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NUCLEAR MECHANISMS OF PROTEIN SYNTHESIS.

by

SUSAN WADDINGTON.

Thesis presented for the degree of Doctor of Philosophy, in the University of Glasgow.

April, 1964.

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NUCLEAR MECHANISMS OF PROTEIN SYNTHESIS

by

S. Waddington, B.Sc.

A study has been made of ribonucleic acid (RNA) metabolism in the liver cell nuclei obtained from rats receiving various diets. Rats receiving a normal intake of protein and rats on a protein-free diet were used, and in the former group some animals were studied in the fasting state and some during active absorption after a meal of protein.

A new technique for isolating pure liver nuclei in bulk was devised and the nuclei were then fractionated by successive extraction with phosphate buffer and molar sodium chloride, leaving a final "nucleolar" residue. The RNA content of the two extracts was reduced by feeding a protein-free diet, whereas the amount in the nucleolar residue was increased, in agreement with histochemical examination. This was taken as evidence of storage of RNA in the nucleolus during protein depletion. Nucleolar RNA also increased during active absorption after a protein meal, but in this case the change may be due to stabilization by incoming amino acids of an unstable nuclear RNA species. Attempts to demonstrate a specially labile type of RNA were unsuccessful.

The effect of diet on incorporation of labelled precursor

into nuclear RNA was also explored. Uptake of ^{14}C-adenine into whole nuclear RNA was augmented by giving a protein-free diet. When the RNA of the nuclear subfractions was compared, the feeding of protein appeared to cause a preferentia stimulus of labelling in the molar sodium chloride fraction.

Specimens of nuclear RNA and whole liver RNA were prepared by the phenol procedure and examined for heterogeneity by various methods. Both nuclear and whole liver RNA separated in the analytical ultracentrifuge into four components (4-7S, 17-19S, 23-28S and 32S or heavier). When obtained from animals receiving the protein-free diet, the amount of the 4S component was reduced, whereas the heaviest component increased. These changes due to protein depletion were confirmed when whole liver RNA or cytoplasmic RNA was separated by centrifuging in a sucrose density gradient. The density gradients obtained with nuclear RNA were not satisfactory.

Finally, the metabolism of nuclear and cytoplasmic membrane was studied using incorporation of ^{14}C-choline. The results show that nuclear membranes cannot be the precursor of the cytoplasmic membranes. Moreover, feeding protein appeared to stimulate ^{14}C-choline uptake by the nuclear membrane, but not by cytoplasmic membranes.

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SECTION I : INTRODUCTION.

INTRODUCTION.

The concept of the cell as a structural basis of living tissues dates from the studies of Robert Hooke in the 17th century, but it was not until 1838 that the botanist Schleiden and the zoologist Schwann recognised the cell as the fundamentally important "elementary organism" of life. Seven years earlier, Robert Brown had described the nucleus as an integral structure in the cell, and in 1838 Schleiden observed nucleoli in the nucleus. The importance of the nucleus in the cell was first realised by Max Schultz, who, in 1861, put forward the "cell concept", in which he considered the cell to be a unit of a nucleus surrounded by protoplasm.

A chemical characterisation of nuclei was first attempted by Miescher whilst at Hoppe-Seylor's laboratory in 1868-1869. Using a pepsin-hydrochloric acid digestion technique, he was able to separate the nuclear material from pus cells, and from this preparation he isolated an acid material which he termed "nuclein", and which he found contained a high proportion of phosphorus. The high phosphorus content was a fortunate coincidence, since at that time phosphorus was virtually unknown in physiological material, and its presence in Miescher's"nuclein" resulted in considerable interest being focused on the new compound.

During the next 40 years, the approximate composition of "nuclein" (or "nucleic acid" as it was later termed by Altmann) was established, mostly by workers in the laboratories of Kossel and Levene, and by 1924 two types of nucleic acid were generally recognised: "plant nucleic acid", containing uracil, and "animal" or "thymus" nucleic acid", containing thymine, with the three bases

adenine, guanine and cytosine common to both. The sharp division between these two was blurred when Feulgen, in 1924, demonstrated by a histochemical reaction that "thymus nucleic acid" was widespread throughout both plant and animal kingdoms. By 1930 the terms "ribonucleic acid" (RNA) and "deoxyribonucleic acid" (DNA) were in use, and the chemical components of the two types had been identified. (See Davidson and Chargaff, 1955, for a review). Further histochemical studies by Brachet (1933 et seq.) and spectrophotometric analyses by Caspersson (1936 et seq.) culminated in the independent observations by these workers (1942 and 1941 respectively) that the highest concentrations of RNA occurred in those cells which are most actively engaged in protein synthesis, and a relationship between the two was suggested (see Caspersson, 1950). The genetic role of DNA was first put on an experimental basis by Avery _et al_ (1944) from observations on bacterial transformation, and in 1945 Schneider demonstrated that the DNA of the cell was confined to the nucleus, although RNA occurred throughout the cell. It was later discovered by Boivin, Vendrely and Vendrely (1948), and by Thomson _et al_ (1953) that the amount of DNA in the diploid nucleus was constant, and double that of the haploid cell, for any given species. This finding made the presumed genetic role of DNA even more probable. A turning point was reached in 1953 when Watson and Crick proposed a double helical structure for DNA, based on analyses performed mainly in Chargaff's laboratory. By suggesting a complementary structure formed by base pairing, Watson and Crick were able to give a possible explanation as to how DNA could replicate itself.

During the last 10 years, a great deal of interest has been focused on the cellular sites of nucleic acid

synthesis and function, particularly the genetic regulation of nucleic acid and protein synthesis. As a result of these studies, the cell nucleus has emerged as an organelle of major importance.

The Cell Nucleus.

Under the light microscope the living interphase nucleus of the rat liver cell appears as a roughly spherical body, about 7µ in diameter, containing one or more refractile nucleoli. The general *in vivo* appearance of the intranuclear material is granular, although in isolated nuclei this may be drastically affected by changes in the medium (see Philpot and Stanier, 1956). Little more of the intranuclear structures may be resolved under the light microscope.

Electron microscopy reveals the nucleus as a very much more complex structure, bounded by a double membrane (see Fig. 5). Within the nucleus the nucleolus is seen to be made up of various structures, and many smaller particles are visible. Chromatin is distributed throughout the nucleus, although there seems to be some doubt about its presence within the nucleolus. The structure, composition and function of the nuclear organelles are considered in more detail below.

1. The Nuclear Membrane.

The nuclear membrane is a double layered envelope surrounding the nucleus, with pores passing at intervals through the membrane from the nucleus to the cytoplasm (Watson, 1954, 1955; Hoffman and Grigg, 1958). In pancreatic cells these pores appear large enough to allow molecules the size of RNA and proteins to be freely permeable (Watson, 1955). Similar sized pores have also been observed in neuron cells (Dawson *et al*, 1955), although some workers believe these pores to be artefacts

of fixation (Kautz and De Marsh, 1955). More recent work shows that these pores, or annuli, are possibly genuine cytological structures, and electron micrographs have been published showing the fine structure of the pores, with a small membrane present in the waist of the pore itself, providing a thin barrier between the nucleus and cytoplasm (Afzelius, 1955). From the electron micrographs of Gay (1955) and others, it appears that the nuclear membrane is continuous with the endoplasmic reticulum, and it is now widely thought that the endoplasmic reticulum arises from the nuclear membrane, which is held to be a specialised part of the cell membrane system (Rebhun, 1956; Brachet, 1957; Waddington and Perry, in Sirlin, 1961; and Mirsky and Osawa, 1961).

Studies by Merriam (1961), however, suggest that RNA is not a component of the nuclear membrane. An interesting study of the nuclear membrane and its origin has been made by Davis (1963), from observations on the mitotic nucleus of the regenerating rat liver cell. He finds that the nuclear membrane re-forms at telophase, and suggests that this re-formation is brought about by *de novo* synthesis of the membrane structure rather than by a re-orientation of the double-membraned elements of the endoplasmic reticulum, as Porter and Machado (1960) suggest, since most of the cytomembranes have disappeared by early prophase. Similar conclusions on the origin of the nuclear membrane have been reached by Merriam (1962) on the basis of comparative uptake of ^{14}C-lysine by the nuclear and cytoplasmic membranes, and by Jones (1960), who found that the nuclear membrane of foetal rat haemoblasts was formed *de novo* after mitosis.

The composition of the nuclear membrane is uncertain,

since it has never been isolated, but it almost certainly contains lipoprotein. There is evidence that the composition of the nuclear membrane is different from the cytomembranes, in that it contains more protein than lipid (Palmer, Hodes and Warren, 1961; Merriam, 1962). If one assumes that the majority of the nuclear lipid is contained in the nuclear membrane (Gurr, Finean and Hawthorne, 1963), then comparative studies on the lipid composition of the nucleus and the cytomembranes suggest that the nuclear membrane has a very different composition from the cytomembranes (Patterson and Touster, 1962; Levin and Thomas, 1961; Biezenski et al 1963; Gurr, Finean and Hawthorne, 1963). Despite these apparent differences in composition between the nuclear and cytomembranes, the general consensus of opinion seems to favour the findings of the electron microscopists, that is, that the nuclear membrane gives rise to the cytomembranes, and RNA is possibly passed from the nucleus to the cytoplasm in this process.

It is obvious, from these considerations, that the nuclear membrane must be a most important factor in controlling the passage of material from the nucleus to the cytoplasm and vice versa. Few experiments have been conducted on the transport activity of the nuclear membrane, but some facts emerge. The many in vivo experiments using labelled amino acids or RNA precursors show that small molecules can easily pass through the membrane. Experiments by Allfrey, Mirsky and Osawa (1957) and Allfrey and Mirsky (1959) suggest that the nuclear membrane contains a selective sodium-potassium transport mechanism, and there is abundant evidence in the literature to suggest that proteins such as ribonuclease, and even larger proteins such as anti-human-γ-globulin, can pass intact into the

nucleus from the cytoplasm (Beck, 1963). This indicates that the nuclear membrane must contain a very powerful active transport system (see Mirsky and Osawa, 1961). The spectrophotometric studies of Caspersson (1950) suggest that RNA passes from the nucleus to the cytoplasm across the nuclear membrane, since the uniformity of the RNA concentration at the cytoplasm-nuclear membrane interface is striking, and this uniformity would be unlikely if the RNA were transferred via the pores. Indeed, the electron microscopy studies of Afzelius (1955) would suggest that there is a complete barrier between the nucleus and cytoplasm, and one must therefore conclude that all material to and from the nucleus to the cytoplasm is passed via the nuclear membrane; either it is transferred by a transporting system within the membrane, similar to that in mitochondria, or else the nuclear material must peel off rapidly to form endoplasmic reticulum, and the material is carried out into the cytoplasm on the newly formed reticulum. It is possible that both these mechanisms may operate.

The Nucleolus.

Within the nucleus, the nucleolus is the most prominent structure, and is characterised by its density due to its high protein content. It has been suggested by Estable and Sotelo (1954) on the basis of a silver staining technique, that the interphase nucleolus is composed of two parts: an organised series of fibrous bundles, termed the "nucleolonema", surrounded by amorphous material, termed the "pars amorpha". These areas have been found in rat liver nucleoli by Davis (1960), but there is considerable discussion as to whether the nucleolonema and pars amorpha are made up of small ribonucleoprotein particles, as suggested by Sirlin (1961) or of fibres.

A detailed critique of the literature is given by Davis (1963). Serial electronmicrographs by Bernhard and Granboulan (1963) show the rat liver nucleolus to have a sponge-like structure, with innumerable interconnecting vacuoles. It is clear, however, that there is no nucleolar membrane.

The nucleolus has been considered by Sirlin (1961, 1962) to be a differentiated locus of a particular chromosome, since it is formed during telophase at a particular chromosomal site, the "nucleolar organiser" (Heitz, 1931; McClintock, 1934). Ohno, Weiler and Skenius (1961) have found that there may be 6 chromosomal sites participating in the formation of nucleoli, but one of a pair of chromosomes may remain dormant. It has been suggested by Lin (1955) that the RNA content of the nucleolus of maize depends on the number of organisers present. Reviews on the origin of the nucleolus have been presented by Vincent (1955); Stich (1956); Brachet (1957); Mirsky and Osawa (1961) and Sirlin (1962). In the normal cell the size and activity of the nucleolus are very closely correlated with the synthetic activity of the cell (Vincent, 1955). Thus, in cells which are not actively synthesising RNA or protein, the nucleoli are small, and often multiple. For instance, during cell division, when the cells have temporarily ceased synthetic activity, the nucleoli are either very much reduced in size (Austin and Braden, 1953), or have disappeared altogether (Sirlin, 1961), and in plant cells which have been kept in the dark, and are therefore relatively inactive, the nucleoli are also small (Fisher, 1934, quoted by Lin, 1955). On the other hand, cells with large nucleoli are usually very active in protein synthesis: indeed, Casperson (1950) has

stated that "the increase of nucleolar masses is a most conspicuous phenomenon during protein synthesis". Thus Stowell (1949) and Castro and Foraker (1962) both found that in regenerating rat liver, where the activity of the cell is very much greater than normal, the mean mass of the nucleolus is also significantly increased, and Stöcker (1963) has shown that the increased nucleolar volume induced with thioacetamide is associated with a marked uptake of ^{3}H-cytidine by the nucleolus. In larvae of Rhodnius prolixus, treatment with moulting hormone caused an increase in the size of the nucleolus which coincided with a rise in the RNA content of the cytoplasm (Wigglesworth, 1963). In rats, corticotrophin treatment induces an enlargement in the mean size of the nucleoli in the fascicular zone of the adrenal gland (Miller, 1962), which may correspond to an increase in the protein synthetic activity of the cell (Bransome and Reddy, 1963), but the situation is as yet unclarified. Protein withdrawal from the diet of the rat produces an enlargement of the liver cell nucleoli (Stenram, 1953) and a concomitant increase in the activity of nuclear RNA (Stenram, 1962). It is clear, from these considerations of structure alone, that the nucleolus has a major function in controlling the synthetic activity of the cell, but since the functions and metabolic activity of the nucleolus are so closely correlated to those of the nucleus as a whole, further discussion of this topic is reserved until the metabolism of the nucleus generally is considered. A more detailed account of the effects of diet on the liver cell and nucleus will also be given later in this Introduction.

3. Nuclear ribosomes.

It has been known for some time that the nucleus contains small particles ranging in diameter from 100 - 300Å. Thus Bernhard et al (1955) reported the existence of

granular particles of 100 - 150Å in diameter in the nucleolus and on the internal surface of the nuclear membrane of rat liver and Ehrlich ascites tumour cells and suggested that they were made up of ribonucleoprotein. Other particles have been described by Gall (1956) in lamp-brush chromosomes, and by Callan (1956) in the nuclear sap (see Allfrey, 1963, for a review). A review of the electron microscopy of particles which have been observed in nuclei is provided by Swift (1963).

The first suggestion that some of these granules might correspond to the ribonucleoprotein particles in the cytoplasm, described by Palade and Siekevitz (1956), came from studies by Frenster et al (1960), who extracted lymphocyte nuclei with neutral tris buffer and showed that this extract contained particles which could be sedimented by high-speed centrifugation; the resulting pellet was made up of 60% RNA and 20% protein. Furthermore, studies on intranuclear protein synthesis showed that the uptake of radioactive amino acids into nuclear protein required the participation of these particles (Allfrey, 1963). Further evidence for the existence of nuclear ribosomes has come from the work of Samarina and Georgiev (1960) who isolated ribonucleoprotein particles from rat liver nuclei using 0.14M KCl and neutral tris buffer, and found that this "microsomal" material accounted for 20% of the nuclear RNA, and was capable of incorporating ^{14}C-tyrosine, although not as actively as cytoplasmic microsomes. In addition, Wang (1960, 1961) has isolated a ribonucleoprotein fraction from calf thymus nuclei, and has shown that this fraction is capable of incorporating ^{14}C-serine in the presence of a nuclear "pH 5 enzyme", an ATP-generating system, GTP, and Mg^{2+} ions, and is composed of nucleoprotein particles.

The existence of nuclear ribosomes is now well established, and Wang (1963a) has recently demonstrated that nuclear ribosomes from calf thymus contain two RNA components of 26S and 17S respectively, and possibly a 5S subunit. These figures correspond well with the values of 28 - 30S and 16 - 18S for mammalian cytoplasmic ribosomes (Scherrer, Latham and Darnell, 1963; Staehelin *et al*, 1964). A detailed review of the physical properties of nuclear ribosomes from calf thymus has been presented by Wang (1963b). Pogo *et al* (1962) have purified calf thymus nuclear ribosomes on a sucrose density gradient, and found that they have a sedimentation coefficient of 78S; about 60% of their mass is RNA. Electron microscopy of the preparation shows that these ribosomes are about 200Å in diameter, and, like cytoplasmic ribosomes, they dissociate into 64S and 42S components in the presence of small amounts of EDTA, and at 0.01M EDTA only 49S and 33S components are present. From these experiments one may conclude that nuclear ribosomes from calf thymus are similar in behaviour to ribosomes from rat liver cytoplasm (Petermann and Hamilton, 1961).

