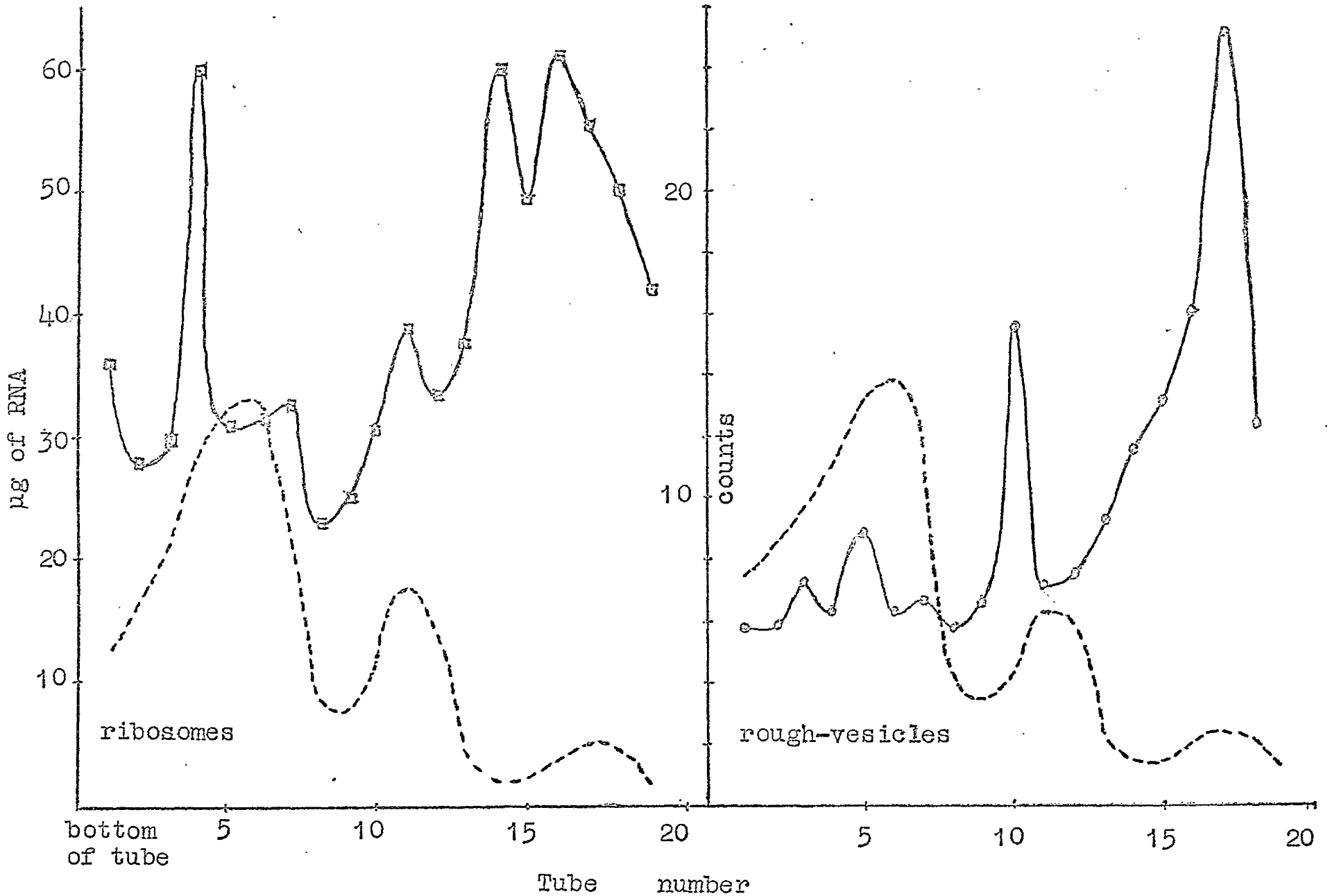
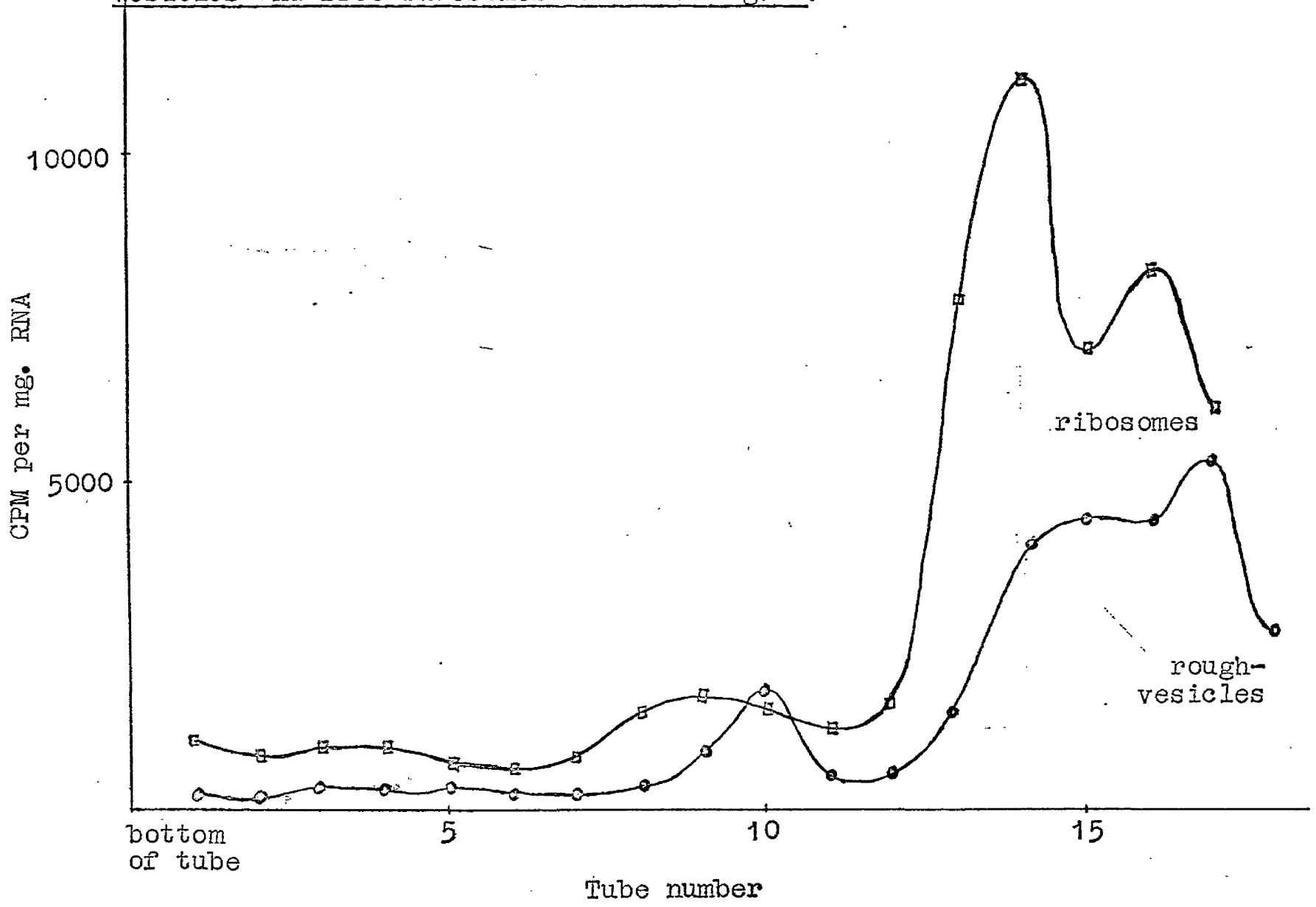

60
50
40
30
20
10
µg of RNA
ribosomes
20
10
counts
rough-vesicles
bottom of tube
5
10
15
20
Tube number
5
10
15
20

Fig. 23. The specific activity of the RNA components from rough- surfaced vesicles and free ribosomes shown in Fig. 22.



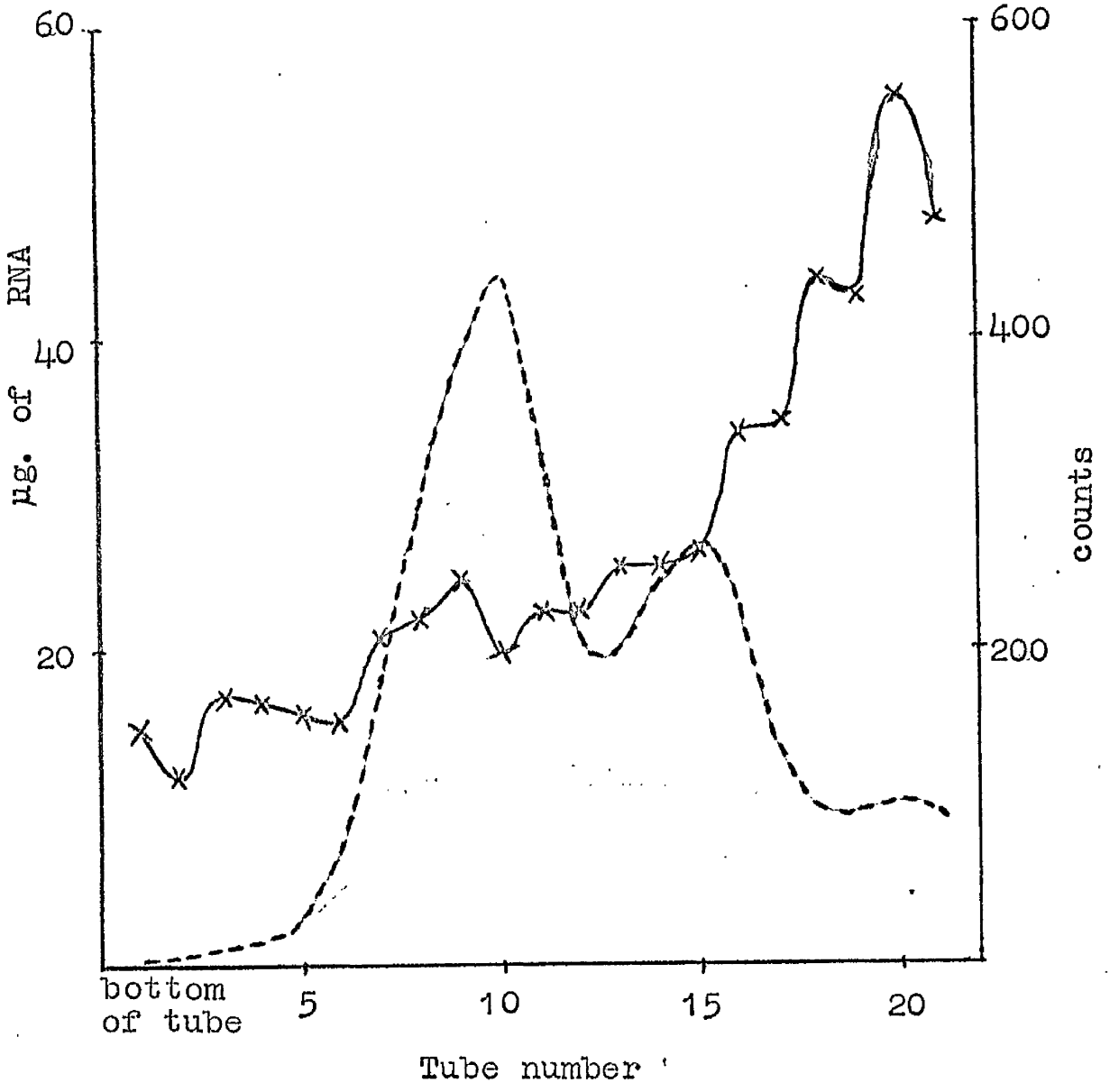
of Munro and Korner (1964) for whole rat liver microsomes labelled 1 hr. with ^{32}P. Expression of this as specific activity (counts per min. per mg. RNA) smooths out irregularities and shows more clearly the relationship between the labelling of the ribosomal RNA components, of the two sub-fractions. (Fig. 23).

The 29s and 18s ribosomal-RNA components seem to become about twice as highly labelled in free ribosomes as they do in rough-surfaced vesicles, which parallels the labelling of the total RNA of these two fractions (Hallinan and Munro, 1963, a,b.) There is never any marked difference in the labelling of the RNA which sediments at less than 18s in the two subfractions, except that RNA from free ribosomes tends to have a biphasic peak of activity in this region more often that RNA from rough-surfaced vesicles. The activity of all the fractions is reduced about 30% after extraction with cold 0.2n PCA, in the presence of bovine serum albumin carrier; however, acid extraction does not affect the pattern of labelling.

The labelling of smooth-surfaced vesicle RNA components from fasted rats follows the basic pattern observed for the other two fractions (Fig. 24), except that smooth-vesicles contain a very small amount of very highly-labelled RNA heavier than 29s, as indicated by the tail of counts over this region and the specific-activity plot (Fig. 24). This highly labelled, heavy RNA is always observed in smooth-surfaced vesicles from fasted rats and its /

Fig. 24. Distribution of radioactivity amoung the RNA components of smooth-surfaced vesicles from fasted rats.

RNA from smooth-surfaced vesicles of fasted rats, injected with 5 mC of ^{32}P, 40 mins. before killing was separated by centrifugation for 8 hrs. as described above.
The dotted lines represent RNA and the solidlines, counts. Note the proportionately large amount of radioactivity associated with the very small amount of RNA towards the bottom of the tube. This radioactivity was not removed by extraction with 0·2N PCA, which removes acid-soluble material.