It has already been mentioned that the nuclear ribosomes are required for incorporation of ^{14}C-amino acids into nuclear protein, but the metabolic requirements of nuclear ribosomes show several divergences from those of cytoplasmic ribosomes. Thus, the uptake of ^{14}C-amino acids into nuclear proteins appears to be greatly stimulated by the addition of sodium ions to the nuclear suspension (Frenster, Allfrey and Mirsky, 1960), as opposed to the requirement of cytoplasmic incorporating systems for potassium. Secondly, the ability of nuclear ribosomes to incorporate protein is inhibited by deoxyribonuclease (Allfrey, Mirsky and Osawa, 1957), but the addition of DNA

from various sources to the DNase treated ribosomes almost completely restores the incorporation of amino acids into nuclear protein. This requirement for DNA is probably related to the requirement of the system for an ATP-generating system, since nuclear ATP synthesis in calf and rat thymus nuclei has been found to be DNA-dependent (Allfrey, Mirsky and Osawa, 1957; Betel and Klouwen, 1963, respectively). In addition, Naora (1962) has found that ribosomes of calf thymus nuclei form a multiribosomal complex with DNA. On the basis of this, he suggests that nuclear protein synthesis can be regulated by a mechanism which involves association or dissociation of the ribosomes with a particular DNA in the genome. His results, however, indicate that the binding is non-specific, since any number of from 3 to 27 ribosomes can bind to one DNA molecule, and the ribosomes can also bind with other polyanions. This suggests that Naora's findings are compatible with non-specific ionic binding between two highly charged particles. It seems probable, however, on the basis of the work of Allfrey and co-workers, that the requirement of nuclear ribosomes for DNA-dependent ATP-synthesis is a real one. Allfrey's group also find that chloramphenicol and puromycin inhibit the incorporation of amino acids into nuclear protein (Allfrey, Hopkins, Frenster and Mirsky, 1960). It seems that the amino acids incorporating systems of the nucleus are in the nuclear sap, since the nucleolus does not incorporate amino acids to such a great extent (Allfrey, Hopkins, Frenster and Mirsky, 1960); it is possible that ribonucleoprotein particles in the nucleolus comprise ribosomal material which is shortly to be transferred to the cytoplasm (Birnstiel and Chipcase, 1963; Edstrom and Gall, 1963; Korden and Morgenstein, 1963).

Although most of the work on nuclear protein

synthesis has been performed with calf thymus nuclei, Rees and Rowland (1961) and Rees, Rowland and Varcoe (1963) have shown that isolated rat liver nuclei can also incorporate amino acids into nuclear protein. The uptake is greatest in a fraction sedimenting after 30 minutes at 105,000*g* . The system, however, does not show a specific requirement for sodium, since potassium and sodium are interchangeable, but uptake is inhibited by uncoupling agents, indicating a requirement for ATP synthesised by oxidative phosphorylation.

Nucleic Acid turnover in the liver cell nucleus.

(1) DNA.

The metabolic inertness of rat liver DNA was established by Hevesey and Ottoson in 1943, and the constancy of the DNA content of rat liver nuclei was shown by Thomson *et al* (1953), who found that the amount of DNAP per nucleus is about 9.3 picograms. This figure is higher than that of 6.5 to 7.0 picograms for the DNAP content of diploid cells from other organs of the rat, due to the number of tetraploid and octaploid nuclei in the liver (Thomson, 1953). The cell DNA is contained almost entirely in the chromatin (see Thorell, 1955), but there is some argument as to its presence in the nucleolar substance. Thus Brachet (1957), and Sirlin (1961), state that the nucleoli themselves do not contain DNA, but are surrounded by condensed chromatin which forms the "nucleolus-associated chromatin". Davis (1960), on the other hand, has found that the "fibrous bundles" of nucleoli from regenerating liver stain densely with Feulgen staining, and suggests that these nucleoli contain DNA throughout their substance. Certainly, most preparations of isolated nucleoli contain DNA (e.g. Monty *et al*, 1956; Rees, Rowland and Varcoe, 1963; Muramatsu *et al*, 1963; Birnstiel and Chipcase, 1963). It is possible that in these preparations

the DNA may represent contaminating "nucleolus-associated chromatin", but recently Ro *et al* (1964) have reported the separation of the DNA in their preparations into intranucleolar DNA of the "perichromatin fibres", the perinucleolar "nucleolus-associated chromatin", and an unidentified "adventitious DNA". It seems likely from these more recent studies, and also from studies on the DNA-dependence of nucleolar RNA synthesis (Ro *et al*, 1964), that the nucleolus does indeed contain intranucleolar DNA.

(2) RNA.

On the other hand, it has been known for some time that the RNA of the nucleus is distributed throughout the nuclear sap, the nucleolus, and the chromatin (Caspersson, 1950). Experiments on the uptake of isotope by the RNA of the nucleus were first performed by Marshak and Calvet (1949), on liver. These authors showed that after an *in vivo* injection of ^{32}P, the specific activity of the nuclear RNA was higher than that of cytoplasmic RNA for up to 73 hours after injection. Other workers have since confirmed their results using ^{32}P (Jeener and Szarfarz, 1950 ; Barnum and Huseby, 1950; Smellie *et al*, 1953), and using ^{14}C-orotic acid (Hurlbert and Potter, 1952). These and other experiments have led many workers to the conclusion that the nucleus is the site of synthesis of cytoplasmic RNA (see, e.g. Goldstein and Plaut, 1955). Certainly, experiments by Prescott (1960), have shown the nuclear dependence of RNA synthesis in *Acanthamoeba* species, and the dependence of cytoplasmic protein synthesis on the nucleus has been well shown by the experiments on enucleated *Acetabularia* (Stich and Plaut, 1958). More recent experiments with actinomycin D (to be discussed later) have provided strong support for these theories. The presence of an RNA polymerase in the cytoplasm,

however, does not exclude the possibility of cytoplasmic RNA synthesis: this aspect has been well reviewed by Harris (1963).

The distribution of RNA precursor label within the nucleus was first studied by Ficq (1955) from autoradiographs of amphibian eggs which had been exposed to ^{14}C-adenine: Ficq was able to show that the heaviest labelling occurred over the nucleolus. This observation is now a classic one in all species studied (e.g. Fitzgerald and Vinijchaikul, 1959; Ficq, 1959; Woods and Taylor, 1959; and Tandler and Sirlin, 1961), and indicates that the nucleolus is a particularly active centre of synthesis and/or turnover of RNA. Other autoradiographic experiments by Goldstein and Micou (1959) using human amnion cells which had been exposed to ^{3}H-cytidine showed that label in the chromatin appeared within five minutes, whilst label in the nucleolus did not appear until ten minutes after exposure. These observations led these authors to conclude that the heavily labelled RNA in the nucleolus is RNA which has been synthesised in the chromatin and is passed rapidly to the nucleolus, whose function is to act as a "store". Similar observations by Rho and Bonner (1961), using plant tissues, would support this theory. On the other hand, studies by McMaster-Kaye and Taylor (1958); Perry and co-workers; Srinivasan et al (1963); and by Stenram (1963) seem to indicate that RNA synthesis occurs independently in the nucleolus and in the chromatin. The experiments of Perry and co-workers are particularly interesting. They irradiated the nucleolus with a microbeam of U.V. light, causing a 90% decrease in nucleolar uptake of RNA precursor, and found that damage to the nucleolus inhibited incorporation into non-nucleolar RNA by 30%, and ultimate incorporation into cytoplasmic RNA by 60-70% (Urota and Perry, 1957; Perry and Errera, 1960; Perry,

1960). Irradiation of the non-nucleolar part of the nucleus did not inhibit incorporation of labelled RNA precursors into the nucleolus, however. These observations provide strong evidence for independent nucleolar and non-nucleolar nuclear RNA synthesis, and also for nucleolar RNA being an important precursor of cytoplasmic RNA. More recent experiments by several authors do, in fact, suggest that the nucleolus is the site of synthesis of cytoplasmic ribosomes. Thus, Birnstiel, Chipcase and Hyde (1963) have shown that nucleolar ribonucleoprotein particles, extractable with deoxycholate, are similar in size, and have sedimentation constants approximating to those of ribosomes in the cytoplasm. Furthermore, the dissociation and association of these nucleolar particles relative to magnesium concentration resembled that of the cytoplasmic particles. Birnstiel and Chipcase (1963) have also shown that the amino acid composition of the "residual protein" of pea nucleoli is almost identical to that of cytoplasmic ribosomal protein, and Edstrom and Gall (1963), who isolated the nucleoli of Triturus oocytes by microdissection, showed that the base compositions of nucleolar and cytoplasmic RNA were very similar; both were richer in guanine and cytosine (G-C rich), than in uracil and adenine. In addition, Georgiev _et al_ (1963) have found highly active ribonucleoprotein particles in the nucleolonema of rat liver nucleoli; the base composition of the RNA component of these particles was also G-C rich ribosomal RNA (r-RNA). These ribosomal-like particles sedimented at a speed characteristic of ribosomes in a sucrose density gradient. Such considerations lead one to postulate that possibly the nucleolus is the site of assembly of r-RNA and protein into whole ribosomes, and this assembly of components is

probably DNA-dependent (Love and Walsh, 1963).

The RNA precursors of r-RNA have been shown by Scherrer, Latham and Darnell (1963), and Tamaoki and Mueller (1962), to be made up of a 45S and a 33 - 35S component, which are converted in the nucleus to 28S and 16S RNA respectively; Girard, Penman and Darnell (1964) find that treatment of HeLa cells with actinomycin D interrupts the conversion of the 45S and 33S ribosomal RNA precursors into 28 and 16S r-RNA: this conversion would thus also appear to be DNA-dependent. Experiments by Yankofsky and Spiegelman (1962) on Escherichia Coli show that synthesis of ribosomal RNA occurs on the genome, but that the actual proportion of the genome involved is very small (0.02% of the total DNA of E. Coli). It is possible that in mammalian cells, the de novo synthesis of precursor ribosomal RNA takes place on the extranucleolar chromatin and passes rapidly to the nucleolus where it is "stored", or immediately assembled (after Goldstein and Micou's (1959) findings) or else that de novo synthesis of ribosomal precursor RNA takes place in the nucleolus itseelf, independently of other nuclear RNA synthesis.

Messenger RNA.

It is now a well-established concept that the major part of cellular protein synthesis occurs at the ribosomes in the endoplasmic reticulum (see, e.g. Hoagland, 1960), and until 1961 it was thought that the genetic coding system required for the synthesis of species-specific protein was a built-in function of the ribosomes. In 1961, however, Brenner, Jacob and Meselson presented evidence from bacterial studies in favour of a separate RNA species acting as a "messenger" in the transfer of genetic information from the gene to the protein-synthesising system in the ribosome, and identified

this RNA species from its extremely high rate of turnover. Since that time, a great deal of attention has been focused on the nature of "messenger RNA" (m-RNA); in particular, bacterial messenger RNA has been well studied (see Lipmann, 1963, for a review). Some of the main characteristics of bacterial m-RNA which are known are its heterogeneity of molecular size (e.g. Otaka, Mitsui and Osawa, 1962), base ratios which more closely resemble those of homologous DNA, than total cell or ribosomal RNA (Volkin and Astrakhan, 1956; Gros et al, 1961; Midgley and McArthy, 1962), and a capacity to associate as a hybrid complex with homologous DNA (Spiegelman, Hall and Stock, 1961; Yankofsky and Spiegelman, 1962). Work by Nirenberg and Matthaei, and by Ochoa and co-workers (see e.g. Crick, 1963, for a review and references), has centred on the use of artificial messenger RNA in a cell-free protein system, and it has been found (Nirenberg and Matthaei, 1961) that a synthetic polyribonucleotide "messenger", e.g. polyuridylic acid, can direct the synthesis of a defined polypeptide (in this case polyphenylalanine). From experiments with Escherichia Coliphage mutants, Crick et al (1961) have calculated that the most probable number of nucleotides required to code for each amino acid of a polypeptide chain is three: they term this nucleotide triplet a "codon".

Reports of the existence of an analogous RNA fraction in mammalian cells have appeared more recently. The mammalian messenger RNA fractions found have properties very like those of the bacterial messenger in that they appear rapidly labelled in the nucleus (Sibatani et al, 1962; Hiatt, 1962; Debellis and Marks, 1963; Harris, 1963; Harris et al, 1963; Scherrer, Latham and Darnell, 1963), have a composition similar to that of DNA (Sibatani et al, 1962; Georgiev et al, 1963; Hoyer et al, 1963), are

heterogeneous in size (8 - 16S) (Debellis and Marks, 1963; Stachelin et al, 1964), and form a complex with homologous DNA (Love and Rabotti, 1963; Kidson et al, 1963). Finally, the addition of this mammalian "messenger RNA" to a cell-free amino acid incorporating system results in considerable activation of the system (Barondes, Dingman and Sporn, 1962; Garren and Howell, 1963). Recently, Stachelin et al (1964) have shown that the number of nucleotides in a messenger RNA coding for a polypeptide chain is three times the number of amino acids in the polypeptide chain, and have thus elegantly confirmed the triplet "codon" for mammalian cells.

The biosynthesis of messenger RNA occurs on the DNA of the chromatin, and is inhibited by actinomycin D (Garren and Howell, 1963; Tamaoki and Mueller, 1962; Scherrer, Latham and Darnell, 1963), and by DNase (Hurlbert et al, 1963). Some evidence has been presented for the biosynthesis of a part of the messenger RNA in the nucleolus. Thus, Sibatani et al (1962) find a rapidly labelled DNA-like RNA in the "nucleolar residue" RNA which remains with the protein after phenol extraction. Similarly, Georgiev et al (1963) find in the "nucleolo-chromosomal-apparatus" an RNA whose base composition is similar to DNA (D-RNA). Although both these findings are open to the criticism that the material studied is almost certainly not entirely nucleolar in origin, the possibility remains that the nucleolus may be the site of synthesis for some part of the m-RNA of the cell, as well as the probable site of synthesis of ribosomal RNA.

In addition to the synthesis of m-RNA and r-RNA, s-RNA (transfer RNA) synthesis has also been reported to take place in the nucleus. Thus, Chipcase and Birnstiel (1963) have shown that isolated pea nuclei can synthesise

amino-acyl s-RNA *in vitro*, and this biosynthesis appears to be DNA-dependent, since both DNase and actinomycin D are inhibitory. It also seems likely, from experiments by Errera *et al* (1963) that the methylation of s-RNA bases (e.g. 5'-methyl cytosine) can take place in the nucleus and nucleolus.

In passing, it is perhaps worth mentioning that actinomycin D-resistant RNA synthesis has been observed in mammalian cells. Thus Paul and Struthers (1963), and Martin and Leslie (1964) have shown the incorporation of labelled RNA precursors into nuclear RNA of cultured cells (LS and HLM cells respectively) in the presence of actinomycin D. Paul and Struthers suggest that this is evidence for the presence of RNA-primed RNA polymerase in mammalian cells, but Gomatos and Tamm (1963) suggest that these findings are compatible with the presence of "reovirus", containing double-stranded RNA, in the HLM culture.

Dietary Protein Level, the Liver Cell, and Liver Nuclear RNA.

a) Changes in the liver and liver cytoplasm with dietary protein level.

It was established by Addis and co-workers, in 1936, that the liver is one of the most responsive organs to protein deprivation and subsequent re-feeding of protein. Addis *et al* showed that if rats are fasted for seven days, the liver will lose 40% of its initial protein content, whereas the blood will lose 20%, the carcass (muscle, skin and skeleton), 8%, and only 5% of the total protein content of the brain is lost. On the other hand, feeding a diet of 74% casein to these animals restored the liver protein very rapidly. It was later shown by Kosterlitz and co-workers (1947), that the losses in liver weight and liver protein are paralleled by losses in liver phospholipid and RNA, although the actual numbers of the cells present in the liver do not decrease as

shown by constancy of DNA. Kosterlitz et al (1947) therefore concluded that the losses and subsequent gain in weight of the liver during protein deprivation and protein feeding are due to changes in the amount of cytoplasm in the cell: they termed this "labile liver cytoplasm".

A comparative study of the changes in composition of the liver cytoplasmic subfractions was made by Wikramanayake et al (1953), who fractionated rat liver cytoplasm into mitochondria, "heavy microsomes" and "cell supernatant". "Cell supernatant" corresponded to "cell sap" together with lighter microsomes (see Munro, 1964). Wikramanayake et al showed from these studies that, when rats are deprived of protein for several days, there is 1) a uniform loss of protein from all the fractions; 2) a loss of phospholipid from the microsomes and cell supernatant; and 3) a loss of RNA from the microsomes. These findings coincide with electron microscopic observations published later, showing, that when rats are starved there is a gross reduction in the amount of endoplasmic reticulum in the liver (Fawcett, 1955; Bernhard and Rouiller, 1956).

Studies on the turnover of RNA in protein-deprived and protein-fed rats were carried out by Munro et al (1953), and by Clark and Munro (1957) (see Fig. 1), and showed that, if rats were deprived of protein for 24 hours, the rate of uptake of ^{32}P into liver RNA is greatly decreased: on the basis of various pieces of evidence, they concluded that the decrease in labelling was due to the increased pool size of free nucleotides, arising from the breakdown of cytoplasmic RNA when dietary protein is withdrawn. Feeding of protein caused immediate reversal of this phenomenon. On the other hand, after four days' protein deprivation, the specific activity of ^{32}P and ^{14}C uptake was markedly

Fig. 1.

Influence of protein intake on the amount of ribonucleic acid in the liver (upper two curves) and on its uptake of radioactive phosphorus (lower two curves). The rats were given either a diet containing protein (●——●) or a similar diet free from protein (o— —o), and were injected with ^{32}P-orthophosphate 24 hours before killing. Each point is the mean of observations on three rats.
(reproduced from Munro *et al*, 1953).