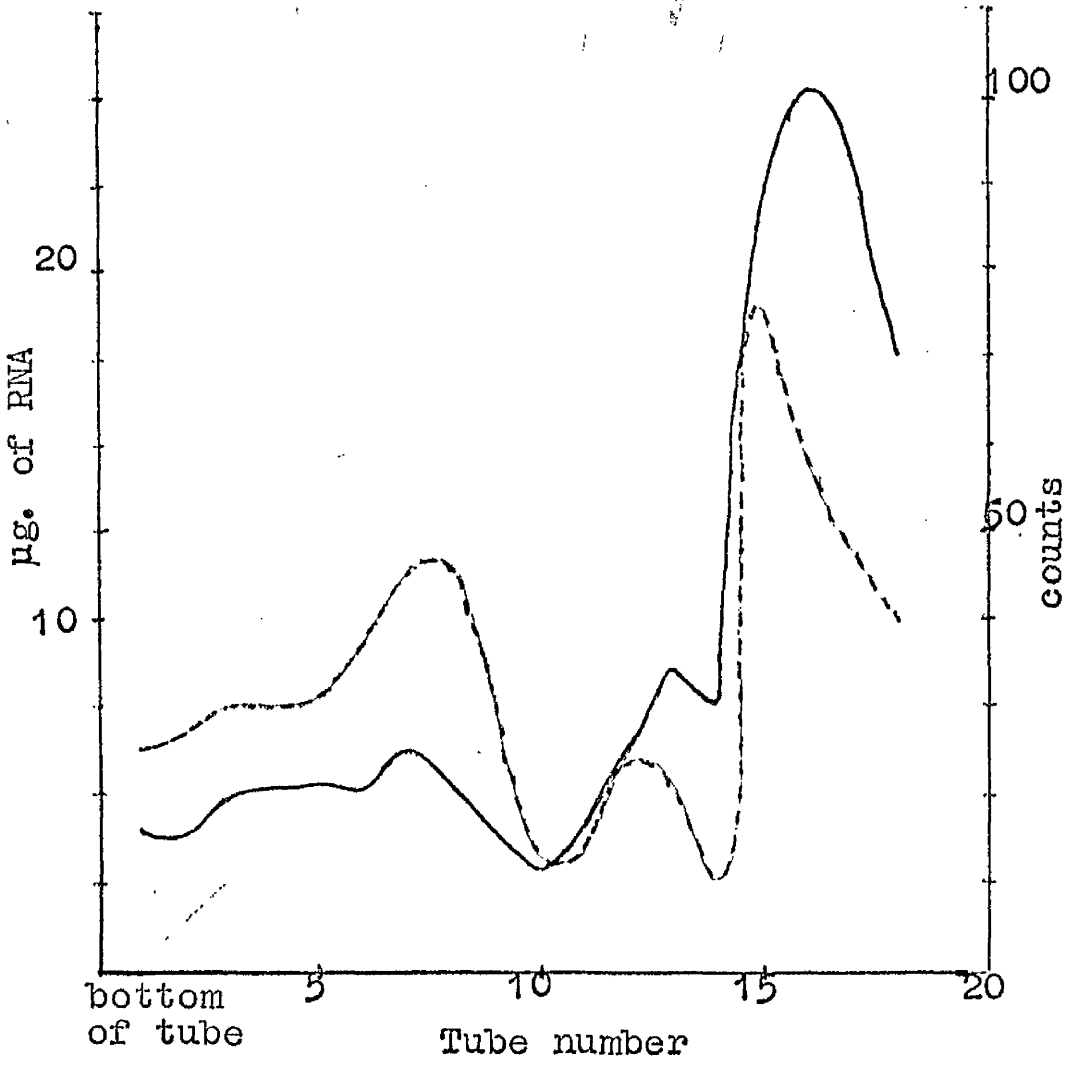
activity is not affected by extraction with cold 0.2N PCA, after the addition of bovine serum albumin as carrier. Similar trace amounts of heavy RNA have been observed in the nuclei of rat liver (Georgiev et al,1963) and HeLa cells (Scherrer et al,1963), where it is believed to consist mainly of precursors of ribosomal RNA having a high guanylic and cytidylic acid content and Kubinski et al,(1963) have observed an accumulation of this heavy-RNA in ascites tumor and tissue culture cells grown on deficient media. We believe that the above is the first case in which such heavy RNA has been found associated with a cytoplasmic fraction.

Attempts to fractionate the RNA of smooth-surfaced vesicles from rats fed casein before injection of precursor have so far been unsuccessful, the separation profile obtained resembling that in Fig. 25, which shows a small 18s ribosomal RNA component and a very large component sedimenting at less than 18s, which is indicative of degradative changes in the RNA prior to or during fractionation. (Staehelin et al, 1964). Consequently, it is not yet known which RNA components in the smooth vesicles are responsible for the dramatic increase in activity in this fraction from protein-fed rats. However it seems, at least in the case of the partially degraded samples of RNA studied so far, that the highly radioactive heavy RNA, found in smooth-vesicles from fasted rats is absent from those from protein-fed animals (Hallinan, Fleck and Munro, 1963).

During experiments on the labelling of the RNA species from the sub-microsomal fractions it has been frequently observed /

Fig. 25. Distribution of radioactivity amoung the RNA components of smooth-surfaced vesicles from protein-fed rats.

Animals were maintained on a protein-free, carbohydrate-rich diet for 4 days and were fed 2 gm. of casein 60 mins. before injection of 40 μC of ^{14}C adenine. 60 mins. later they were sacrificed and the RNA from their submicrosomal fractions was isolated and separated into its components as described in Figs. 22 & 24. Note the large proportion of radioactivity and RNA associated with material sedimenting at less than about 18s. RNA is denoted by dotted lines and radioactivity by solid lines.



that the radioactivity of components increases from the bottom of the tube to the top, with no break corresponding to the trough between 29s and 18s ribosomal-RNA components (eg. Fig. 24). This makes it impossible to determine the degree of labelling of any components accurately. Very recent work of Staehelin et al, (1964) and Munro and Korner (1964) suggest that this peculiar distribution of radioactivity is due to the binding of highly radioactive messenger RNA to the ribosomal RNA components, especially 18s ribosomal RNA. This binding seems to be most marked in the presence of magnesium ions and at high ionic strength. Our sucrose gradient separations were carried out in a buffer containing 50mM sodium chloride, 10mM sodium acetate and 0.1mM magnesium chloride, which does not seem to have caused messenger binding in most experiments since the proportion of total counts in messenger? and other RNA sedimenting at less than 18s was if anything slightly greater than in the experiments of Munro and Korner, (1964).

4) Conclusions on the turnover of RNA in the submicrosomal fractions.

Studies on the turnover of RNA in the submicrosomal fractions are still at such a preliminary stage, that any conclusions drawn must be very tentative and speculative. However, it seems possible, from the shape of the labelling curves at early time courses and the labelling of isolated 29s and 18s ribosomal RNA components, that free ribosomes may be the precursors of ribosomes bound to the rough-surfaced endoplasmic reticulum.

The /

The evidence available that protein feeding may be rapidly followed by a small increase in the polysome population of microsomes and in their ability to incorporate amino-acids into protein *in vitro* (Fleck and Leggate, 1964) suggest that protein feeding may be followed by the release of messenger into the cytoplasm, (Munro and Korner, 1964). The rapid increase under these conditions in labelling of the smooth-surfaced vesicle fraction RNA, which constitutes only about 5% of the total microsomal RNA could possibly be due to this fraction containing part or all of the newly released messenger RNA. This of course is pure speculation at present but can be tested by examining the labelling of different species of undegraded smooth-vesicle RNA after protein feeding.

C) COMPARISON OF PHOSPHOLIPID TURNOVER IN THE MEMBRANOUS SUBFRACTIONS OF THE MICROSOMES AND IN NUCLEI.

1) Rates of incorporation of ^{14}C - choline into the total phospholipid of rough-surfaced vesicles, smooth-surfaced vesicles and nuclei.

Groups of two to five 150 gm. rats were injected with ^{14}C choline and killed at various times thereafter (10 - 65 mins.). The rough and smooth-surfaced vesicle fractions and nuclei were prepared and their total lipids were extracted and purified as described above to remove any phosphorus or labelled choline not associated with phospholipids. Samples of purified lipid were then taken for determination of radioactivity and phospholipid.

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The labelling of the various fractions (counts per min. per mg. phospholipid) at different times is shown in Fig. 26 . The order of initial labelling observed is generally smooth-surfaced vesicles, followed closely by rough-surfaced vesicles and then by nuclei, though in a few experiments nuclei and rough-surfaced vesicles change places. In most experiments with rats on normal diet, uptake of label by the fractions reaches a peak or at least levelsout 20-40 mins. after injection of ^{14}C choline, but Fig.26(1)shows an experiment where a slow increase in activity continues at later times into rough and smooth-vesicles.

A picture very similar to the above is found when, instead of normal rats, animals are used whose reticulum has been seriously depleted and allowed to regenerate. Depletion was brought about (a) by maintenance on a protein-free diet for 4 days (Fleck, 1964) (fig.27) or (b) by 4 days of fasting with water ad libitum (Fawcett, 1955) (see below) and regeneration of reticulum was triggered off by feeding 2 gm. of casein 2 hours before injection of choline (Fawcett, 1955).

In rats regenerating reticulum as with normal animals smooth-surfaced vesicles take up the label most rapidly, followed in order by rough-surfaced vesicles and then nuclei. Feeding protein produces no dramatic increase in the labelling of the phospholipids of the smooth-surfaced vesicles as it does with the labelling of their RNA. However protein feeding constantly prolonged the uptake of label into nuclear phospholipids, so that these attained higher specific activities than the cytomembranes at later times after ^{14}C choline injection (Fig. 27).

Fig. 26. Distribution of radioactivity amoung the total phospholipids of the submicrosomal fractions and nuclei from normal, fasted rats. Rats were fasted 18 hrs. before injection of 6.8 μC of ^{14}C choline.