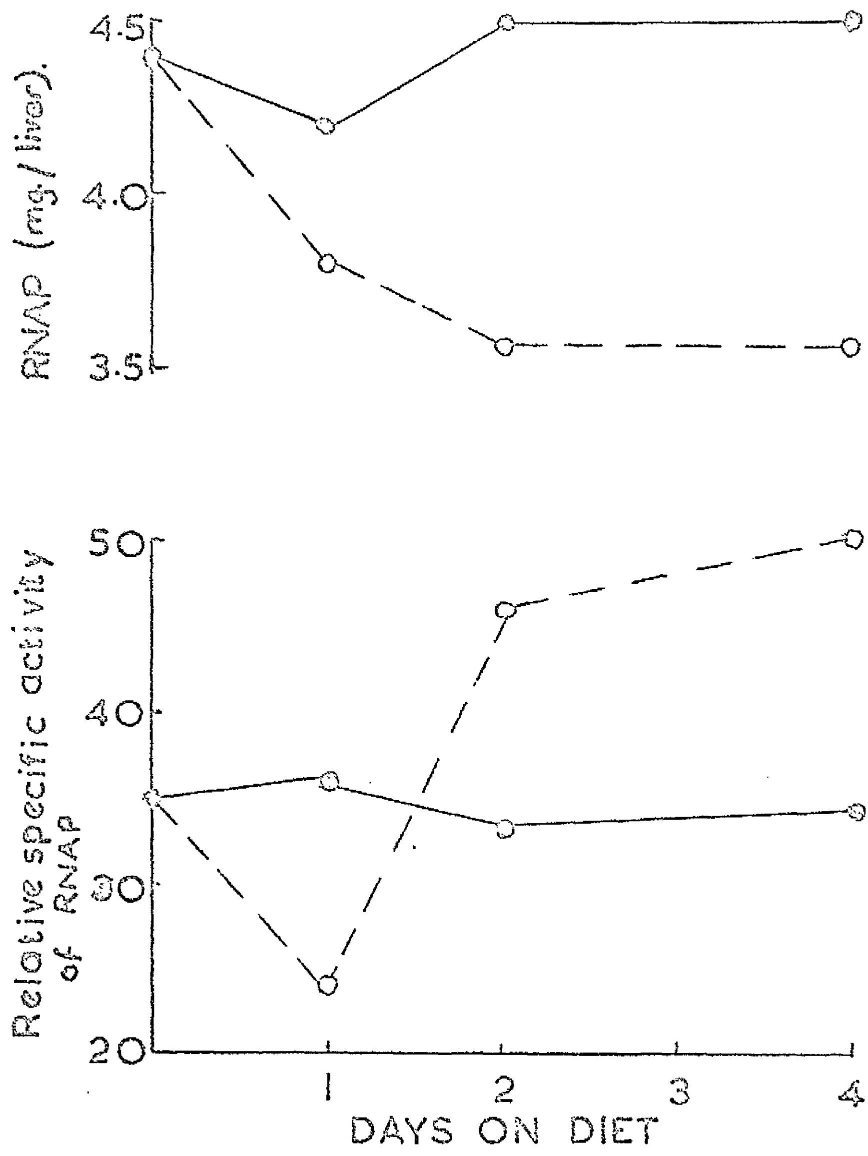


Fig. 1.

increased. It is concluded by Munro (1964) that the eventual increase in RNA turnover after four days' protein deprivation represents the "true" rate of turnover of RNA of the liver, after the excess pool of nucleotides due to the initial breakdown of RNA has disappeared.

On the other hand, similar studies by Clark and Munro (1959) on a transplantable rat hepatoma showed that there was no response of the tumour cell to variations in protein intake, either in RNA loss, or in loss of protein, and the uptake of ^{32}P in the liver was not altered by changes in dietary protein. It will be recalled (Howatson and Ham, 1955; Novikoff, 1957), that most hepatomas have little organised endoplasmic reticulum, and the majority of the ribosomes are free; this was confirmed for the tumour used by Clark and Munro (1959). A comparison of the responses in normal and malignant liver cells to changes in dietary protein leads Munro (1964) to suggest that the change in the RNA metabolism of the normal liver cell with variation in dietary protein level is due to an RNA component in the membranous part of the endoplasmic reticulum of the liver cell, rather than in the ribosomes, since this membrane is absent from the unresponsive hepatoma cells. Goswami et al (1962) did in fact obtain from pyrophosphate-prepared membrane an RNA species of high G-C content and rapid ^{32}P uptake. A detailed review of this, and of the general effects of protein diet on cell metabolism is given by Munro (1964).

b) Changes in the liver cell nucleus with diet.

The literature on this subject is meagre, but some interesting histochemical observations have been made by Stenram. Stenram (1953, 1958a) showed that the nucleoli of rats which had been deprived of protein had an average volume of $3.3\mu^3$, instead of $1.8\mu^3$ for rats fed 25% casein

(Stenram, 1958a). This represents an increase of over 80% above normal, during protein deprivation. Interferometric determinations of the dry matter of those nucleoli showed that the absolute amount of dry matter in the liver nucleoli of protein-deprived rats was increased, although the concentration of material in the nucleolus remained the same for both protein-fed and protein-deprived animals (Stenram, 1957). Further interferometric determinations on these groups indicated that there is an absolute increase in the RNA in the nucleoli of protein-deprived rats, although again the concentration of nucleolar RNA does not alter (Stenram, 1958a,b). In rat hepatoma nuclei, however, the nucleoli are always large, and the amount of dry matter is constantly raised above that of normal hepatocytes (Stenram, 1958a). Moreover, this situation does not revert to that observed in normal liver cells on feeding protein. Thus, neither the nucleolus of the hepatoma cell, nor the cytoplasm, respond to changes in diet, whereas the normal liver parenchymal cell shows a simultaneous response to protein deprivation affecting both nucleolus and cytoplasm. That is to say, in the liver cells of animals receiving adequate protein, the nucleoli are small, the endoplasmic reticulum extends throughout the cell cytoplasm, and the uptake of labelled precursor RNA is constant. During protein deprivation, however, the nucleolus enlarges, the endoplasmic reticulum decreases in abundance, and the level of ^{32}P uptake into RNA at first falls, by precursor dilution, but then rises again after a few days. This suggests that, if a membranous RNA component is involved in the response of the liver parenchymal cell to withdrawal of dietary protein, as suggested by Goswami et al (1962), then it seems that the origin of this membranous RNA may be in the nucleolus. On this hypothesis, the nucleolus of the

protein-depleted animal thus stores up RNA. The relationship of the nucleolus and nuclear membrane, and the possible precursor-product relationship of the nuclear and cytoplasmic membranes (discussed earlier, and see Fig. 40) makes this hypothesis particularly attractive.

The object of this work was therefore to study the changes in nuclear RNA in animals which had either received protein at specified times, or else had been deprived of protein, and to try and establish a relationship between the responses to diet in the cytoplasm, and those in the nucleus and nucleolus. Experiments were therefore carried out on the liver nuclei and sub-nuclear fractions of rats on dietary regimes containing protein or free from protein. Other studies included the separation of the nuclear RNA from these animals on sucrose density gradients, and Schlieren and U.V. optical analysis of the components, using the analytical ultracentrifuge. The metabolic activity of the nuclear and cytoplasmic RNA species were also examined.

SECTION II : MATERIALS AND METHODS.

MATERIALS AND METHODS.

1. General.

(i). Animals and diet.

Young male albino rats weighing between 120g. and 180g. were housed individually, under thermostatic conditions, and given water *ad libitum*. The rats were divided into three dietary groups and fed on diets calculated to provide 1,200 calories/square metre body surface area/day (Clarke and Munro, 1957). The diets of two of these groups contained an adequate supply of protein (26% by weight of the total food intake), whereas the third group was fed on an isocaloric diet free from protein. Details of the diet mixtures are given in Tables 1-5, and quantities of diet allocated to the animals, per day, are given in Table 6. The animals were kept on these diets for at least 4 days, and soon learnt to consume the meals rapidly.

On the morning after the last day of feeding, all groups had been fasting for 18 hours and were in the post-absorptive state. One group of the rats which had received protein in their diet were then fed 2g. casein solubilised with 0.1g.$NaHCO_3$, the other two groups receiving no food at this time. Thus animals in three dietary conditions could be studied: (a) those animals actively absorbing amino acids from the gut (H.P.F. group), (b) animals fasting after receiving a diet containing adequate protein (H.P. group), and (c) animals fasting after a protein-free diet (L.P. group).

Administration of Isotope and time of killing.

For dietary experiments not involving the use of radioisotopes, the rats were killed 2 hours after feeding the H.P.F. group. For dietary experiments in which

TABLE 1.

Composition of high protein diet.

Potato starch	69g.
Glucose	69g.
Margarine	42g.
Casein	240g.
Total	420g.

TABLE 2.

Composition of protein-free diet.

Potato starch	189g.
Glucose	189g.
Margarine	42g.
Total	420g.

TABLE 3.

Vitamin-Mineral-Roughage (V.M.R.) mixture.

Sodium chloride	32.5g.
"446" salt mixture	130g.
Vitamin mixture	250g.
Agar powder	62.5g.
Margarine	77.5g.
Total	552.5g.

1g. α-tocopherol acetate was mixed with 14ml. radiostoleum (B.D.H.). 0.8ml. of this was mixed with the above mixture.

TABLE 4.

VITAMIN MIXTURE.

Pyridoxine hydrochloride	25mg.
Riboflavin	25mg.
Thiamine hydrochloride	25mg.
Nicotinic acid	100mg.
Menaphthone	5mg.
Biotin	5mg.
Calcium pantothenate	200mg.
p-Aminobenzoic acid	500mg.
Inositol	1g.
Choline chloride	10g.
Folic acid	trace.
Potato starch	To 500g.

TABLE 5.

Salt mixture "446".

Sodium chloride	243.2g.
Potassium citrate	533.0g.
Di-potassium hydrogen phosphate	174.0g.
Di-calcium phosphate	800.0g.
Calcium carbonate	368.0g.
Ferric citrate $5H_2O$	360.0g.
Copper sulphate $5H_2O$	0.4g.
Potassium aluminium sulphate $24H_2O$	0.2g.
Magnesium carbonate	92.0g.
Manganese sulphate	2.8g.
Potassium iodide	0.1g.
Zinc carbonate	0.1g.
Cobalt chloride $6H_2O$	0.2g.
Sodium fluoride	0.002g.

TABLE 6.

Table showing diets fed per day to the three dietary groups of rats.

Time	Diet	Dietary group H.P.F.	H.P.	L.P.
9 a.m.	V.M.R.	1.0g.	1.0g.	1.0g.
	Glucose	3.8g.	3.8g.	3.8g.
2 p.m.	High protein	4.2g.	4.2g.	-
	Protein free	-	-	4.2g.

H.P.F. = High protein group, fed 2g. casein 2 hours before killing.

H.P. = High protein group, fasted 18 hours before killing.

L.P. = Low protein group, fasted 18 hours before killing.

radioisotopes were being used, the isotope was injected intraperitoneally one hour after feeding the H.P.F. group, and the rats were killed at varying time intervals after the injection. It was noticed that the stomachs of the H.P.F. group contained food at all these time intervals, so that in their case, a continuous absorption of amino acids had occurred during the labelling period.

Isotopes used.

Either 8-^{14}C-adenine sulphate or ^{32}P-orthophosphate in sterilised saline at pH 7 was used. Both were obtained from the Radiochemical Centre, Amersham. ^{14}C-adenine was used in experiments on salt extracts of nuclei or nuclear RNA. For these experiments, 5 µc were given per rat. ^{32}P-orthophosphate was used during the experiments with sucrose density gradients, when 100 to 700µc ^{32}P was given per animal.

Estimation of RNA-P, DNA-P, and protein.

RNA-P and DNA-P were separated and estimated by the method of Fleck and Munro (1962). The RNA-P content of an alkaline digest was found by measuring the optical density at 260mµ of the acidified digest in 0.1N PCA. (O.D. of 1.000=3.412µg RNA-P.). DNA-P estimations were performed on the acid-precipitated material from the alkaline digest. The DNA precipitate was dissolved in 0.1N KOH and then diluted to a suitable volume (containing about 2-3µg DNA-P per ml). Duplicate 2ml. aliquots of this solution were then taken and the DNA-P estimated using the method of Ceriotti (1952).

Protein was estimated by the method of Lowry et al., (1951), using a solution of 100µg/ml bovine serum albumin as standard.

Radioactivity measurements.

RNA was counted by pipetting aliquots of the RNA solution (either in 0.1N KOH or in sucrose buffer) on to lens paper

discs in stainless steel planchets, and counting for 200, 500 or 1000 counts in a Nuclear-Chicago gas-flow counter with an automatic sample changer.

2. Methods for isolating rat liver cell nuclei.

As Chauveau, Moule, and Rouiller (1956), have pointed out, any attempt to isolate a component of a cell should ideally satisfy the following conditions: (a), preservation of the morphology and biochemical composition of the fraction, (b), purity of the fraction in that it is free from contamination by other cell particles, and (c), a sufficiently high yield to make the method workable, although as Roodyn (1963) has pointed out, it is difficult to reconcile purity of the preparation with a very high yield. As it was necessary to be able to prepare nuclei in as pure a state as possible, several of the many published methods were critically examined. These are: (i), isolation in 0.25M sucrose-0.002M $CaCl_2$, (ii), isolation in glycine-HCl, (iii), the use of Tween 80, and (iv), isolation through 2.2M sucrose.

(i). Isolation in 0.25M sucrose-0.002M $CaCl_2$.

This method was developed by Allfrey, Mirsky and Osawa (1955) for isolating calf thymus nuclei. They homogenised calf thymus in 0.25M sucrose-0.0018M $CaCl_2$, and by repeatedly spinning the nuclei down through 0.34M sucrose, they obtained a preparation which was almost free from cytoplasm. Attempts were made to isolate rat liver nuclei by this method.

Rats were anaesthetised with ether and their livers perfused with ice-cold 0.25M sucrose-0.002M $CaCl_2$, to remove most of the blood. The livers were then excised, blotted, weighed, and homogenised in 10 volumes of 0.25M sucrose-0.002M $CaCl_2$, filtered through nylon gauze, and spun for 5

minutes at 100g to remove fibrous material. The supernatant was then layered over 0.34M sucrose-0.002M $CaCl_2$, and spun for 10 minutes at 600g. The supernatant was discarded, and the precipitate of crude nuclei was resuspended in 0.25M sucrose-0.002M $CaCl_2$ relayered over 0.34M sucrose-0.002M $CaCl_2$, and spun for 5 minutes at 1200 r.p.m. This resuspension and relayering was repeated four to six times, or until no improvement in the purity of the nuclei could be observed under the light microscope.

(ii). Glycine-HCl.

This is essentially a modification of the widely used citric acid method of Dounce (1955). Rat livers were perfused *in situ* with ice-cold 0.05M glycine-HCl buffer at pH 3, containing 0.02M $CaCl_2$ and 0.01M $MgCl_2$ (Paul, 1962), homogenised in ice-cold buffer, and filtered through nylon gauze. The homogenate was spun at 1500g for 10 minutes, the red supernatant discarded, and the pale nuclear pellet washed by resuspending in 10 volumes of buffer and spinning for 5 minutes at 600g. The nuclei were resuspended in 10 volumes of buffer and spun at 250g for 5 minutes; this 250g spin was repeated until the supernatant was clear (three times).

(iii). Use of Tween 80.

Since the nuclei prepared by the methods described above appeared grossly contaminated when examined under the light microscope, a modification of the Tween 80 method of Fisher and Harris (1962) was used in an attempt to solubilise with Tween 80 the cytoplasmic fragments which contaminated the preparation. Nuclei were isolated by the method of Allfrey, Mirsky and Osawa (1955), but 0.1% Tween 80 was added to the sucrose solutions. However, a very high degree of contamination was observed, so this method was rejected.

Tween 80 also tended to rupture the nuclei, causing them to clump.

(iv). Isolation in 2.2M sucrose.

The use of 2.2M sucrose to separate the less dense whole cells and cytoplasm from the denser nuclei has been described by Chauveau, Moule, and Rouiller, (1956), subsequent modifications being introduced by Wilczok and Chorazy (1960), and Zbarskii and Georgiev, (1959). At first, the more recent method of Wilczok and Chorazy (1960) for isolating rat liver nuclei was followed. This employs a discontinuous sucrose density gradient of rat liver homogenate in 0.25M sucrose-0.002M $CaCl_2$ over 2.2M sucrose. The nuclei are purified by sedimenting them through the dense sucrose.

Crude nuclei were prepared from perfused rat livers by homogenising the livers in 0.25M sucrose-0.002M $CaCl_2$, removing the fibrous material, and spinning through 0.34M sucrose-0.002M $CaCl_2$ as before. The sediment of crude nuclei was then resuspended in one volume of 0.25M sucrose-0.002M $CaCl_2$, layered over 2.2M sucrose, and spun at 30,000g for 30 minutes. The last centrifugation was done in an MSE superspeed head. It was necessary to keep the diameter of the tube less than 15mm and the height of the sucrose in the tube at 16mm. The nuclei were recovered as a light yellow pellet at the bottom of the tube. Although pure nuclei were obtained by this method, it was suitable for only very small quantities of nuclei, since the yield was very low. The method was therefore rejected, and the original method of Chauveau et al was followed.

Rat livers were homogenised in 20 volumes of 2.2M sucrose and spun for an hour at 30,000g in the Spinco Model L ultracentrifuge. A pale sediment of nuclei was

precipitated on to the outer wall of the centrifuge tube; these nuclei appeared to be free from any contamination, apart from some fibrous material, when viewed under the light microscope.

The method described by Dingman and Sporn (1962), using 2.39M sucrose containing ATP was also investigated; this gave good nuclei but took longer to execute due to the considerable number of washings of the crude nuclear pellet.

Method finally used.

Since the method described by Chauveau et al proved to be by far the best as far as ease of preparation and purity of the nuclei were concerned, the general pattern of this procedure was adopted. However, the homogenisation of the rat liver in 2.2M sucrose was very difficult to execute, and tended to rupture the nuclei due to the high pressure required to force the homogeniser pestle through the sucrose: this also produced a significant amount of local frictional heat. The following modified procedure was therefore used. Perfused rat liver was homogenised in 10 volumes of 0.25M sucrose-0.002M $CaCl_2$-0.001M $MgCl_2$. $MgCl_2$ was introduced since its presence seems to prevent clumping of the nuclei (Philpot and Stanier, 1956). The homogenate was spun at 100g for 5 minutes, the supernatant layered on to 0.34M sucrose-0.002M $CaCl_2$-0.001M $MgCl_2$ and spun at 600g for 10 minutes. The crude nuclear pellet was resuspended in 0.25M sucrose-0.002M $CaCl_2$-0.001M $MgCl_2$ so that the total volume of nuclei and sucrose combined did not exceed one volume. 19 volumes of 2.51M sucrose-0.002M $CaCl_2$-0.001M $MgCl_2$ were then added to give an overall sucrose concentration of 2.2M (density 1.273), and the homogenate was spun for 1 hour at 30,000g . The nuclei were recovered as a pale smear on the outer tube wall; this smear was gently rinsed several times in either ice-

cold distilled water, or Na acetate-NaCl buffer when sucrose density gradients were to be run, to remove the excess sucrose. The nuclei were harvested by carefully loosening the nuclear pellet from the tube wall with a nickel spatula, and then resuspending in either 0.25M sucrose or Na acetate buffer, before finally precipitating the pure nuclei.

Yields and purity.

The yield obtained by each method was estimated by measuring the DNA content of nuclei recovered from a known volume of liver homogenate. Purity was evaluated by (a) light microscopy, (b) electron microscopy, and (c), RNA/DNA ratios of nuclear preparations.

3. Salt-fractionation of Rat Liver Nuclei.

Nuclei from the liversof dieted rats were prepared from 2.2M sucrose-0.002M $CaCl_2$-0.001M $MgCl_2$ by the method previously described, washed, and resuspended in 0.25M sucrose-0.002M $CaCl_2$-0.001M $MgCl_2$. Duplicate 2ml aliquots of the nuclear suspension, containing 100-300µg DNA-P per ml, were used for determining the RNA and DNA content of whole nuclei, and duplicate 4ml aliquots of the same nuclear suspension were taken for the extraction procedure.

Cytoplasmic fractions.

Duplicate cytoplasmic aliquots of 1ml for the livers of each dietary group of rats were taken from the supernatant fraction obtained after the 0.34M sucrose spin during the preparation of the nuclei (see flow sheet). This supernatant fraction contains the general cytoplasmic components of microsomes, mitochondria, and cell sap. Specific activity determinations on the RNA of these cytoplasmic fractions were carried out, as for the nuclear fractions.

Extraction procedure for nuclei.

Extraction procedure for nuclei.

Nuclei were sedimented from the 0.25M sucrose suspension of pure nuclei by spinning at 1000g for 10 minutes, and the fractionation scheme described by Allfrey, Mirsky and Osawa (1957) and of Logan (1957), for calf thymus nuclei, was followed for each dietary type, as described in the flow sheet.

Nuclei were extracted by stirring them for 5 minutes with 1ml ice-cold 0.1M KH_2PO_4/K_2HPO_4 buffer (pH 7.1) and then spun at 1000g for 15 minutes. This procedure was repeated using a) 1ml and b) 0.5ml of the phosphate buffer, and pooling the extracts. This gave the "phosphate extract" of the nuclei. The residue was then stirred for 10 minutes with 1ml ice-cold MNaCl and spun at 2000g for 20 minutes. This was repeated using a) 1ml and b) 0.5ml MNaCl and pooling the extracts. This gave two further fractions, the "sodium chloride extract" and the "nucleolar residue".

The whole nuclei, the three nuclear fractions, and the cytoplasmic fractions, for each dietary group of rats, were then precipitated with ice-cold PCA to a final concentration of 0.2N PCA, and the precipitate washed twice with ice-cold 0.2N PCA. Any ^{14}C-adenine contaminating the radioactive RNA was diluted out by cold adenine which was dissolved at a concentration of 3mg/ml in the PCA used for the first precipitation. All precipitated fractions were then incubated in 2ml of 0.3N KOH for 1 hour at 37°C to release the ribonucleotides (Fleck and Munro, 1962). After incubation, 1ml aliquots of 1N PCA were added to each of the digestion mixtures, the precipitates of protein and DNA centrifuged down, and washed twice with 0.5ml 0.2N PCA for each fraction, the acidified supernatant and washings, containing the RNA digestion products, were pooled and the

volume noted, and then samples were withdrawn for determination of the RNA-P content. 2N KOH was then added to the remaining RNA solutions to give a final concentration of 0.1N KOH. The potassium perchlorate was centrifuged down, and 0.5ml aliquots from each RNA sample were plated for the determination of ^{14}C-activity. The precipitate from the acidified KOH digest (protein and DNA) was dissolved on 0.1N KOH as described under "Estimation of DNA-P" for the determination of the DNA-P content.

4. Salt extraction of nuclear phenol RNA.

Results of the previous series of experiments on salt extracts of rat liver nuclei showed that, after precipitation with 0.2N PCA, a consistent loss of RNA and counts occurred (Table 27 in Results section). The RNA lost was between 3% and 7% of the total nuclear RNA, whilst the counts lost amounted to 30 - 40% of the total nuclear RNA. Moreover, the amount of unrecovered RNA varied significantly with diet.

As a result of these observations, a series of experiments was performed on nuclear RNA isolated and purified by the phenol method, to see if there was an acid-soluble RNA which was significantly affected by diet. We were also interested to find out if treatment with either of the neutral salt solutions used in the salt fractionation scheme of Allfrey et al (1955) rendered the nuclear RNA more soluble in acid. This would be more likely to occur with the M NaCl extraction which would tend to strip any protein off the nuclear RNA. Isolation and extraction of nuclear RNA were carried out simultaneously on the three dietary groups.

Isolation of Nuclear RNA.

(Unless otherwise stated, all operations were carried out at 0-2°C).

Nuclei were isolated from 2.2M sucrose-$CaCl_2$-$MgCl_2$ and rinsed carefully with acetate buffer (0.01M NaAc, 0.05M NaCl, 10^{-4}M $MgCl_2$, pH 5.25) to remove excess sucrose. They were suspended in acetate buffer and 0.5% "Dupanol" was added to a final concentration of 0.05%. This was to rupture the nuclear membrane and liberate the RNA into the aqueous phase (Hiatt, (1962) and Dingman and Sporn (1962). The suspension was shaken vigorously before adding an equal volume of 90% phenol. The mixture was shaken for an hour at 4°C, and the phases then separated by spinning at 30,000g for half an hour. This vigorous centrifugation precipitates any glycogen present as a pellet at the bottom of the phenol phase (Hall and Doty, 1958). The upper aqueous phase was pipetted off, and aqueous and phenol phases twice re-extracted with equal volumes of phenol and acetate buffer, respectively. The isolation of the RNA from each of the phases is described in the flow sheet.

Fractionation of the nuclear RNA.

The freeze-dried RNA was dissolved in distilled water to a concentration of 100-200µg RNA per ml, and eight 1ml aliquots of the RNA solution pipetted into centrifuge tubes. The eight samples were treated with either neutral salt solution, followed by 0.2N PCA, or 0.2N PCA alone, or else the sample was digested in 0.3N KOH for RNA estimation without any acid pre-treatment. The scheme is given in Table 7.

After precipitation with 0.2N PCA, the precipitates were centrifuged down, and the supernatants transferred to separate tubes. KOH was added to both precipitates and supernatants to a final concentration of 0.3N, and the samples were digested for 1 hour at 37°C. They were then chilled, made up to 2.5ml with distilled water, and 0.5ml samples withdrawn and plated out for counting.

Flow Sheet showing the isolation of nuclear RNA from phenol and aqueous phases.

NUCLEI

Shake with buffer, dupanol and phenol. Centrifuge. Repeat extraction, pool washings.

AQUEOUS LAYER.

(RNA in acetate buffer). Extract 5 times with equal volume of Et_2O. Blow off Et_2O with N_2.

Dialyse overnight against distilled water.

Freeze dry.

AQUEOUS RNA I.

PHENOL LAYER.

Wash with buffer and cold adenine (3mg/ml). Add 3 volumes ice-cold EtOH:Et_2O 3:1. Spin 1000g for 30 minutes; wash white ppt. with buffer and cold adenine. Add EtOH containing 2% cold K acetate. Wash with EtOH; EtOH:Et_2O 3:1; Et_2O. Boil 13 minutes in 10% NaCl at pH 7. Repeat twice. Dialyse pooled NaCl supernatant against distilled water overnight.

Freeze dry.

PHENOL RNA II.

TABLE 7.

Table showing scheme for testing effects of buffers used in salt fractionation on the degree of sensitisation of nuclear RNA to treatment with ice-cold 0.2N PCA.

Phase	Fraction number	Treatment
Phenol	1	None
	2	Neutral 0.1M Phosphate buffer + 0.2N PCA.
	3	M NaCl + 0.2N PCA.
	4	0.2N PCA.
Aqueous	5	None
	6	Neutral 0.1M Phosphate buffer + 0.2N PCA.
	7	M NaCl + 0.2N PCA.
	8	0.2N PCA.

The rest of the alkaline digest was acidified with HCl to 0.1N, any DNA and protein precipitating out was spun off, and the optical density of the supernatant was read at 260mμ in order to determine the RNA-P content. Protein contamination was checked by reading the O.D. at 240 and 280mμ in some samples.

Methods for preparing nucleoli.

Following the experiments on the incorporation of ^{14}C-adenine into nuclear subfractions of rat liver, and the experiments with the nucleolar antibody, attempts were made to isolate pure nucleoli from rat liver nuclei, in order to study their role in the nucleus during protein feeding and withdrawal. A complete account of the methods tried, with the results obtained, together with a review of the work done by others in this field, is presented here.

The three major problems to be overcome before a convincing method of isolating pure nucleoli can be elaborated are 1) the lack of a specific marker for identifying nucleoli; 2) the difficulty of lysing the nuclear membrane and dispersing the intranuclear contents without damaging the nucleoli; and 3) the separation of the nucleoli from the rest of the nuclear lysate: whole nuclei would probably prove to be a major source of contamination.

1. Identification of the nucleoli.

Both light and electron microscopy have been used in the identification of nucleoli. The identifying characteristics have been nucleolar size and shape (Maggio, Siekevitz and Palade, 1963) refractivity (Vincent, 1952; Maggio, Siekevitz and Palade, 1963), and the reactions of nucleoli with a number of histochemical stains, all of which give coloured products with RNA or DNA or both. For example, methyl green-pyronin has been used by Rees, Rowland and Varcoe (1963), for rat liver nucleoli, and methyl green-pyronin and Feulgen staining has been used by Monty, Kay and Dounce (1956), for rat liver and by Poort (1961) for beef pancreatic nucleoli. Fluorescence in U.V. light after staining with acridine orange was used by Morton (1961) with mouse liver and plant nucleoli, and azure C staining was used by Busch and

co-workers (Busch et al, 1963; Muramatsu et al, 1963), with rat liver and Walker carcinoma nucleoli. Maggio, Siekevitz and Palade (1963), identified nucleoli under the electron microscope by uranyl acetate staining followed by lead hydroxide.

Of the histochemical stains used in light microscopy, methyl green-pyronin is probably the most useful, since the RNA in the nucleolus stains pink with the pyronin, whilst the DNA in the surrounding nuclear chromatin stains green with the methyl green. However, experiments with the fluorescent nucleolar antigen of Beck, both in this laboratory, and in collaboration with Dr. Beck, (p.71), suggest that the technique of staining the nucleolus with an immunologically specific marker could be very profitably exploited as an indicator for nucleoli during attempts at their isolation. This technique was therefore used during these experiments as a final means of identifying possible preparations of nucleoli. Preliminary examinations of the nucleoli were on preparations stained with Pappenheimer's stain and viewed under the light microscopes.

2. Lysis of the nuclear membrane.

Complete removal of the nuclear membrane so that the intra-nuclear contents can be dispersed without damaging the nucleoli has proved to be very difficult to achieve. Several approaches to this problem have been tried. These are a), direct dissection, b) use of hypotonic media, c) high-speed homogenisation, d) rupture of the nucleus under pressure, e) effect of calcium and magnesium ions, f) effects of pH and ionic strength, g) use of detergents, h) use of trypsin, and i) ultrasonic disintegration.

a) Direct dissection;

Edström and co-workers have reported isolation of the

nucleoli from the giant cells in the salivary glands of Chironomids (Edström and Beerman, 1962), and oocytes of Triturus species (Edström and Gall, 1963), by direct microdissection. These cells are exceptional types, however, and such a method would not be generally practicable.

b). Use of hypotonic media.

The use of hypotonic media for the lysis of nuclei does not appear to be widespread, although this technique is routinely used in lysing red blood cells (see Ponder, 1948). Weiss (1960), however, found that rat liver nuclei isolated in sucrose-$MgCl_2$ were lysed after being allowed to stand for 10 minutes at $0^{o}C$ in 20 volumes of this buffer at pH 7.4.

During experiments in this laboratory, rat liver nuclei isolated in 2.2M sucrose were rinsed thoroughly with ice-cold distilled water to remove excess sucrose, and then suspended in 5-10 volumes of either distilled water or weak buffer with or without Ca^{2+} or Mg^{2+} ions. The nuclei were allowed to stand for 20 minutes at $0^{o}C$, before a small volume was taken and examined under the light microscope. A drop of crystal violet was added to the suspension to aid in identifying the nuclei. Following the 20 minutes standing, the nuclear suspensions were frozen rapidly in methanol-solid CO_2, and thawed; this process was repeated about five times, and the nuclei were then examined microscopically.

The results are given in Table 8, where it will be apparent that nuclei do not lyse in hypotonic media, whether calcium or magnesium is present or not; the small percentage of lysed nuclei observed after extensive freezing and thawing was felt to be unsatisfactory, and the method was therefore rejected.

c). High-speed homogenisation.

High-speed homogenisation as a method of breaking nuclei has been used in several laboratories. Norton (1961) disrupted

TABLE 8.

Table showing effects of hypotonic media on the integrity of isolated rat liver cell nuclei.

Hypotonic solution	Appearance of nuclei after 20 minutes suspension	Appearance of nuclei after 5 times freezing and thawing
Distilled water	Less than 5% broken nuclei.	About 30% broken nuclei. Some nuclear "ghosts". Very few nucleoli.
Distilled water and 0.002M Ca^{2+}	Very few broken nuclei.	30% broken nuclei but very few nucleoli.
Distilled water and 0.001M $MgCl_2$	Same as above	Same as above but nuclei frequently mis-shapen.
1% EDTA pH 7.1	Very shrunken, mis-shapen nuclei but little or no lysis.	Nuclear "ghosts", about 30% broken nuclei. Very few nucleoli.
Distilled water + 0.002M $CaCl_2$ + 0.001M $MgCl_2$.	Same as for Ca^{2+} and Mg^{2+} alone.	Same as for Ca^{2+} or Mg^{2+} alone.
10^{-4}M phosphate buffer pH 7.1.	Less than 1% lysis.	Some nuclear lysis. Few nucleoli.
10^{-4}M phosphate buffer pH 7.1 + 0.002M $CaCl_2$ + 0.001M $MgCl_2$.	No lysis.	Mis-shapen nuclei. Some nucleoli. Not more than 30-40% lysis at most.

mouse liver nuclei in 0.4M sucrose in the presence of Mg^{2+} using a very high-speed blendor. He found that the disruption of the nuclei required a fairly high concentration of nuclei and very high speeds of homogenisation. Finamore (1961), also found that homogenisation was critical during attempts to isolate nucleoli from amphibian oocytes, and Poort (1961) used a high-speed homogeniser in a dense medium of 70% glycerophosphate for preparing beef pancreas nucleoli. High-speed homogenisation has also been used in conjunction with small glass beads (Ballotini beads) by Baltus (1954) to shatter whole starfish oocytes and liberate the nucleoli. In this laboratory, high-speed homogenisation with both the Potter-Elvehjem type of homogeniser, and the Nelco blendor was tried; a few experiments using glass beads were also carried out. The nuclei were homogenised in both isotonic and hypotonic solutions in both the Potter-Elvehjem homogeniser and the Nelco blendor, at top speeds for time intervals up to 20 minutes, and were then examined microscopically. The volume of suspending medium to nuclei was about 2:1. With the Ballotini bead treatment, beads equivalent in volume to the nuclei were added, and top speed blending in the Nelco blendor was continued for intervals of up to 20 minutes.

Table 9 shows the results of these experiments, from which one may conclude that the Potter-Elvehjem homogeniser is too gentle, whilst the Nelco blendor shatters both nuclei and nucleoli. This method of blending did not show an optimal time at which the largest number of nuclei would be broken and the fewest number of nucleoli would be damaged. Damage to both occurred concurrently. Homogenisation in all its forms was therefore rejected as an unsatisfactory method.

TABLE 9.

Table showing effect of high-speed homogenisation of rat liver nuclei with either the Potter-Elvehjem (P-E.) homogeniser or the Nelco blendor.

Suspending medium	Appearance of nuclei after 20 minutes P-E. homogenisation.	Appearance of nuclei after 20 minutes Nelco blending.
0.25M sucrose + 0.002M $CaCl_2$	About 10-20% nuclei observed were broken.	About 60-70% nuclei broken. Nucleoli disintegrating.
Distilled water.	10-20% nuclei were broken. Nucleoplasm did not separate from nucleoli.	Nuclei broken into pieces, but nucleoplasm adhering to disintegrating nucleoli.
Distilled water + 0.002M $CaCl_2$.	Same as for distilled water.	Same as for distilled water.
10^{-4}M phosphate buffer pH 7.1.	Nuclear "ghosts" or nuclei with ruptured membranes seen; nucleoli not liberated.	Nuclei "sliced". About 60-70% microscopic field appeared to be broken nuclei. Nucleoli not often liberated.
0.25M sucrose + 0.002M $CaCl_2$ + Ballotini beads	-	Nuclei and nucleoli shattered. Nucleoplasm adhering to nuclear pieces.

d). Rupture of the nucleus under pressure.

In one of the earliest attempts at isolating cell nucleoli, made by Vincent in 1952, starfish oocyte nuclei were ruptured by forcing the oocyte homogenate under high pressure through a fine hypodermic needle. The liberated nucleoli were then purified. Poort (1961) has also reported the isolation of nucleoli from beef pancreas after rupturing the nuclei using a specially designed press, and Busch *et al* (1963) obtained a 50-70% yield of rat liver nucleoli after rupturing the nuclei under pressures of 5000-8000lbs/sq. inch in a French press.

We did not attempt to rupture nuclei by these means during the course of this work.

e). Effects of calcium and magnesium.

The effect of calcium and magnesium on the behaviour of isolated nuclei in general, and particularly in connection with the isolation of nucleoli seems far from clear. Busch and co-workers (Muramatsu *et al*, 1963) state that a concentration of 0.0033M calcium is optimal for the isolation of nucleoli from rat liver and Walker carcinoma; at concentrations below 0.0026M calcium, the nuclei remain unbroken and the nucleoli are broken, and at concentrations above 0.005M the nuclei become very resistant to sonication. In agreement with Dounce (1955) and Hogeboom and Schneider (1952), Maggio, Siekevitz and Palade (1963) found that citrate-EDTA buffer lysed the nuclei almost completely, but the nucleolus and chromosomes formed a gel which was difficult to disperse, while in saline-EDTA the liberated nucleoli swelled and burst. Philpot and Stanier (1956) also emphasise that the integrity of the liver cell nucleolus in the isolated nucleus requires the presence of calcium.