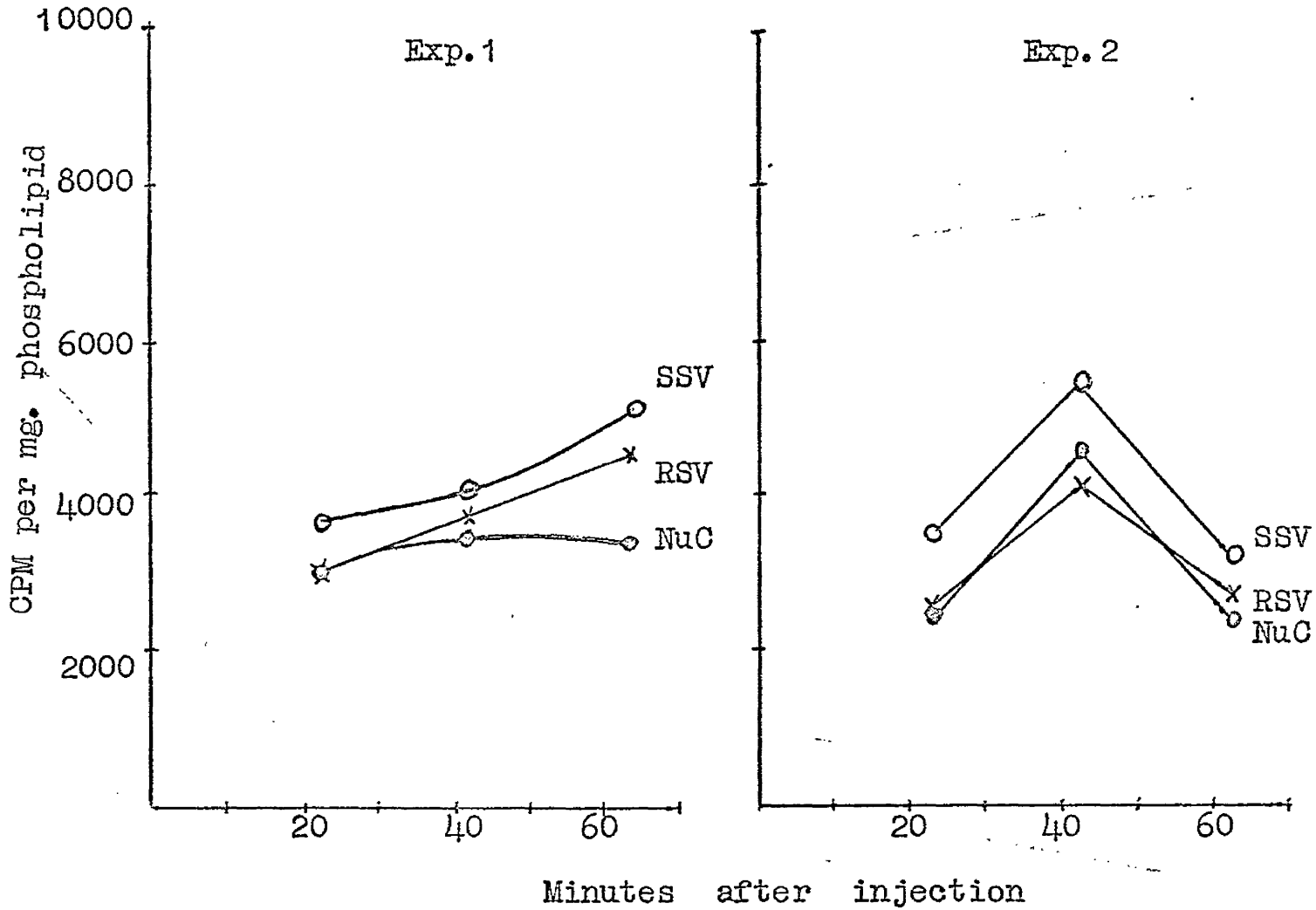
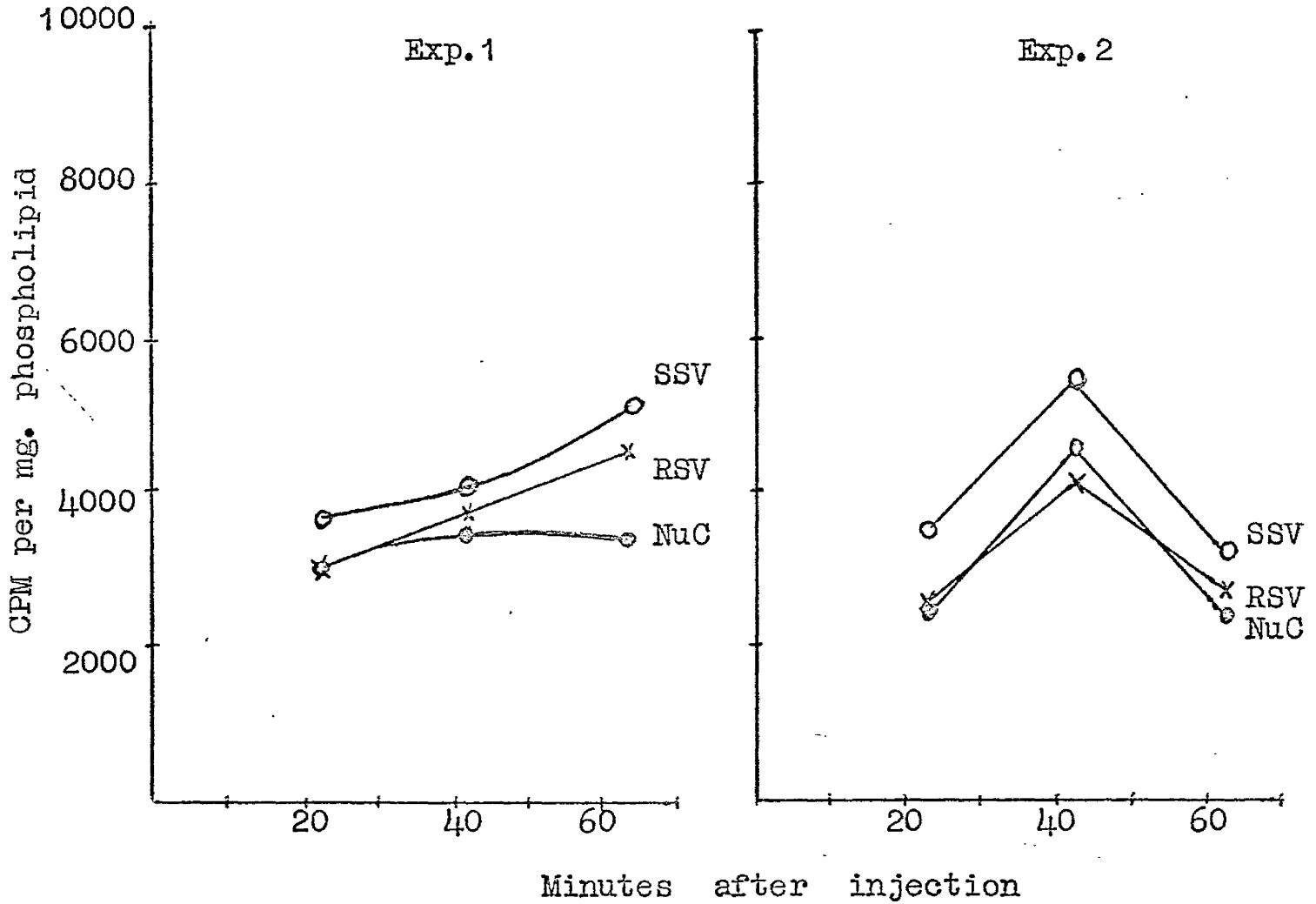


Fig. 26. Distribution of radioactivity amoung the total phospholipids of the submicrosomal fractions and nuclei from normal, fasted rats. Rats were fasted 18 hrs. before injection of 6.8 μC of ^{14}C choline.