On the other hand, studies by Leeson and Kalant (1961)

TABLE 10.

Table showing effect of presence or absence of added calcium and/or magnesium on isolated rat liver nuclei.

Suspension medium.	Microscopic appearance.	After 20 minutes P-E. homogenisation.	Addition of 0.05% Sodium lauryl sulphate.
0.25M sucrose or 0.25M sucrose + 0.002M $CaCl_2$ + 0.001M $MgCl_2$.	Nuclei are spherical and appear normal. Very few broken nuclei.	About 10-20% nuclei were broken, a few nucleoli visible.	A few nuclei in clumps, and unrecognisable; others unaffected. Free nucleoli not recognisable.
10^{-4}M. phosphate buffer pH 7.1	No lysis	Nuclear "ghosts" or nuclei with membranes ruptured but contents not extruded. Nucleoli surrounded with nucleoplasm.	Nuclei clumped; no free nucleoli visible
10^{-4}M phosphate buffer pH 7.1 + 0.001M $MgCl_2$ + 0.002M $CaCl_2$	Less than 10% lysis.		Nuclei clumped; no free nucleoli visible.
1% EDTA.	Nuclei very shrunken and do not take up stain very well.	-	-
M phosphate buffer, pH 6-7.	Nuclei fairly well broken, some nucleoli.	-	-
M phosphate buffer + 5% Na_3 citrate	Nuclei 90% lysed. Many nucleoli, no clumps.	-	-

showed that when rat livers were perfused in situ with 0.5% EDTA, "blebs" were seen on the nuclear membrane and the cells themselves separated, but the cell organelles appeared normal. Rees, Rowland and Varcoe (1963) report the isolation of viable rat liver nucleoli in a medium free from calcium, and calcium was also omitted from the final medium elaborated by Maggio, Siekevitz and Palade (1963). Johnson, Setterfield and Stern (1959) and Rho and Bonner (1961) break isolated pea nuclei by removing the calcium with citrate, and isolate the nucleoli in dense sucrose. A brief survey of the literature shows that many methods of isolating nucleoli do not include calcium in the isolation medium: (Vincent, 1952; Baltus, 1954; Litt et al.,1952; Monty et al. 1956; Finamore, 1961; and Poort, 1961).

During the present series of experiments attempts were made to lyse nuclei using media with and without calcium. The results are given in other tables, but are summarized collectively in Table 10. From these results it will be seen that the absence of added calcium or magnesium does not appear to cause lysis of the nuclei, but removal of any remaining calcium by addition of EDTA causes some intranuclear disintegration.

f). Effects of pH and ionic strength.

The effect of increasing pH and ionic strength on the intact liver cell nucleus has been described in detail by Philpot and Stanier (1956). Maggio, Siekevitz and Palade (1963), studied the effect of increasing pH on the rupture of guinea pig liver nuclei in 0.88M sucrose, and found that at pH 7.5-8.0, only occasional nuclei remained intact; the freed nucleoli retained their size and refractivity, and the nucleoplasm was finely dispersed and did not agglutinate even after prolonged storage. Finamore (1961), working with amphibian oocyte nucleoli, found that the pH of the

TABLE 11.

Table showing effect of pH and ionic strength on lysing of isolated rat liver nuclei.

Buffer used	pH			
	5.4	6.6	7.0	9.5
0.1M PO_4	Very little lysis	Unruptured	Unruptured	Nuclei appear shrunken.
M PO_4	About 20% breakage	90% ruptured. Many nucleoli visible, no clumping.	90% ruptured. Nucleoli visible, no clumping.	-
M PO_4 + 5% Na_3 citrate	20% breakage.	90% ruptured, no clumping, many nucleoli visible.	90% ruptured, many free nucleoli, no clumping.	-

isolation medium drastically affected the purity of the preparation.

Maggio and co-workers (1963) also found that, when they added 0.1M phosphate buffer, (pH 7.1), to the sucrose medium, there was a marked increase in the numbers of broken nuclei. In the present work, the effect of varying the pH from 5.4 to 9.5 was studied, using both 0.1M and 1.0M phosphate buffer. The effect of citrate-M phosphate buffer was also investigated. Nuclei were prepared from 2.2M sucrose, rinsed thoroughly with distilled water, and then shaken with about 5 volumes of ice-cold buffer before being examined microscopically. The results are shown in Table 11.

Although the use of M phosphate buffer at pH 6 and 7 appeared promising, the nuclei did not lyse to the same extent when the experiment was repeated, and the method was rejected on the grounds that it was not reproducible.

g). Detergents as agents for solubilising the nuclear membrane.

The action of detergents in solubilising lipid-containing material is well-known, and the effects of surfactants on cell membranes has been widely described. Palmer, Hodes and Warren (1961) studied the action of synthetic detergents on tumour cell membranes, including the nuclear membrane, with anionic (sodium lauryl sulphate), cationic (lauryl pyridinium chloride) and non-ionic ("Igepal-DN-710") detergents. They found that the anionic detergent lysed both the nuclear and cell membranes, although the cell membrane was more easily broken, whilst the non-ionic detergent caused cytolysis only, and the cationic detergent induced "blebbing" of the cell membrane but did not lyse either the nuclear membrane or the cytomembrane. The effectiveness of sodium lauryl sulphate as a lysogenic agent was also observed by Ponder (1946), who found that of the

sulphated straight chain alcohols, the C_{16} and C_{14} alkyl sulphates were the most powerful haemolytic agents. Amongst the many other detergents used, deoxycholate has been extensively employed for a number of years as a reagent for solubilising the endoplasmic reticulum to release the ribosomes (Palade and Siekovitz, 1956; Littlefield, Zamecnik and Keller, 1957), and more recently for preparing polysomes from rat liver without damaging the polysome-attached messenger RNA. (Wettstein, Staehelin and Noll, 1963). Rendi (1960) used a mixture of deoxycholate and Lubrol to prepare a "DOC-soluble" fraction from plant nuclei isolated in 0.25M sucrose, but no intranuclear structures appeared to remain at the concentration of detergent used during his experiments.

In the present series of experiments, four detergents were used in an attempt to lyse the nuclear membrane selectively. These were the two anionic detergents sodium lauryl sulphate and sodium deoxycholate, and two non-ionic detergents, Lubrol W and Tween 80. Tween 80 has been used as cytolytic agent by Fisher and Harris (1962), but previous experiments during the course of this work indicated that this detergent might prove a useful nuclear lysin. (p.27)

Nuclei isolated in 2.2M sucrose were rinsed with distilled water and suspended in either 0.25M sucrose or 10^{-4}M phosphate buffer pH 7.1. The detergents were added so that their final concentrations were either 0.05% or 1%. Calcium ions were either included or omitted from the medium. The results are given in Table 12, from which it was concluded that, although sodium lauryl sulphate, Lubrol W and sodium deoxycholate were efficient lysins, the irreversible clumping of the nuclei showed that these agents were unsuitable as a means of liberating nucleoli.

TABLE 12.

Table showing effect of 0.05% and 0.1% detergent on isolated rat liver nuclei in 0.25M sucrose or 10^{-4}M phosphate buffer.

Detergent used	Microscopic appearance of nuclei at detergent concentration.	
	0.05%	0.1%
Sodium lauryl sulphate in sucrose	Nuclei in clumps, others unaffected. About 30% clumped.	About 50-60% nuclei clumped; could not be separated by homogenisation. Others occasionally lysed. Few free nucleoli.
Sodium lauryl sulphate in sucrose-$CaCl_2$	Same as above.	Same as above.
SLS* + 10^{-4}M PO4 buffer pH 7.1	A few "ghosts" around outside of large nuclear clumps.	Irreversible clumping. Little or no signs of nucleoli.
SLS in 10^{-4}M phosphate + 0.002M $CaCl_2$	Same as without calcium.	Same as without calcium.
Sodium deoxycholate in 0.25M sucrose	30% nuclei clumping.	Clumping. (about 70%).
NaDOC** in sucrose - 0.002M $CaCl_2$	Same as without Ca^{2+}.	Same as without calcium.
Lubrol W + sucrose ± 0.002M $CaCl_2$	Complete solubilization.	Solubilization of all nuclear particles.
Tween 80-sucrose ± 0.002M $CaCl_2$	No lysis.	Small amount (c.10%) of lysis.

* SLS = sodium lauryl sulphate
** NaDOC = sodium deoxycholate

h). Trypsin.

Trypsin is routinely used in tissue culture techniques for separating the cells of a piece of tissue by selectively digesting the connective tissue between the cells. The cell walls are apparently left undamaged during this procedure. A few experiments of trypsinising nuclei were therefore carried out to see if a similar selective digestion technique would be applicable at the nuclear membrane level, such that the membrane would be digested away whilst the intranuclear material would be left unaffected.

Nuclei were digested for 1 minute at 37°C with varying concentrations of trypsin in 10^{-4} M phosphate buffer (pH 7.1). The results are shown in Table 13.

Trypsin at 0.005% concentration was then incubated with rat liver nuclei for varying times under the same conditions of pH. The results are shown in Table 14.

From these two simple experiments, it was concluded that, for nuclei, trypsin was obviously highly unsuitable, since the nuclei clumped as soon as even partial digestion of the membrane occurred. This clumping was irreversible.

i). Ultrasonic oscillation techniques.

Disintegration in an ultrasonic oscillator is probably the most widely used technique for rupturing nuclei. Reports on the time taken and conditions used to rupture the nuclei vary very widely, however. Muramatsu et al. (1963) found that 99.8% of rat liver or Walker carcinoma nuclei were disrupted in 25-35 seconds at 10K/cycles per second, but they stated that the calcium concentration and sonication volume were critical. Litt et al (1952) and Monty et al (1956), ruptured rat liver nuclei suspended at pH 6.2 in 7-7½ minutes at a frequency of 9K/cycles per second, and Rees, Rowland and Varcoe (1963) also report 7 minutes as the optimal time, but they were operating at a frequency of 25K/ cycles per second. The nuclei were not completely ruptured

TABLE 13.

Table showing efficiency of varying concentrations of trypsin on selective removal of the membrane of isolated rat liver cell nuclei. Time of reaction for each digest was one minute.

Trypsin concentration	Appearance of nuclei
0.5%	Complete digestion of nuclei. No nucleoli visible.
0.1%	Digestion of nuclei complete: no nucleoli.
0.05%	Incomplete digestion: nuclei clumping. No free nucleoli.
0.01%	Some nuclear clumps; some unidentifiable small, irregular bodies. No nucleoli.
0.005%	Very little clumping; few or no nucleoli.

TABLE 14.

Table showing effect of varying time of digestion of isolated rat liver cell nuclei with 0.005% of trypsin.

Incubation time	Appearance of nuclei
1 minute	Nuclei single, some membrane damage, little extrusion of contents; no clumping.
2 minutes	Nuclei forming into clumps. No nucleoli visible.
3 minutes	Large clumps of nuclei. Small particles visible; nuclear damage extensive.
4 minutes	Fewer clumps, small, unrecognisable particles.
5 minutes	Very few clumps, large number of unrecognisable small fragments.

at this time, however, and often times up to at least 20 minutes were necessary before 70% or more of the nuclei were broken (Rowland, 1962). Maggio, Siekevitz and Palade (1963), using 10K/cycles per second frequency, required 20-30 minutes oscillation to rupture 70% of guinea pig liver nuclei suspended in 0.88M sucrose at pH 7.8.

Experiments performed by us using ultrasonic oscillation indicated that 30-80% disintegration of rat liver nuclei occurred at times varying from 5 to 30 minutes, at a frequency of 10K/cycles per second. Often, nuclear disintegration did not start until 15 minutes of sonic disintegration. Nuclei were suspended in 0.25M sucrose containing 0.002M $CaCl_2$ and the suspension was placed in the cylinder of a "Raytheon" sonic oscillator with ice-cold water circulating in the jacket. The nuclei were subjected to 10K/cycles per second and examined microscopically at five minute intervals.

As indicated above, the extent of nuclear disintegration varied considerably, and often needed 20 minutes or more. When the nuclei did rupture, however, the nucleoli were easily recognisable as small, spherical refractile bodies, and attempts could be made to isolate them and test their antigenicity using the Beck antigen.

Conclusions on lysis of nuclear membrane.

The nuclear membrane appeared to be a much tougher proposition than one would predict. There are two possible reasons for this. The first is that the nuclear membrane may be damaged during the isolation procedure using sucrose. Ponder (1948) states that most sugars in isotonic solutions irreversibly inhibit haemolysis of red blood cells by bile salts or other lysins, and he suggests that this phenomenon "can be thought of as due to an injury on the cell surface". A similar, irreversible effect may operate with sucrose-

isolated nuclei. The second, more plausible proposition is that the nuclear membrane has a different composition from the cytomembrane. This was suggested by Palmer et al (1961), who proposed that the reason for the observed different behaviour of nuclear membranes and cytomembranes with detergents was that nuclear membranes contained more protein and less lipid. This would explain the comparative resistance to detergents, and also the comparative resistance of the cell membrane to trypsin digestion, and the high degree of sensitivity of the nuclear membrane to this treatment. Of the other methods attempted, sonic disintegration was probably the most efficient at liberating nucleoli from nuclei, but this was also highly variable. The variability might be a consequence of the denaturation and consequent precipitation of the membrane protein during the first minute or so of oscillation. A very profitable line of approach might be the use of phospholipase, which would selectively attack the nuclear membrane alone. Isolation of the nucleoli might then resolve on whether the nuclei would clump or not - this could be controlled by ionic strength and pH.

3. Isolation of the nucleoli from disrupted nuclei.

Since the nucleoli are the densest particles in the nucleus, the subsequent separation of the nucleoli from the rest of the nucleoplasm should not be particularly difficult. Rees, Rowland and Varcoe(1963) used low speed centrifugation for separating the nucleoli from the nuclear sonicate, and Maggio, Siekevitz and Palade (1963), used high density sucrose for separating the dense nucleoli from the less dense nucleoplasm. In the present work, both methods were used.

Low speed centrifugation.

The method followed was that of Rees, Rowland and

Varcoe (1963). Rat liver nuclei from 6 male albino rats were isolated in 2.2M sucrose, and suspended in 15-20ml sucrose - 0.002M $CaCl_2$. They were subjected to ultrasonic oscillation at 10K/cycles per second in a 60 watt Raytheon disintegrator for 20-25 minutes when about 50% of the nuclei were broken, and were then centrifuged at 2,100g for 20 minutes. The sediment was washed in distilled water and recentrifuged at 2,100g for 20 minutes. This process was repeated once. Microscopic examination showed the preparation to be grossly contaminated with whole nuclei. It was felt that, unless a method could be evolved for quantitatively lysing the nuclei, it would not be possible to remove nuclear contamination by this method.

2. High density centrifugation.

Nuclei were isolated from the livers of 6 male albino rats, and the procedure indicated in Fig. 2 was followed. Each fraction was examined microscopically with Pappenheimer's stain, and with the fluorescent nucleolar antibody. The results are given in Table 15.

Conclusions.

From the final experiments with the fluorescent nucleolar antibody, it would appear that once the nuclei have been lysed successfully and the nucleoli liberated, it should not be too difficult to elaborate a method for isolating nucleoli. Unfortunately, the methods available for lysing the nuclei did not prove reproducible, or satisfactory, and very little progress could be made.

It is possible, however, that the proposed method of digesting the nuclear membrane with a phospholipase may eliminate this variability in resistance to disintegration, and place the technique on a satisfactory basis. Until this can be achieved, there is no point in studying the biochemical features of isolated nucleoli, and this approach has therefore not been developed further.

Fig. 2. Flow Sheet giving details of isolation of rat liver nucleoli.

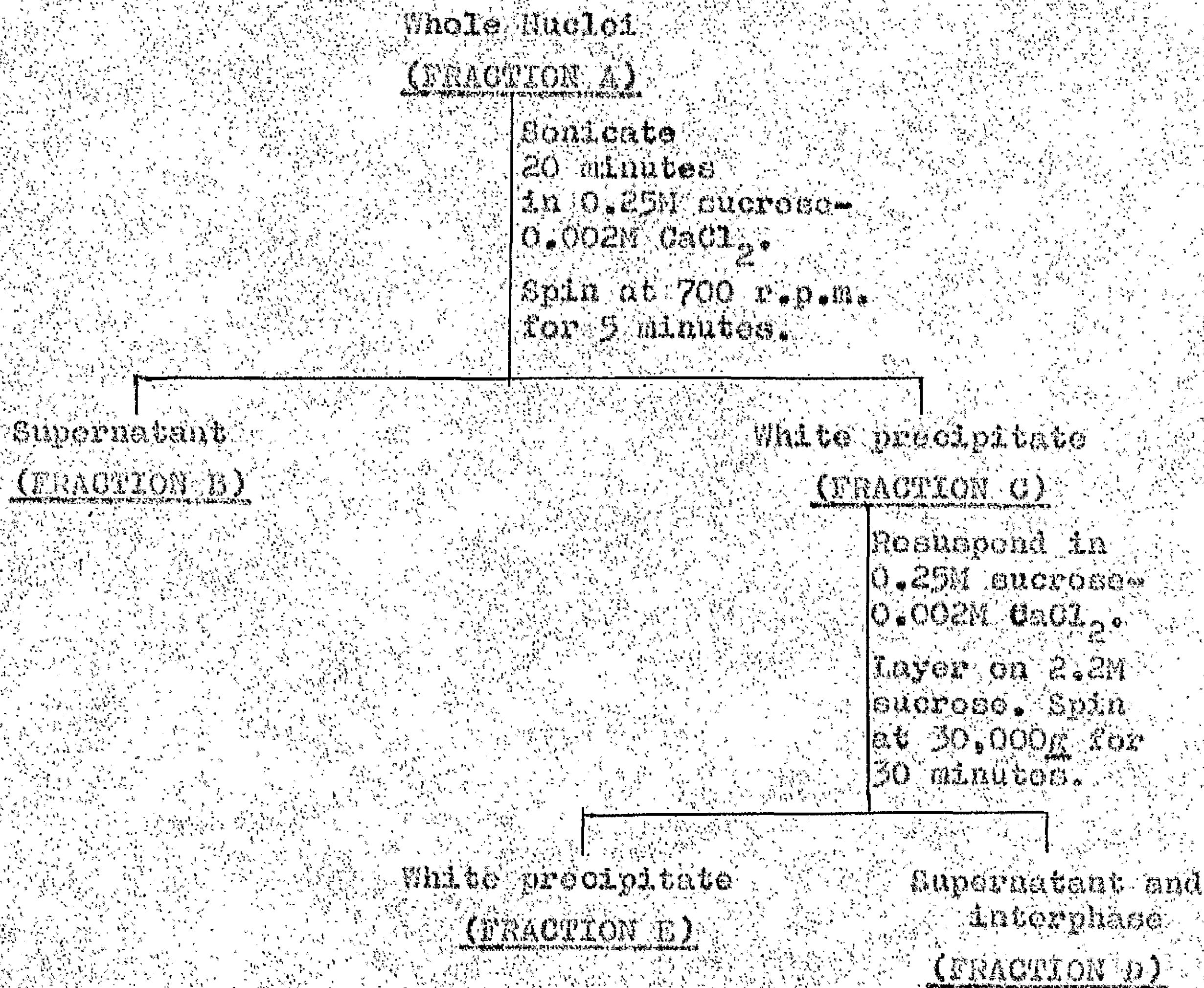


TABLE 15.