2) Rates of Incorporation of ^{14}C choline into lecithin and sphingomyelin isolated from rough and smooth-surfaced vesicles and nuclei.

The labelling pattern of lecithin, isolated from the total lipids by thin layer chromatography as described above, resembles the labelling of the total phospholipids very closely as was expected, since it is quantitatively the most abundant choline-containing phospholipid in liver (Figs. 28,29) . It generally exhibits about 2-3 times the specific activity of the total phospholipids. Also as for the total phospholipids, nuclear lecithin continues to take up label at an approximately linear rate for 60 mins in protein-fed animals, in distinction to the two cytomembrane fractions.

Isolated sphingomyelin again gives a very similar labelling pattern to the total phospholipids and lecithin (Fig. 30). It attains about 15-20% of the specific activity of lecithin as found also by Johnson et al, (1954) in whole liver. Fig. 30 seems to show it turning over a little faster in nuclei than lecithin does in the same experiment (Fig. 29). However smooth-surfaced vesicles contain the highest specific activity sphingomyelin at the peak of the labelling curve as they do of all the other phospholipids. The above figures were obtained from very small quantities of sphingomyelin, owing to the limited amount of material which can be separated by thin layer chromatography; consequently they may be much more in error than figures for lecithin or total phospholipids.

Fig. 28. Distribution of radioactive lecithin amoung the submicrosomal fractions and nuclei from normal rats.

Animals were fasted 18 hrs. before injection of 6.8 μC of ^{14}C choline. Lecithin was isolated from other phospholipids by thin layer chromatography on silica gel.

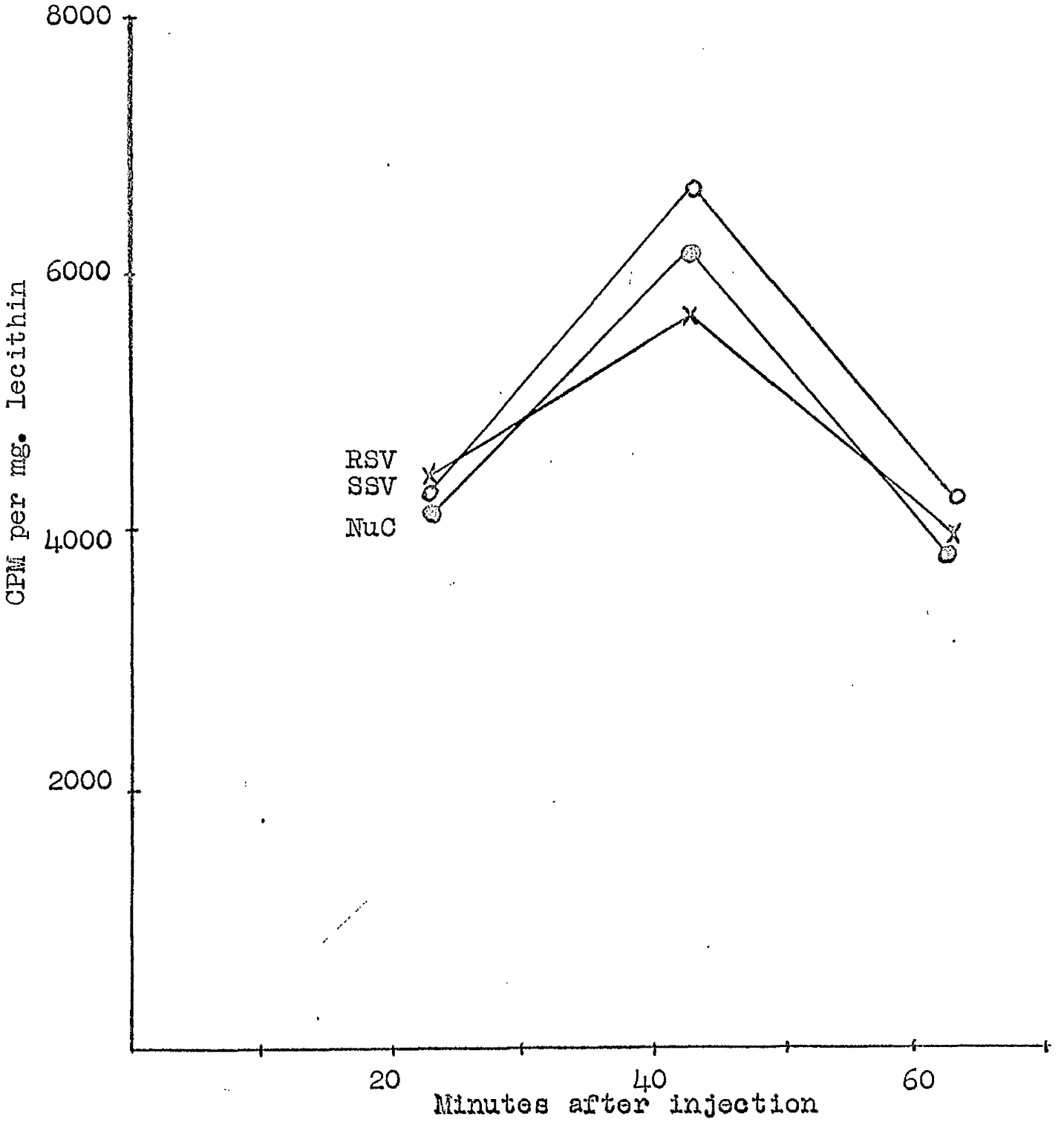


Fig. 29. Distribution of radioactive lecithin amoung the submicrosomal fractions and nuclei from rats regenerating endoplasmic reticulum after experimental depletion.

Animals were depleted of reticulum by maintainance on a diet of water, only, for 4 days. They were injected with 6·8 μC of ^{14}C choline 120 mins. after receiving 2 gm. of casein.