The reaction of subnuclear fractions obtained from sonicated nuclei with a) Pappenheimer's stain, and b) the fluorescent nucleolar antigen.

Fraction	Pappenheimer's stain	Fluorescent nucleolar antigen
A.	Blue, round nuclei, few broken.	Fluorescent nucleoli in nuclei.
B.	Irregular, bluish bodies.	No reaction.
C.	Pale, round bodies, some unidentifiable material.	Fluorescent round bodies with some non-fluorescing material.
D.	Irregular blue bodies.	Some fluorescing material.
E.	Palish, regularly shaped small spherical bodies.	Concentrated field of fluorescing bodies. Apparently pure nucleoli.

<u>Sedimentation Analysis of whole liver RNA and liver nuclear and cytoplasmic RNA, from dieted rats.</u>

<u>a) Separation on a sucrose density gradient.</u>

RNA from homogenates of whole liver, liver cytoplasm, and nuclei was isolated by various modifications of the phenol method of Kirby (1956), and was separated on either a 5-20% or 3-20% sucrose density gradient using the technique developed by Bolton <u>et al</u> (1959).

<u>Preparation of the RNA.</u>

In preliminary experiments with liver nuclear RNA, the RNA was isolated by the method of Reiner, Bain and Groth (1963). This method was originally adopted because the procedure incorporates a preliminary precipitation and extraction with organic solvents, to destroy RNase. The RNA is then extracted from the residue using sodium lauryl sulphate and 90% phenol.

Nuclei from the livers of male albino rats were isolated in 2.2M sucrose - 0.002M $CaCl_2$ - 0.001M $MgCl_2$, rinsed in ice-cold distilled water, and samples were withdrawn for RNA and DNA analysis. The nuclei were then dispersed in cold absolute ethanol, centrifuged down, and the precipitate redispersed in cold ethanol-ether 3:1 for 45 minutes at 4^oC, with occasional shaking. The extracted nuclei were spun down and drained in the cold for 15-20 minutes before being redispersed in 0.3% sodium lauryl sulphate in 0.05M tris buffer pH 6.9 (7-8 mls per mg RNA). The nuclei were shaken at 25^oC for 1½ - 2 hours to complete the extraction of RNA, an equal volume of 90% phenol was added and the mixture was stirred for a further 1½ hours. The phases were separated by centrifugation, the upper layer removed, and the phenol layer and interphase were washed once with tris buffer. The washings were added to the upper phase, and the RNA was precipitated from the

combined aqueous layers with 2 volumes of ethanol - 2% acetate. The RNA was reprecipitated, then redissolved in tris buffer and treated with 40 μg/ml DNase I in 0.005M $MgCl_2$ overnight in the cold. The RNA was again precipitated and finally dissolved in tris buffer to a concentration of 50 O.D. units per ml. A volume of 0.2ml of this solution (10 O.D. units) was layered on a linear sucrose density gradient of 3 - 20% sucrose buffered with 0.01M tris pH 7.3. The final yield of RNA by this method was 70%, with respect to both the optical density and counts recovered, and the final preparation contained less than 1% protein. In our hands, however, there were a number of disadvantages. The DNA contamination was of the order of 10%, and this was not removed by the DNase treatment in the cold. The method seemed unnecessarily lengthy and the final RNA precipitate was difficult to dissolve, probably due to the contaminating DNA. Finally, the RNA profile obtained from the gradient after centrifugation suggested that the RNA was degraded (see Fig. 16). It was felt that possibly some RNase might survive the ethanol treatment, in which case the RNA could easily be degraded during the long extraction at 37°C. A method was therefore devised in which this treatment was avoided.

Nuclei were isolated from livers which had been homogenised in sucrose containing a few drops of bentonite prepared by the method of Fraenkel-Conrat _et al_ (1961), to inhibit RNase. The nuclear pellet from the 2.2M sucrose spin was taken up in acetate buffer containing 0.05M NaCl, 0.01M Na acetate, 0.001M $MgCl_2$ and 0.5% naphthalene - 1:5 disulphonate (NDS), with a few drops of bentonite added. This was brought to pH 5.25 with N acetic acid. In experiments where whole cell RNA was studied, the homogenisation was done directly in the acetate buffer

described above. Cytoplasmic RNA was prepared from the supernatant left after spinning whole liver homogenate in 0.25M sucrose - 0.003M $MgCl_2$ at 1,000g for 10 minutes. Sodium lauryl sulphate was then added to all suspensions to a final concentration of 1.0% (see page 80), and the suspension shaken immediately. An equal volume of 90% phenol containing 0.1% 8-hydroxyquinoline was added to the opalescent suspension, the mixture shaken at 4°C for an hour, and then centrifuged at 30,000g for 30 minutes in the Spinco Model L Ultracentrifuge to separate the layers and precipitate DNA and glycogen (Hall and Doty, 1958). The aqueous layer was removed and bentonite added, the phenol layer washed once with acetate buffer, and the two aqueous layers were combined and extracted 3 - 5 times with an equal volume of ether to remove the phenol. The ether was blown off with nitrogen, and the RNA was precipitated with ethanol - 2% acetate. The RNA was resuspended in acetate buffer, with the pH adjusted to 6.8 - 7.0, and incubated for 20 minutes at 37°C with 40 µg/ml DNase I. The RNA solution was shaken with chloroform to precipitate the DNase, the layers separated by centrifugation, and the RNA reprecipitated with ethanol. The pellet was suspended in a small volume of acetate buffer pH 5.25 without the NDS and the suspension dialysed overnight against acetate buffer pH 5.25 without NDS. It was found that this treatment not only removed traces of ethanol, etc., but also the continuous agitation of the suspension during dialysis caused the RNA polymer to dissolve in a matter of hours. Nuclear RNA seemed to be more difficult to dissolve than cytoplasmic or whole cell RNA. The yield obtained by this method was 70% for both the RNA and the counts; the preparation contained less than 1% protein, and the high

DNA contamination brought about by the high detergent concentration used was reduced to 5% or less during dialysis. It was found that the yield of RNA in the aqueous phase, both of O.D. and of counts, fell to well below 90% when the concentration of SLS was below 0.2% (see p. 80 and Table 32).

<u>Preparation of sucrose density gradients.</u>

Linear gradients of 3-20% or 5-20% sucrose, buffered in tris at pH 7.3 or in acetate buffer pH 5.25 were used. The gradients were prepared by the method of Bolton et al (1959) from a device working on the principle of Bock and Ling (1954) for producing a smooth linear gradient.

Two types of gradient could be prepared: neither were really linear (see Fig. 3). The first type of gradient was S-shaped (Fig. 3a), and was prepared by keeping the outflow tube just beneath the surface of the sucrose as it rose up the tube. The gradient obtained was nearest to a linear gradient when the mixing in the tube directly connected to the outflow tube, and containing the dense sucrose, was very efficient. This was achieved by passing a fairly rapid thin stream of air through the tube. It was also necessary to maintain the flow of sucrose at a very slow rate, and to keep the outflow tube exit just below the surface of the sucrose gradient. The second type of gradient was more linear (Fig. 3b), and more easily reproducible. It was prepared by fixing the outflow tube at the side of the centrifuge tube, near the top, and allowing the successively less dense sucrose to flow down the side of the tube and layer on to the more dense sucrose. Again, the gradient formed closely approximated to linear when the mixing was efficient and the flow of sucrose very slow. Sucrose density gradients were made to

Fig. 3.

Sucrose density gradients obtained when a) the outflow tube is kept just under the surface of the sucrose and b) the sucrose is allowed to flow gently down the side of the tube.

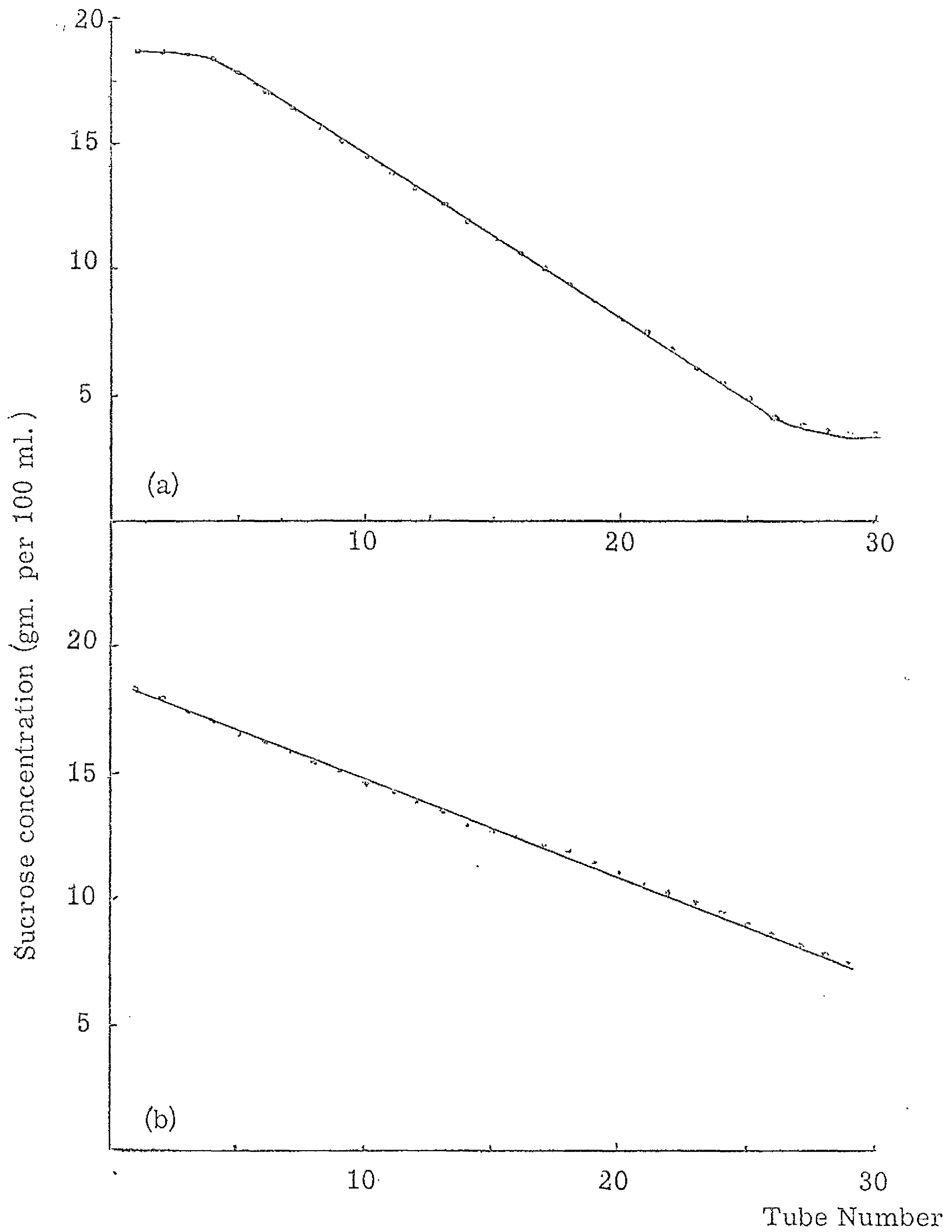


Fig. 3.

a volume of 4.8ml, and 0.2ml of RNA solution, containing RNA at a concentration corresponding to 10 O.D. units at 260mμ, in acetate buffer at pH 5.25, was layered on top, and spun for 12 hours at 25,000r.p.m. in the SW39 rotor of the Spinco Model L ultracentrifuge.

Collection of fractions.

Tubes were pierced at the bottom by a modification of the device of Szybalski (1960), using a No.14 gauge hypodermic needle, and 15-drop fractions were collected, diluted to 1.0ml, and the optical densities read at 260 mμ on the Unicam SP 500 Spectrophotometer. 0.5 ml aliquots of the fraction were then pipetted on to lens paper discs in stainless steel planchets, and counted for 500 counts.

b) Determination of Sedimentation Constants of Liver RNA.

The sedimentation constants of whole liver, cytoplasmic, and nuclear RNA were determined for all three dietary groups, using both the Schlieren and U.V. optical systems of the Spinco Model E ultracentrifuge. In both cases, the RNA for the analysis was prepared by the same procedure as for sucrose density gradient experiments.

(i) Schlieren optics.

Schlieren patterns were obtained for whole liver RNA, cytoplasmic, and nuclear RNA for all three diets. The RNA was dissolved in acetate buffer, pH 5.25, to a concentration of 8 - 15 mg/ml, and the solution centrifuged in the Spinco Model E ultracentrifuge at 44,770 r.p.m. for 40 minutes, in a quartz cell, at 20°C. Exposures were taken at 0 minutes and at every 8 minutes thereafter.

(ii) U.V. optics.

Since the S values obtained from the Schlieren patterns would be inaccurate, due to concentration effects, some

analyses of RNA were carried out using U.V. optics. RNA was dissolved in acetate buffer to a concentration corresponding to 2 - 1.5 O.D. units, and centrifuged at 44,770 r.p.m. for 40 minutes at 20°C. Exposures were taken at 0 minutes and at 4 minute intervals thereafter.

SECTION III : RESULTS.

Evaluation of Methods used for preparing Nuclei.

The work reported in this thesis represents studies on the liver nucleus, and it was therefore essential to be able to prepare pure nuclei in reasonable quantity. The numerous published methods for isolating nuclei have been well reviewed (Dounce, 1955; Brachet, 1957; Siebert and Smellie, 1957; Allfrey, 1959; and Roodyn, 1963), and can generally be classified into three main types (see Siebert and Smellie, 1957). They are 1) isolation of nuclei using non-aqueous solvents after completely drying the tissue, 2) isolation of nuclei from homogenates of tissues in dilute acids, and 3) isolation of nuclei from media containing sucrose. In addition, glycerol or glycerophosphates have been used by some workers (e.g. Poort, 1961). Our own experience with some of these procedures is summarised below.

Alternative methods of preparation.

1. Isolation of nuclei using non-aqueous solvents.

The first method for isolating nuclei in organic solvents was described by Behrens in 1932. The medium employed was benzene, and the principle was to float off the lighter cytoplasmic material, leaving the denser nuclei as a sediment. Allfrey and co-workers (1952) used cyclohexane for benzene, and reported preparations of fairly pure rat liver nuclei. Kay, Smellie, Humphrey and Davidson (1956), describe a method for the preparation of nuclei from tissues of young rabbits: liver, thymus, intestinal mucosa, and appendix, using cyclohexane-carbon tetrachloride, with the specific gravity adjusted so that only the nuclei could sediment through the medium. By this method, nuclei could be prepared with a minimum amount of contamination, but in poor yield. Kay et al (1956) were unable to prepare nuclei from adult rabbit liver by this method.

The chief advantage of non-aqueous solvents for preparing nuclei is that there is a minimal amount of water-soluble material lost from the nucleus (Dounce, 1955). However, these organic solvents will remove most or all of the lipid components of the nuclear membrane, and it is possible that other materials in the membrane may be dissolved with it.

In this work, organic solvents as media for isolating rat liver cell nuclei were not used, due to the doubtful purity of previous preparations (Roodyn, 1963, and Dounce, 1955), the poor yield, lengthy procedure and destruction of the potentially interesting nuclear membrane.

2. Isolation procedures involving acid media.

Citric acid has been used for some considerable time as a medium for isolating liver nuclei. (Dounce, 1943a, b, c), although acetic acid has also been employed (Stedman and Stedman, 1951). Many modifications of the citric acid procedure have since been published, 1% and 2% citric acid being the most commonly used media (Barnum *et al* 1950; McIndoe and Davidson, 1952; Dounce, 1955; Gurr, Finean and Hawthorne, 1963). The strong citric acid methods employed by Mirsky and Pollister (1946) and Frazer and Davidson (1953) have been quoted by Dounce (1955) as being satisfactory for experiments where either nucleic acids or lipids only were to be studied. The appearance of such nuclear preparations under the light microscope show very pure preparations of nuclei (Davidson, 1960).

Treatment of nuclei with acid extracts a large proportion of low molecular weight material such as minerals, nucleotides, and amino acids (Siebert and Smellie, 1957), whilst Dounce *et al* (1950), Allfrey *et al* (1952), and Kay *et al* (1956), report losses of the nuclear protein and RNA.