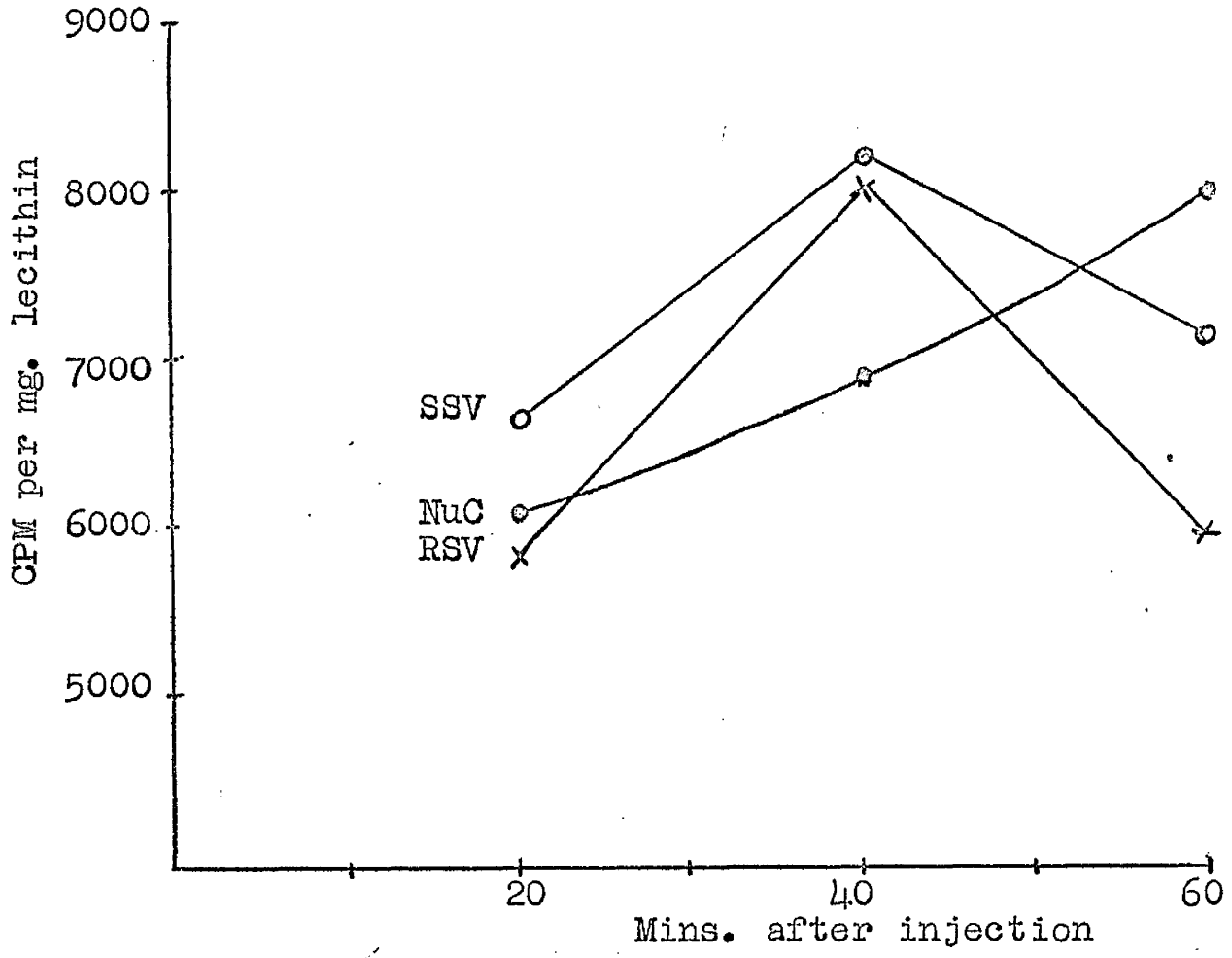
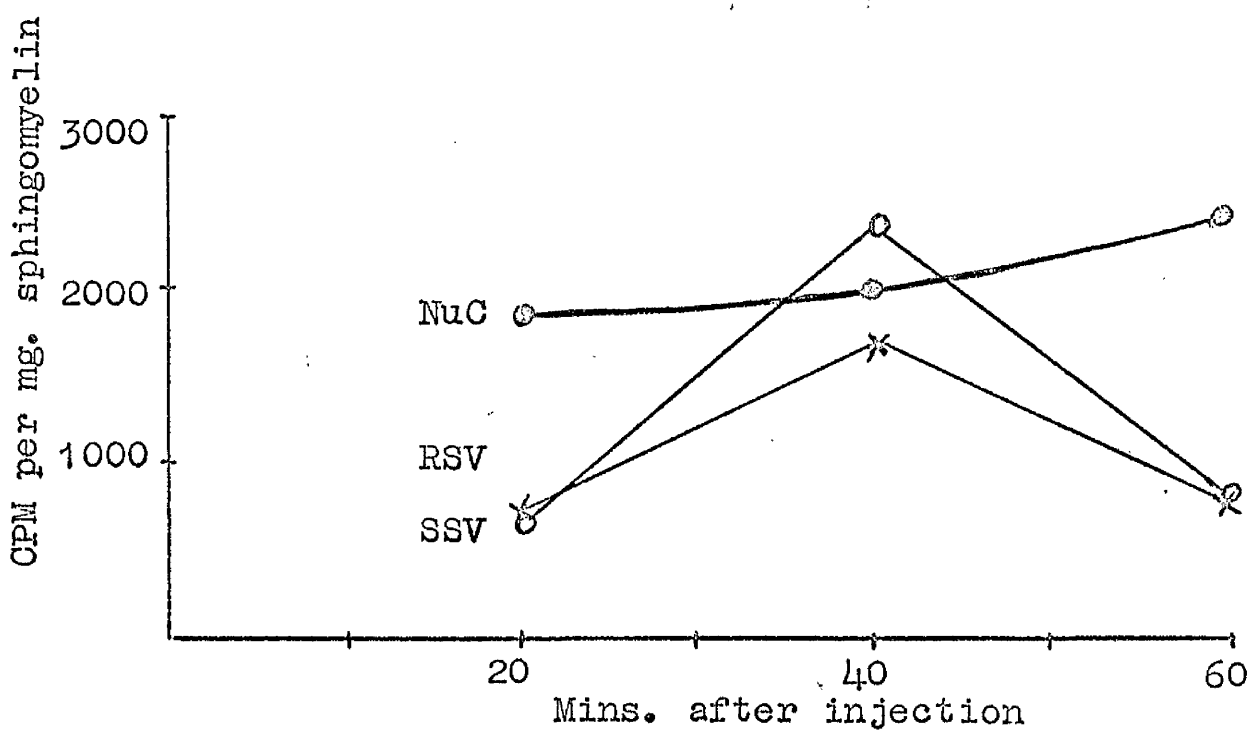


Fig. 30. Distribution of radioactive sphingomyelin amoung the submicrosomal fractions and nuclei from rats regenerating endoplasmic reticulum after experimental depletion.

Animals were starved 4 days to deplete them of reticulum and were fed 2 gm. of casein to trigger off its regeneration. 120 mins. later they were injected with 6·8 μC of ^{14}C choline and the sphingomyelin was separated from other phospholipids by thin layer chromatography.



3) Conclusions on the turnover of phospholipids in the membranous subfractions of the microsomes and in nuclei.

It was assumed in this study that the nuclear phospholipid is all contained in the nuclear membrane and hence that the turnover of total nuclear phospholipid represents the turnover of both layers of the nuclear membrane. Evidence that most if not all of the phospholipid of nuclei is contained in their membranes is presented by Gurr, Fiȟean and Hawthorne (1963). They found that the amount of phospholipid present was just enough to form two membranes in nuclei isolated in sucrose as used in this study and that citric-acid nuclei, lacking the outer layer of the nuclear membrane, contained exactly half as much phospholipid as sucrose nuclei. Also, there are no authenticated descriptions of intra-nuclear membranes in nuclei from liver cells.

If the nuclear phospholipid is contained in the two layers of the membrane, there is very strong evidence from the labelling curves in the above experiments that this membrane is not the precursor of the phospholipid portion of the two cytomembrane fractions in liver. Lipid turnover gives an accurate estimate of the turnover of the mitochondrial membranes in rat liver (Fletcher and Samadi, 1961), so it should provide a measure of the turnover of the nuclear and endoplasmic reticulum membranes also. If total phospholipids, lecithin and sphingomyelin do turn over at the same rate as their parent membranes, then the nuclear membrane is unlikely to be the precursor of the rough or smooth-surfaced reticulum membranes. Merriam (1962)/

showed by autoradiography that incorporation of ^{14}C-lysine occurs more slowly into the protein of the nuclear membrane of oocytes than into the cytomembranes, so on this evidence the nucleus is not the source of the protein component of these membranes either (see however, Sirlin and Elsdale, 1959).

Indeed there is no evidence for a precursor-product relationship between any of the various membranous structures studied, each seeming to label in parallel, and the best interpretation at present of the preliminary data available seems to be that each structure synthesizes its own phospholipids independent of the others. It is assumed in this interpretation that these structures are incorporating choline from a single pool or from several pools in which the precursor has approximately the same specific activity. This assumption common to most turnover studies in whole organs, is difficult to validate experimentally.

It is possible though unlikely that one layer of the two-layered nuclear-membrane turns over very rapidly and is a precursor of the cytomembranes, while the other layer turns over very slowly, so that the whole structure appears to turn over a little less rapidly than the cytomembranes. However, unpublished work of Gurr (1963) on nuclei prepared in citric acid and having only a single-layered membrane, agrees substantially with that reported above, for sucrose nuclei with two layered membranes, in that he found that the phospholipids of his nuclei turned over a little less rapidly than those of whole microsomes, which contained both rough and smooth surfaced vesicles. /

Hence, it seems unlikely that the two layers of the nuclear membrane turnover at very different rates.

It is more plausible however that the smooth-surfaced vesicle fraction may contain structures turning over at different rates and it is proposed in subsequent experiments to sub-fractionate these particles further into small and large vesicles, using the method of Dallner (1964). Fawcett (1955) and Merriam (1962) have proposed that the endoplasmic reticulum membranes are formed by fusion of small, smooth-surfaced cytoplasmic vesicles, while Manton (1962) has proposed that it originates from the Golgi apparatus, which also consists partly of small, smooth-surfaced vesicles; each of these possible precursors would be expected to be in the 'small' sub-fraction of the smooth-surfaced vesicles. This is an area susceptible of further exploration.

GENERAL DISCUSSION.

a) The Cellular Origin of the Submicrosomal Fraction Components.

Because of their very close morphological resemblance to the rough-surfaced endoplasmic reticulum (Palade and Siekevitz, 1956a; Moule et al, 1960) and the high correlation between the yield of rough-surfaced vesicles and the amount of rough-surfaced reticulum in tissues under various conditions (Henshaw et al, 1963; Campbell and Lowe, 1964), it is generally agreed that the rough-surfaced vesicle subfraction of microsomes consists of fragments of the rough-surfaced endoplasmic reticulum. These components make up the bulk of the sub-fraction sedimenting in 60 mins. at 78,000g. in this study, as shown by electron micrographs of this fraction, sectioned at various levels from the top to the bottom of the pellet. It is considered likely though not yet proven that the rough-surfaced reticulum in liver consists largely of polysomes bound in some way to the lipoprotein membrane (Henshaw et al, 1963; Campbell and Sargent, 1964).

It is believed that the free ribosome fraction, isolated by homogenizing the rough-surfaced vesicle supernatant with isooctane and centrifuging it for 120 mins. at 150,000g, consists largely if not entirely of ribosomes initially free in the liver. These contained an average of 34% of the total microsomal RNA over a series of five experiments. This is similar to the figure of 37% calculated from the data in Table 1 of Wettstein et al, (1963) for the proportion of RNA in 'monomer ribosomes' (presumably identical with free ribosomes) from /

whole rat liver microsomes, disrupted with deoxycholate, and is slightly lower than the approximate figure of 44% calculated from the amount of material absorbing at 260 mμ under the three apparent free-ribosome peaks, obtained when whole rat liver microsomes were separated into rough surfaced vesicles and free ribosomes on a sucrose gradient (Fig. 4, Munro and Korner, 1964). Henshaw et al (1963) estimated that free ribosomes could not account for more than 10% of the RNA of rat liver microsomes, but it now seems apparent that this low figure is due to the use of calcium chloride in their homogenization medium, which probably causes most of the free-ribosomes to aggregate and be discarded with the premicrosomal particles.

It has been suggested in discussion by Munro and Korner, (1964), that free ribosomes isolated in 0.88 M sucrose could be contaminated with ribosomes artefactually released from the rough surfaced endoplasmic reticulum due to the low concentration of magnesium ions. No evidence of such contamination was found when this question was investigated previous to their suggestion; however it is obviously of the utmost importance to establish beyond question that contamination does not occur. Another test for contamination could be made by labelling the liver microsomes with an RNA precursor (e.g. adenine or uracil) and then isolating rough-surfaced vesicles and free ribosomes in 0.88 M sucrose containing magnesium ions in increasing concentrations. Rough-surfaced vesicle ribosomal RNA only attains /

about half the specific activity of that of free ribosomes, at early times after injection of an RNA precursor. Hence, if rough-vesicle ribosomes seriously contaminate the free ribosome fractions isolated at low magnesium concentrations, these should contain lower specific activity RNA than fractions isolated in the presence of sufficient magnesium to prevent this hypothetical detachment of ribosomes from the rough-surfaced vesicles. It is intended to carry out this experiment in the near future, using ^{14}C uracil to label the RNA of the microsomes.

Electron micrographs show that the smooth-surfaced vesicle fraction, isolated in this study consists largely of smooth membrane fragments but that these exhibit a considerable range of size and form. A similar fraction of smooth membranes from rat liver microsomes has been shown by Dallner, (1963) to consist of at least two distinct species of particles. These differ in their morphology, in their susceptibility to aggregation by magnesium ions and their content of several electron-transport enzymes (Dallner, 1963). It is likely that the smooth-surfaced vesicles examined in this study, are also heterogeneous in cellular origin and function and it is proposed shortly to test this by applying Dallner's fractionation scheme to the particles.

b) Role of the Submicrosomal Fractions in Protein Synthesis.

It is now reasonably well established that the rough-surfaced endoplasmic reticulum (which undergoes fragmentation to form rough-surfaced vesicles when liver cells are homogenized) is the site of /

synthesis of most of the protein in rat liver microsomes (Henshaw et al, 1963, Hallinan and Munro, 1963,1964; Campbell and Lowe, 1964). Smooth-surfaced endoplasmic reticulum does not seem to contribute at all significantly to total protein synthesis (less than 4%, Hallinan and Munro, 1963,1964), while free ribosomes may be responsible for 10-25% of the total synthesis (Hallinan and Munro, 1963,1964), with the lower figure the more likely one.

As a consequence of the high correlation between the amount of protein which cells synthesize for secretion and the extent of their rough-surfaced reticulum (Palade, 1958; Birbeck and Mercer, 1961), and the change in state of ribosomes from free to rough-reticulum bound as tissues cease to undergo rapid cell division and begin to secrete protein (Eschner and Glees, 1963), it has been postulated that the rough-surfaced endoplasmic reticulum may be the site of synthesis of proteins for 'export' by secretion, while the cells own structural and enzymic protein might be synthesized on free ribosomes (Siekevitz and Palade, 1958; Eschner and Glees, 1963).

It has recently been reported that free ribosomes are activated to incorporate phenylalanine into polyphenylalanine by adding the artificial messenger-RNA, polyuridylate, much more than rough-surfaced vesicle ribosomes are. This has been interpreted to mean that the free ribosomes contain much less natural messenger-RNA to compete with the added polyuridylate for binding sites (Henshaw et al,1963; Campbell /

and Cooper, 1963). However, this work is open to serious criticism, for although the same amount of polyuridylate was added to both free and rough-vesicle ribosomes, the concentration of ribonucleoprotein particles in the assay system for phenylalanine incorporation was much higher in the case of rough-surfaced vesicle ribosomes. It is clear from the work of Fessenden et al, (1963) that incorporation of amino acids by artificial polysomes made with polyuridylate is very strongly dependent upon ribonucleoprotein particle concentration, being much higher at low particle concentrations. This was shown also in this study for rough-surfaced vesicle ribosomes, without added polyuridylate. Hence the observation of less activation by polyuridylate of rough-vesicle ribosomes by Henshaw et al and Campbell et al may merely be a reflection of this damping effect of high concentrations of ribonucleoprotein particles upon amino acid incorporation. If, however, their interpretation is still correct and free ribosomes do in fact contain only small amounts of endogenous messenger-RNA, it is unlikely that they could be the site of synthesis of the non-secreted cellular proteins as Siekevitz et al (1958) and Eschner et al (1963) postulate. Plainly there is a need for further investigations in this area, using well-characterized rough-surfaced vesicles and free ribosomes.

c) The Turnover of different RNA Species in the Submicrosomal Fractions.

Rat liver microsomes have been shown by Munro and Korner, (1964) and Staehelin et al, (1964) to contain rapidly labelled /

messenger-RNA and transfer-RNA as well as ribosomal structural RNA, which turns over much more slowly. Hiatt's group initially failed to demonstrate messenger-RNA in rat liver cytoplasm (Hiatt, 1962) but recently have referred briefly to such a species associated with the rough-surfaced vesicles (Henshaw et al, 1963 b).

The submicrosomal fractions all contained a small amount of rapidly labelled RNA sedimenting slower than the 18s ribosomal RNA peak. This rapidly labelled RNA, which constituted about 10% of the total in each fraction, turned over at least five times faster than the two species of ribosomal structural RNA sedimenting at about 29s and 18s. This rapidly labelled submicrosomal RNA may also contain some messenger-RNA, though the present investigation has not been extended to this question yet.