Gurr, Finean, and Hawthorne (1963) give convincing electronmicrographic and chemical evidence (see Table 17) that the outer nuclear membrane is stripped off by these acid procedures, whilst Siebert and Smellie (1957), and Dounce (1955), report that acid hardens the nuclear membrane. This could be due to the denaturation of protein in and inside the nuclear membrane. Certainly, attempts during our present work, to disintegrate nuclei ultrasonically, showed that nuclei isolated in glycine-HCl buffer at pH 3 resisted sonication for 30 minutes or more, after which it might be assumed that most of the protein would be denatured anyway. Davison and Mercer (1956) have examined nuclei obtained by different methods under the electron microscope, and report that nuclei isolated in citric acid, which looked pure under the light microscope, showed cytoplasmic contamination under the E.M., whilst nuclei obtained by Stedman's method were free from cytoplasm but looked very empty in appearance, with ruptured nuclear membranes. Their experiments were carried out using nuclei from calf, mouse, and rat liver, and calf and mouse thymus and spleen.

A method employing glycine-HCl buffer at pH 3 has been described by Paul (1962). When attempts were made to isolate rat liver nuclei by this procedure, the nuclei were found to be grossly contaminated with cytoplasm and fibrous material, and it was very difficult to rupture the nuclear membrane during attempts at isolating subnuclear particles. As Paul has reported that this method works very well with cells grown in culture, it seems that these methods are suitable for tissues which are free of extra-connective tissue, but are less satisfactory for a heterocytic organ such as liver.

3. Methods using sucrose.

3. Methods using sucrose.

Sucrose solutions are probably the most recently developed media for isolating nuclei. Dounce (1955) reports that nuclei isolated in 0.25M (isotonic) sucrose alone were destroyed, probably due to some degree of autolysis. A more successful attempt was made by Schneider and Peterman (1950), and Hogeboom et al (1952) when they introduced $CaCl_2$ into their 0.25M sucrose and were able to isolate metabolically active nuclei from this medium. Dounce (1955) found that 0.0018M $CaCl_2$ seemed to be the minimal concentration for preventing autolysis, and preserving the nuclear membrane. Philpot and Stanier (1956) also stress the importance of the presence of Ca^{2+} or Mg^{2+} ions for isolating unbroken nuclei, and a report by Leeson and Kalant (1961) on the effect of in vivo decalcification on the ultrastructure of rat liver showed that 'blebs' were formed from the nuclear membrane when the livers were perfused in vivo with Krebs-Ringer-EDTA. Allfrey, Mirsky and Osawa (1955), used a discontinuous gradient of 0.25M sucrose and 0.34M sucrose containing 0.0018M $CaCl_2$ for isolating calf thymus nuclei. These were reported to be minimally contaminated with cytoplasm, thymocytes being the major source of contamination, with 45-77 cells per 1,000 nuclei as typical levels of contamination. These nuclei were also capable of incorporating radioactive amino-acids into protein (Allfrey et al, 1955 et seq), and the yield of nuclei was fairly high. Many other workers have since used discontinuous gradients of 0.25M sucrose-0.34M sucrose for isolating nuclei (Logan and Davidson, (1957), for mouse thymus nuclei; Branster and Morton, (1957), for mouse mammary gland nuclei; Logan, (1957), and Tung-Yue Wang, (1961), for calf thymus nuclei; and Scholtissek, (1962), and Rees and Rowland, (1961), for rat liver nuclei).

However, in our experience with liver, the method described by Allfrey, Mirsky and Osawa (1955), for calf thymus nuclei, gave a very impure preparation, which, when viewed under the light microscope, showed at least 50% contamination with cytoplasmic material. It has been calculated by Chauveau, Moulé and Rouiller, (1956) that the isolation of completely pure nuclei from 0.25M or 0.34M sucrose is unattainable.

A very dense medium has been used by Chauveau, Moulé and Rouiller, (1956), Zbarskii and Georgiev, (1959), Wilczok and Chorązy, (1960), and Sporn and Dingman, (1962), who have all reported the successful isolation of pure rat liver nuclei from 2.2M sucrose. When each of these methods was investigated, during the present work, it was found that the nuclear preparations were very free from cytoplasm; the only contamination being some fibrous material in the Chauveau preparation. This contaminant was insignificant, since it was most likely to be fibrous material derived from connective tissue, and as such would not interfere with the RNA studies under investigation.

In our hands, the method of Wilczok and Chorązy, (1960), was found to give very poor yields, and was lengthy and tedious for large-scale preparations, although better yields were obtained when the sucrose concentration was reduced from 2.2M to 2.0M. A concentration of 1.8M sucrose gave contaminated nuclei. The best method found was the original one described by Chauveau and co-workers, (1956), although difficulty was experienced with the homogenisation in 2.2M sucrose. This was overcome by first homogenising the livers in 0.25M sucrose, and isolating a crude preparation of nuclei from this homogenate using a discontinuous sucrose density gradient of 0.25M-0.34M sucrose; the nuclei could then be purified by resuspending them in 0.25M sucrose and

adjusting the concentration to 2.2M with 2.31M sucrose before centrifuging. We found that these modifications of Chauveau *et al*'s method enabled us to prepare large amounts of pure nuclei fairly rapidly.

Properties of the preparations.

A comparison of the methods of nuclear isolation tested is given in Table 16. The most significant criteria of purity were taken to be a) microscopical examination and b) the RNA/DNA ratio. This latter is a particularly useful criterion for the constancy of the purity of the preparations, since the nucleus has been shown to contain a constant amount of DNA for each species, (Boivin, Vendrely and Vendrely, 1948; Thompson *et al*, 1953), and the RNA content can thus be related to a standard figure. A perusal of the literature indicated that microscopically clean nuclei have a low RNA/DNA ratio, whilst the more contaminated preparations contain large amounts of RNA (Roodyn, 1963). Since the RNA/DNA ratio for the rat liver cell is between 3.6 and 4.0 (Davidson, 1960), and the RNA/DNA ratios for the cleanest nuclei are given as 0.2-0.3, (Roodyn,(1963), and see Table 16), it follows that if 5% of the volume of the preparation is cytoplasmic in origin, then this will represent an increase of 50% in the observed 'nuclear' RNA. However, as Roodyn has observed, low RNA/DNA ratios may also be due to the loss of RNA from the nucleus into the aqueous medium during isolation.

The evidence for loss of RNA during our isolation procedure is slight. The first part of the procedure is identical to that described by Allfrey, Mirsky and Osawa, 1955, who compared their sucrose nuclei to nuclei isolated in non-aqueous media (Allfrey, Mirsky, Stern and Saetren, 1952; Osawa, Allfrey and Mirsky, 1957; and Stern and Mirsky, 1953), and found no significant differences in enzyme

activity, DNA content, or all-over protein composition, (Stern and Mirsky, 1953). Further evidence against loss of RNA from the nucleus into the cytoplasm is the observations made during the course of this work where it was found that, when RNA was labelled *in vivo* with ^{14}C-adenine, the greatest activity was in the nuclei, and very low counts were found in cytoplasm separated from the nuclei after short-term labelling. This would not occur if the highly labelled nuclear RNA leaked into the cytoplasm. In addition, the constancy of the RNA/DNA ratio (between 0.25 and 0.29 over 22 estimations) suggest that, if RNA were leaking out from the nucleus, it would be RNA of low activity leaking at a constant rate over 22 experiments. This is highly unlikely. It is also unlikely that material which, according to the evidence of Mirsky *et al*, cited above, is not lost during isolation in 0.25M sucrose, would be dissolved during subsequent isolation in 2.2M sucrose.

4. Other media used in isolating nuclei.

Glycerol has been used by Poort (1957), and Zbarskii and Georgiev (1959), and Philpot and Stanier (1956) both added glycerophosphate to their sucrose media. Philpot and Stanier, in fact, added 40% glycerol in addition, since they found that nuclei clumped in the absence of either Mg^{2+} or glycerol. Clumping of nuclei was not observed during the procedures used here, however, and electron micrographs published by Davison and Mercer (1956) showed that nuclei isolated by the Philpot and Stanier technique had no observable nuclear membrane. Glycerol and glycerophosphate were therefore omitted from the isolation medium employed.

General conclusions on methods of isolation.

These are summarized in Table 16, which shows a comparison of microscopic appearance, RNA/DNA ratios, yields and other

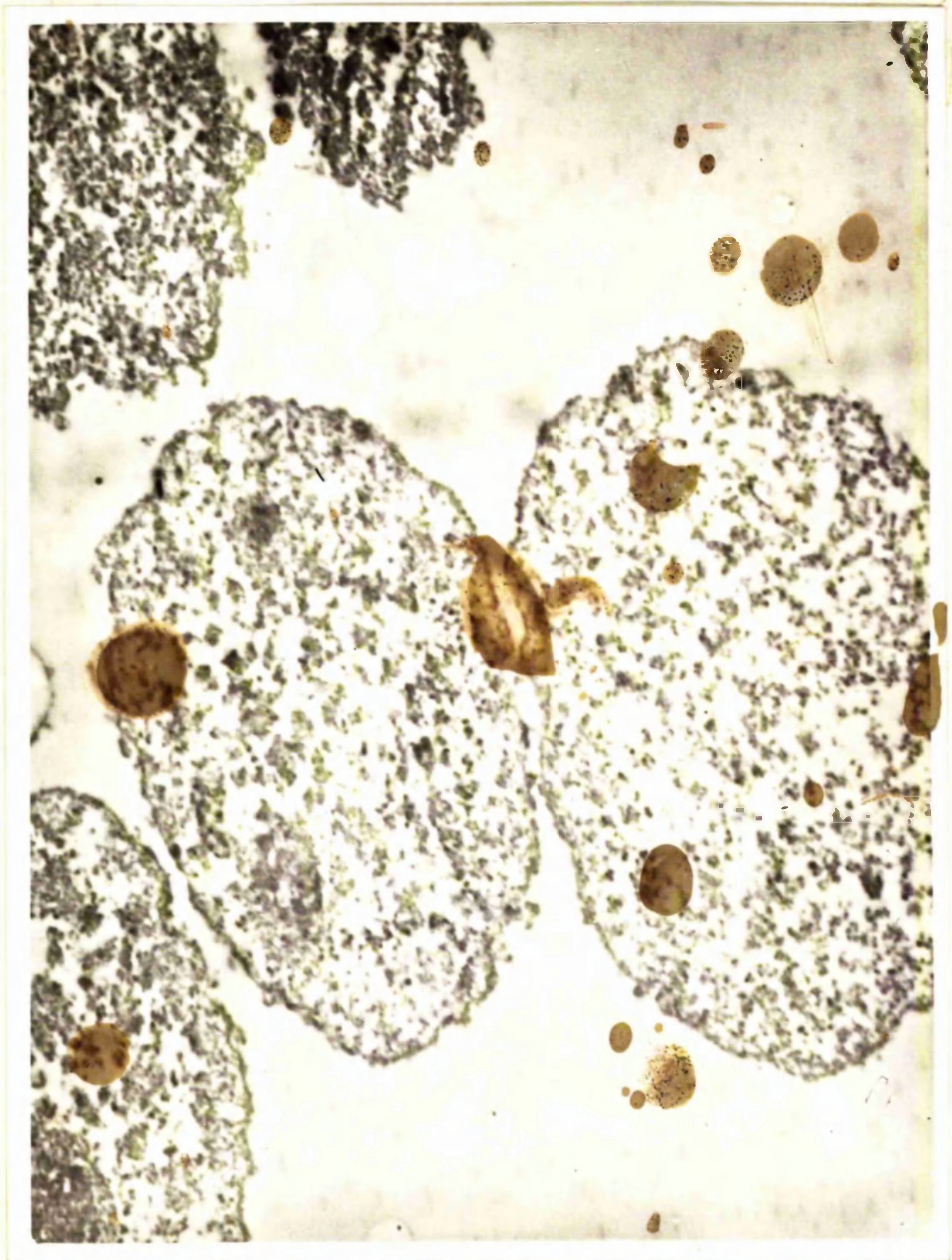


Fig. 4. Electronphotomicrograph of rat liver nuclei prepared in 2. 2 M sucrose by modified Chauveau method. Magnification x 12, 000.

Fig. 5. Electronphotomicrograph of section of isolated rat liver nucleus, showing double nuclear membrane. Magnification x 100, 000.

TABLE 16.

Table showing survey of the various methods examined for preparing rat liver nuclei.

Method	Microscopic appearance	RNA/DNA ratio.	Yield	Comments
Allfrey, Mirsky and Osawa (1955) cf. Rees and Rowland (1961).	Variable amounts of cytoplasmic contamination.	0.3-1.0	50-70%	Preparation very impure.
Modification of Fisher and Harris (1962) Tween 80 method.	Very contaminated with considerable rupture of nuclear membranes.	-	-	Unsatisfactory for liver.
Glycine-HCl method of Paul (1962).	Cytoplasmic contamination. Hardened nuclear membrane.	0.8	not determined.	Impure preparation, nuclear membrane probably denatured.
Method of Wilczok and Chorazy (1960).	Very clean nuclei.	0.22-0.24	5-15%	Yield too low for large scale work.
Method of Dingman and Sporn (1962)	Very clean nuclei.	0.24 (one experiment)	Seemed good, but not determined.	Good method, although rather lengthy.

(continued overleaf)...

TABLE 16 (continued).

Method	Microscopic appearance	RNA/DNA ratio.	Yield	Comments
Method of Chauveau *et al* (1956).	Clean nuclei; some ruptured. Some fibrous material.	0.28 (mean of 2).	45-55%	Clean nuclei, although some ruptured. Homogenis-ation procedure difficult.
Modified Chauveau method.	Nuclei appear clean under E.M.	0.22-0.28 (mean of 22).	40-50%	Reliable over a large number of experiments. Rapid for large scale.

TABLE 17

Table showing phospholipid content of rat liver nuclei. (All figures except those from work done in this laboratory are taken from Gurr, Finean and Hawthorne, 1963).

Author	Isolation technique	μg lipid-P. per nucleus x 10^9 /10^7	Lipid - P. / DNA - P.
Chauveau *et al.* (1956)	2.2M sucrose	104	0.11
Rees and Rowland, (1961)	0.25M sucrose + 0.34M sucrose + 1.8mM $CaCl_2$	590	0.81
Barnum *et al.* (1950)	0.85% NaCl - 2% citric acid	46	0.048
Dounce (1943)	Citric acid, pH 3.8	83	0.088
McIndoe and Davidson, (1952)	5% and 2% citric acid	35	0.037
Gurr, Finean and Hawthorne, (1963)	1%, 2% citric acid	47	0.049
	2.2M sucrose - 2mM $CaCl_2$ + glycerophosphate	123	0.13
Present work	2.2M sucrose. 2mM $CaCl_2$ - 1mM $MgCl_2$	94	0.10

comments for a variety of methods which we have personally experienced. The advantage of the method finally elaborated in this laboratory is clear - nuclei of low RNA/DNA ratio, clean by electron microscopy, (Fig. 4), are obtained in reasonable yield. No other method was so satisfactory in our hands.

A point of further importance in selection of method is shown in Table 17, where the literature on the phospholipid content of nuclei prepared in different media is summarized. This demonstrates that nuclei isolated in sucrose retain their double membrane whilst nuclei isolated in acid media have lost the outer membrane. Fig. 5 shows that nuclei isolated by our method have both inner and outer membranes present in a good state of preservation.

RNA and DNA estimations on salt fractions of nuclei.
Rat liver nuclei were extracted with (a) 0.1M phosphate buffer, pH 7.1, and (b) molar sodium chloride (MNaCl), the residual material (c) being the "nucleolar residue", as described in the experimental section.

In order to obtain RNA from each fraction, the extracts were precipitated with acid, followed by alkaline digestion by the method of Fleck and Munro (1962); the extracted RNA was acidified with PCA and the solutions read at 260mμ to determine the RNA concentration. (P. 31)
A brief examination of the spectra of a few samples of the "RNA" from each nuclear fraction was also made, to check the purity of the RNA samples with respect to protein contamination. The spectra showed that the PCA extracts of the whole nuclei, and of all the fractions were free of protein contamination (see Fig. 6).
There was no DNA contaminating the acidified digests of the whole nuclei or nucleolar residue, by the Ceriotti (1952) reaction.

Effect of Diet.
The results obtained from analysis of liver nuclear RNA of rats fed protein-rich or protein-free diets are in general agreement with those of other workers (see below). Since results for each dietary group did not vary much between experiments, it was considered justifiable to collate the results by analysis of variance in a two way classification for a) total nuclear RNA; b) RNA of phosphate extract; c) RNA of sodium chloride detract; d) RNA of phosphate and sodium chloride extracts collectively, e) RNA of "nucleolar residue"; and f) unrecovered RNA. The unrecovered RNA was defined as the total nuclear RNA minus the sum of the DNA recovered from the nuclear sub-fractions.

Fig. 6.

U.V. absorption spectra for digests of salt extracts of nuclear RNA.