Attention has been concentrated instead on the turnover of the ribosomal structural RNA, which constitutes up to 90% of the total RNA in each of the submicrosomal fractions and seems to turn over approximately twice as fast in the free ribosome fraction as in the rough surfaced vesicles, under different dietary regimen. This suggests that the free ribosomes may provide the precursors of the ribosomes bound to the rough-surfaced endoplasmic reticulum and definitive experiments are presently being sought to test this possibility.

d) Significance of the Nuclear Membrane as a Precursor of the Endoplasmic Reticulum.

Transcription /

Investigations so far carried out show that it is highly unlikely that the whole nuclear membrane is the precursor of the major structural phospholipids, lecithin and sphingomyelin, in either the rough or smooth-surfaced endoplasmic reticulum. If structural phospholipids turn over at a rate commensurate with the turnover of the other components of these membranes, as indeed they appear to in liver mitochondria (Fletcher and Sanadi, 1961), our results indicate that the nuclear membrane does not give rise to any significant fraction of the endoplasmic reticulum. It is proposed shortly to test the effect of puromycin, a known inhibitor of protein synthesis, on the turnover of the phospholipids of the endoplasmic reticulum and nuclear membranes. Puromycin should inhibit the synthesis of the protein component of the membranes and, if the turnover of phospholipid is closely linked to that of other membrane components, it should inhibit this too.

Structures such as annulate lamellae lying near the nucleus and vesicular blebs attached to the outer nuclear membrane, which are frequently put forward as likely intermediates between the nuclear membrane and newly synthesized endoplasmic reticulum, are much more abundant in the undifferentiated, rapidly dividing cells found in embryonic tissues, carcinomas and meristematic tissue in plants (Wheley et al, 1960; Porter, 1961; Kessel, 1963). It could therefore be argued that the nuclear membrane may contribute much more to the formation of /

endoplasmic reticulum in undifferentiated and rapidly dividing cells than it does in, say, normal hepatocytes. However, Gurr, (1963) has shown in unpublished work that the nuclear membrane phospholipids in regenerating rat liver turn over about 50-70% as fast as those of the whole microsomes (rough and smooth-surfaced reticulum), exactly as they do in normal liver in both his experiments and ours. Hence all available evidence from tracer studies of the labelling of phospholipids suggest that the nuclear membrane does not contribute significantly to the formation of the membranes of the endoplasmic reticulum either in rapidly-dividing or non-dividing cells.

SUMMARY.

(1) Rat liver microsomes are made up of three major components:- rough-surfaced vesicles, derived from fragments of the rough-surfaced endoplasmic reticulum; free ribosomes which are unassociated with membranes in intact hepatocytes; and smooth-surfaced vesicles, derived from fragments of the smooth-surfaced endoplasmic reticulum and probably from other smooth-surfaced membranes also. Several existing procedures were investigated for isolating these three components in a metabolically active state, suitable for studies *in vitro*, however none was found entirely satisfactory. Therefore a new procedure was devised for isolating the three submicrosomal components. This requires only 3·5 hours centrifugation and enables all three components to be isolated in high yield, with minimal cross-contamination as shown by chemical analysis and electron microscopy. The chemical composition and yield of the subfractions is given.

(2) The yield of free ribosomes is markedly higher with the new procedure than in previous methods and the reasons for this are discussed. Evidence is presented to show that the free ribosomes are not significantly contaminated with particles released artefactually from the rough-surfaced endoplasmic reticulum or from nuclei during cell breakage and fractionation.

(3) Rough-vesicles contain about 60% of the microsomal RNA, free ribosomes about 34%, and smooth-vesicles about 5%. Examination of native RNA by means of sucrose gradient centrifugation shows that all the subfractions contain the same major RNA species. These consist of 50-60% of ribosomal structural RNA sedimenting at about 29s about 30% of ribosomal RNA sedimenting near 18s and about 10% of rapidly labelled RNA, sedimenting slower than 18s. This latter fraction probably contains transfer-RNA and possibly also messenger-RNA and some degradation products of ribosomal RNA.

(4) All three of the subfractions are metabolically active and suitable for studies of amino acid incorporation into protein *in vitro*. Comparison of their ability to incorporate amino acids into protein *in vitro* and *in vivo* shows that rough-surfaced vesicles are able to incorporate at 2-5 times the rate of the other subfractions. Assuming that most of this incorporation is due to protein synthesis and taking into account the relative amounts of the different subfractions in microsomes, it can be calculated that the rough-surfaced vesicles could account for as much as 85-90% of the protein synthesis by liver microsomes. Smooth-surfaced vesicles on the other hand could not be responsible for more than 3-4% of the synthesis, the rest being performed by free ribosomes (6-11%). However this calculation, based on *in vivo* rates of incorporation, may underestimate the contribution made by free ribosomes, which could account for as much as 20% of the synthesis.

(5) Comparison of RNA-turnover in the submicrosomal fractions shows that free ribosomes exhibit the most rapid, initial uptake of RNA-precursors into their RNA *in vivo* with fasted rats. They are generally followed by smooth and then by rough-surfaced vesicles, in that order. Separation of purified, radioactive, native RNA from rough-surfaced vesicles and free ribosomes, shortly after injection of ^{14}C-adenine or ^{32}P, shows that the ribosomal structural RNA of free ribosomes turns over about twice as rapidly as that from rough-surfaced vesicles. It is suggested therefore that free ribosomes may be precursors of those bound to the rough-surfaced endoplasmic reticulum. Smooth-surfaced vesicles from fasted rats contain radioactive ribosomal structural RNA, shortly after injection of an RNA precursor; they also contain a very small amount of highly-radioactive RNA, which sediments more rapidly than ribosomal structural RNA and is probably heavier than 29s. This is the first known demonstration

of such a heavy RNA in a cytoplasmic fraction from mammalian cells.

(6) Feeding protein to animals induces a burst of labelling in the RNA from smooth-surfaced vesicles. It has not yet been possible to separate this labelled RNA adequately into its various species, to determine whether protein feeding specifically stimulates the turnover of any particular RNA species in smooth-surfaced vesicles.

(7) The small amount of RNA in smooth-surfaced vesicles is made up of about 90% of 28s and 18s ribosomal structural RNA and about 10% of RNA sedimenting slower than 18s, exactly like that from rough-vesicles and free ribosomes .It is tempting therefore to suppose that it arises from minor contamination of the smooth-surfaced vesicles by the other two subfractions. However the individual pattern of precursor uptake by smooth-vesicle RNA, especially in protein fed rats, tends to overrule this possibility, leaving the origin and significance of the RNA still unexplained.

(8) , It is widely held that the membranes of the endoplasmic reticulum may arise by delamination or vesiculation from the nuclear membrane. Smooth-surfaced membranes are initially formed and some of these subsequently acquire adherent ribosomes and become rough-surfaced reticulum. A preliminary test of this pathway for the formation of endoplasmic reticulum in normal liver and liver regenerating reticulum after depletion, was carried out using a tracer technique. ^{14}C-choline was used to specifically label lecithin and sphingomyelin in nuclei and rough and smooth-surfaced vesicles,its initial rate of uptake into those phosphotides being taken as a measure of their rate of turnover. Nuclear phosphatides, which are located mainly if not exclusively in the nuclear membrane, showed no signs of being precursors of the phosphatides of the endoplasmic reticulum sub-fractions indeed they always turned over less rapidly than those from the smooth-

surfaced vesicles. Hence if phosphatides turn over at a rate comparable to that of the other chemical components of the membranes of nuclei and the endoplasmic reticulum, it can be concluded that the nuclear membrane does not give rise to the endoplasmic reticulum membranes in normal liver. This seems to be the case also in regenerating rat liver.

(9) Several common methods of extracting and estimating RNA, phospholipid and protein were investigated and modifications were introduced to improve their accuracy and to permit them to be applied more readily in radioactive tracer experiments.

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