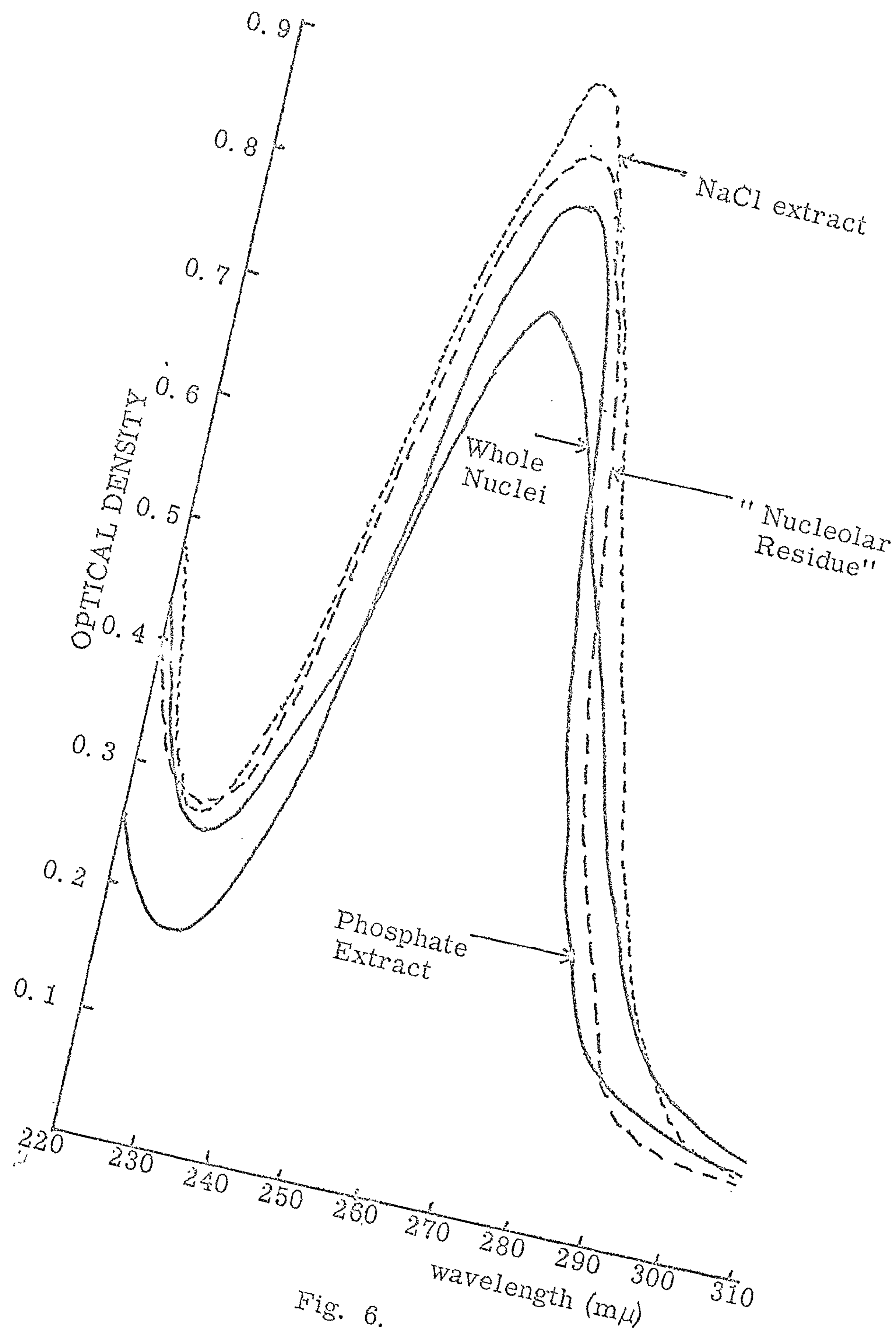


Fig. 6.

Tables 18, 19 and 20 show the results from individual experiments, and Table 21 gives the mean figures from these experiments, with the results of the statistical analyses. The figures are expressed as µg RNAP per 100 µg DNAP, so that the RNA in each group and for each experiment is being compared with an invariant standard, since diet has no effect on the DNA of the liver cell (Thomson et al, 1953). Table 22 shows the RNA of each fraction as a percentage of the total nuclear RNA, in order to demanstrate more clearly the effect of diet. Several points emerge from Tables 18-22.

(a) From Table 21 the total nuclear RNA of the protein depleted (L.P.) and H.P. groups is significantly less than that of the H.P.F. group. This would coincide with the general decrease in cell RNA on protein withdrawal observed by Thomson et al (1953), and by Munro and Clarke (1959).

(b) About 25% of the nuclear RNA is extracted by the neutral phosphate buffer. This figure does not agree with that given by Maggio, Siekevitz and Palade (1963a) who were able to extract only 15% of the nuclear RNA from guinea pig liver nuclei prepared by the Chauveau method, and only 3% from nuclei prepared by their own method. Although they suggest that "phosphate RNA" is contaminating cytoplasmic RNA, it is conceivable that the methods used by them for estimating RNA may incur some loss of RNA into organic solvents (see Hallinan, Fleck and Munro, 1963), and this might account for some discrepancy between their values and our own. Also, Osawa, Takata and Hotta (1958), found that 33% of the RNA of calf thymus nuclei prepared by the method of Allfrey, Mirsky and Osawa, (1955), was associated with the phosphate pH 7.1 fraction, and Hotta and Osawa (1958) found that 43% of rat liver nuclear RNA was removed by phosphate buffer. It is obvious, therefore, that there is

TABLE 18.

Table showing amounts of RNA recovered from nuclear sub-fractions of livers of H.P.F. rats. RNA expressed as µg RNAP per 100 µg DNAP.

Expt. No.	Total nuclei	Fractions				
		PO_4 fraction	NaCl fraction	PO_4 + NaCl fractions	Nucleolar residue	Unrecovered RNA.
1	24.0	4.8	4.5	9.3	14.3	0.4
2	28.0	5.4	4.2	9.6	18.0	0.4
3	27.8	5.2	7.5	12.7	15.0	0.1
4	27.0	8.1	4.7	12.7	14.2	0.1
5	24.7	5.1	5.3	10.4	13.9	0.4
6	28.0	8.5	5.1	13.6	14.0	0.4
7	26.6	7.2	6.3	13.5	13.0	0.1
8	25.0	5.3	4.7	10.0	14.9	0.1
9	25.7	5.8	5.1	10.9	14.7	0.1
10	27.6	6.1	10.2	16.3	11.2	0.1
11	27.8	6.9	8.9	15.8	11.5	0.5
12	28.3	8.0	8.7	16.7	11.1	0.5
13	24.6	5.6	4.9	10.5	13.7	0.4
14	25.3	8.2	5.5	13.7	11.6	0.2
15	28.6	9.3	9.3	18.6	9.2	0.8
16	27.8	8.9	8.7	17.6	9.1	1.1
17	27.2	8.5	9.4	17.8	8.8	0.6
18	28.6	9.2	7.6	16.8	11.4	0.4
19	29.5	4.6	8.0	12.6	16.8	0.1
20	26.0	7.6	5.4	13.0	12.9	0.1
21	24.1	8.2	6.7	14.8	9.1	0.2
22	24.5	5.3	5.3	10.6	13.5	0.4
Mean value	26.7	6.9	6.6	13.5	12.8	0.3

TABLE 19.

Table showing amounts of RNA recovered from nuclear sub-fractions of livers of H.P. rats. RNA expressed as µg RNAP per 100µg DNAP.

Expt. No.	Whole nuclei	Fraction					
		Phosphate extract	NaCl extract	PO_4^- + NaCl extracts	Nucleolar residue	Unrecovered RNA
1	25.3	5.4	5.2	10.6	14.0	0.7
2	23.4	5.6	5.4	11.0	11.3	1.1
3	23.0	5.2	5.7	10.9	11.4	0.7
4	25.9	7.3	5.8	13.1	12.0	0.8
5	26.0	8.9	4.4	13.3	11.4	1.3
6	27.4	7.4	5.4	12.8	11.8	2.8
7	27.2	5.8	7.5	13.3	12.9	1.0
8	25.7	3.9	7.8	11.7	13.8	0.2
9	24.5	4.7	5.1	9.8	13.6	1.1
10	26.2	6.7	7.0	13.7	11.6	0.9
11	29.0	6.7	6.1	12.8	12.8	3.4
12	27.8	8.2	5.3	13.5	11.7	2.6
13	23.0	6.1	4.7	10.8	10.9	1.3
14	26.4	7.1	8.4	15.5	9.7	1.2
15	26.1	7.9	8.2	16.1	9.4	0.6
16	23.7	7.5	7.5	15.0	7.7	1.0
17	28.1	6.1	11.7	17.8	7.5	2.8
18	26.1	9.3	7.3	16.6	6.1	3.4
19	25.8	5.2	10.6	15.8	7.8	2.2
20	27.1	5.7	7.9	13.6	11.3	2.2
21	25.0	7.3	6.2	13.5	10.9	0.6
22	25.0	7.4	4.7	12.1	11.8	0.3
Mean value	25.8	6.6	6.7	13.4	11.0	1.5

TABLE 20.

Table showing amounts of RNA recovered from nuclear sub-fractions of livers of L.P. rats. RNA expressed as μg RNAP per 100μg DNAP.

Expt. No.	Whole nuclei	Fraction				
		Phosphate extract	NaCl extract	PO_4 + NaCl extract	Nucleolar residue	Unrecovered RNA.
1	23.5	4.0	4.7	8.7	13.5	1.3
2	29.5	6.0	5.0	11.0	16.8	1.7
3	26.8	2.4	4.8	7.2	18.0	1.6
4	27.5	4.9	4.3	9.2	15.6	2.7
5	25.8	5.0	4.1	9.1	14.9	1.8
6	27.1	4.4	4.7	9.1	16.3	1.7
7	26.8	4.5	5.6	10.1	15.8	0.9
8	24.2	1.5	5.3	6.8	17.0	0.4
9	25.1	6.2	1.7	7.9	16.3	0.9
10	27.8	8.0	4.3	12.3	11.6	3.2
11	26.6	8.1	4.3	12.4	11.8	2.4
12	26.8	2.8	6.9	9.7	15.2	1.9
13	22.5	5.6	5.4	11.0	9.9	1.6
14	22.1	4.5	6.7	11.2	10.5	0.4
15	24.1	5.1	4.0	9.1	14.6	0.4
16	24.8	7.8	6.3	14.1	8.2	2.5
17	22.9	6.0	6.4	12.4	8.6	1.9
18	25.2	6.8	7.0	13.8	9.2	2.2
19	27.2	7.0	8.1	15.1	8.4	3.7
20	25.6	5.9	5.0	10.9	13.2	1.5
21	22.0	3.0	5.6	8.6	12.2	1.2
22	25.5	5.3	5.3	10.6	13.8	1.1
Mean value	25.4	5.2	5.3	10.5	13.2	1.7

considerable variation amongst the published data, which may be partly explained by differences in techniques of extraction, partly by cytoplasmic contamination, and in the case of calf thymus, possibly by species differences. The findings of Logan and Davidson (1957), that the base composition of "phosphate RNA" differs from cytoplasmic RNA would eliminate the possibility of the "phosphate RNA" being entirely cytoplasmic contamination. We also found that the variation in RNA content of the phosphate fractions from each group were not significant with respect to diet, although there was a tendency for the value from the L.P. animals to be lower than than for either the H.P. or H.P.F. group. (Table 22).

(c) Treatment with molar sodium chloride (MNaCl) also removed 20 - 25% of the nuclear RNA, but no DNA was removed by this treatment in 66 samples. This does not agree with the findings of Allfrey et al (1955) and of Logan (1957), who found all the DNA from calf thymus nuclei released into the MNaCl extract. However, neither Logan (1957) nor Allfrey et al (1955) report on RNA in the MNaCl fraction, and although in a later paper (Allfrey et al, 1957), mention is made of a very active RNA in the MNaCl extract, from the presentation of their results it is impossible to estimate the amount as a percentage of the total nuclear RNA. Maggio, Siekevitz and Palade (1963b) found that 60 - 80% of guinea pig liver DNA was dissolved by MNaCl after vigorous stirring for about 15 hours, while only 10% of the nuclear RNA was released, and Hotta and Osawa (1958) were apparently able to dissolve the entire post-phosphate pellet of rat liver nuclei in MNaCl after very vigorous homogenisation.

It would seem, therefore, that the mild conditions of extraction employed in our experiments were insufficient to release the DNA into the supernatant, but about 20 - 25%

of the nuclear RNA was consistently solubilised. In addition, an antigenic protein specific for the nucleolus was invariably removed by the MNaCl during this treatment. (see later). This gentler method of extraction was adopted routinely since it gave reproducible results, and the failure to remove DNA into the MNaCl was of no significance in our studies. In our experiments, the variations in amount of RNA in this fraction did not alter significantly with diet, although the value for the L.P. group again showed a tendency to be lower.

(d) The RNA in both the phosphate and MNaCl fractions was then considered collectively for each diet, and it was found that the protein-depleted animals contained significantly less RNA ($P < 0.001$) than those of the two high protein groups in these combined fractions. Thus the extractable RNA in the liver nuclei of L.P. animals is considerably smaller than in H.P. animals, among which there is no difference between those fasting and those fed protein just before killing.

(e) The amount of RNA left in the "nucleolar residue" after extraction was about 50% of the total nuclear RNA for all three diets. The amount of this RNA, however, showed a small but significant variation between each dietary group, with the amount of RNA in the nucleolar residue of the L.P. diet being significantly greater than in the other two diets. This would agree with the interferometric determinations of Stenram (1958), who observed an absolute increase in the RNA of the liver nucleoli of rats which had been deprived of protein or various essential amino acids. Stenram's results, however, would suggest that a greater difference between the RNA in the nucleolar residue of the H.P.F. and L.P. groups might be expected, and it is probable

TABLE 21.

Summary of mean values given in Tables 18-20, showing the effect of dietary protein on the RNA content of salt extracts of rat liver nuclei. Results are expressed as µg. RNAP per 100µg. DNAP.

Dietary Group	Whole nuclei	Fraction					
		Nucleolar residue	PO_4 extract	NaCl extract	PO_4 + NaCl extracts	Unrecovered RNA.
H.P.F.	26.7	12.8	6.9	6.6	13.5	0.3
H.P.	25.8	11.0	6.6	6.7	13.3	1.5
L.P.	25.4	13.2	5.2	5.3	10.5	1.7
Significance	$<$ 5.0%	$<$ 0.1%	$>$ 5.0%	$>$ 5.0%	$<$ 0.1%	$<$ 0.1%

TABLE 22.

Table giving the mean values of the RNA recovered in nuclear subfractions of rat liver as a percentage of total nuclear RNA.

Dietary group	Fraction				
	Nucleolar residue	Phosphate extract	NaCl extract	PO_4 + NaCl extracts	Unrecovered RNA
H.P.F.	48%	25%	25%	51%	2%
H.P.	43%	26%	26%	52%	5%
L.P.	52%	20%	21%	41%	7%

that, in the extraction procedure used, the "nucleolar residual RNA" was made up of RNA from the nuclear membrane, and chromosomal RNA, as well as true nucleolar RNA. This has also been proposed by Samarina and Georgiov, (1960). This contamination would tend to mask the effect of diet on true nucleolar RNA.

(f) The recovery of RNA by this extraction procedure varies with the dietary group. With H.P.F. animals, 98% of the nuclear RNA is recovered, but with H.P. and L.P. animals only 95% and 93%, respectively, is recovered. These variations of RNA recovery were statistically highly significant, (Table 21). It was considered that this "unrecovered RNA" might be a fraction of rapidly turning over RNA, which is stabilised by incoming amino acids. This thesis will be discussed in more detail later in this presentation.

(g) The effect of a meal of protein is summarized in Table 23, and shows some dramatic effects occurring within two hours. It is apparent that protein taken 2 hours before killing causes (i) an increase of 0.9µg RNA-P per 100µg DNAP in the total RNAP of the nucleus (ii) a much larger increment in "nucleolar RNA", namely 1.8µg RNAP per 100µg DNAP, and (iii) a reduction in unrecovered RNA. Thus the smaller loss of RNA on fractionation coincides with a larger RNA content of the nucleolus. This is compatible with stabilisation of an unstable RNA which is being rapidly synthesized in the nucleus. Its rapid synthesis would allow for the absolute increase of 4% in total nuclear RNA within 2 hours of feeding; also, the much greater gain (16%) in nucleolar RNA can be accounted for by a reduction in the RNA lost (1.2µg RNAP/100µg DNAP) during salt extraction.

TABLE 23.

Table summarising effect of feeding 2g. casein to H.P. rats 2 hours before killing, on the RNA of whole nuclei, nucleolar residue, and unrecovered RNA.

Dietary group	Whole nuclei	Nucleolar residue	Total RNA recovered	Unrecovered RNA
H.P. group	25.8	11.0	24.3	1.5
H.P.F. group	26.7	12.8	26.4	0.3
Difference:				
Absolute	+ 0.9	+ 1.8	+ 2.1	- 1.2
%	+ 4%	+ 16%	+ 8%	-

In Vivo Incorporation of ^{14}C-adenine into Rat Liver RNA.
In vivo uptake of ^{14}C-adenine into sub-fractions of nuclear RNA from dieted rats was studied for time intervals of 15, 30, 45, 60, 120 and 180 minutes after injection of the isotope. Because of limitations on the amounts of centrifuges, the experiments had to be carried out in two series: i) for time intervals of 15, 30 and 45 minutes, and ii) for intervals of 60, 120 and 180 minutes. Although this division of a time course is far from ideal, statistically, the results nevertheless showed that the effects of diets were reproducible for each series, and correlation between the results for the two sets of experiments was possible.

The figures given in Table 24 are the mean values for the specific activities of RNA from whole nuclei and from subfractions obtained in several experiments. The specific activity of the cytoplasm was also determined. The number of individual animals used to obtain each figure is given in the Table. The results are expressed as counts/minute/100µg RNA. It was considered justifiable to express the results from individual experiments as a mean, since variations in specific activity between corresponding fractions from different experiments were small.

Since the activity of the precursor pools was not examined, the uptake of ^{14}C-adenine by whole nuclei and by nuclear fractions from different groups cannot be directly compared. A high activity in RNA of nuclei might merely mean a more highly labelled precursor. However, in order to compare groups, it is legitimate to compare uptake of isotope into individual fractions with that of the whole nuclear RNA. The ratio specific activity of RNA of nuclear fraction/specific activity of RNA of whole nucleus is termed here the "relative specific activity", and the mean

TABLE 24.

Mean specific activities of RNA in salt extracts of rat liver nuclei after injection of 5uc ^{14}C-adenine into each rat. Results are expressed as counts per minute per 100ug RNAP.

Number of Animals		2	2	2	8	4	4
Minutes after Injection		15	30	45	60	120	180
Diet	Fraction	Specific Activity					
H.P.F.	Whole nuclei	112.4	130.0	180.0	227.2	408.1	456.0
	Nucleolar residue	81.4	105.1	176.0	297.0	600.0	759.0
	PO_4 extract	17.9	27.0	39.3	51.5	102.0	192.2
	NaCl extract	206.0	239.2	242.0	50.5	74.6	33.5
	Cytoplasm	5.3	13.4	31.2	46.0	86.4	119.0
H.P.	Whole nuclei	111.0	174.1	201.1	262.0	303.0	393.0
	Nucleolar residue	112.1	251.0	324.0	447.0	644.2	850.3
	PO_4 extract	20.8	19.9	36.6	54.2	69.1	154.0
	NaCl extract	83.4	170.0	244.0	47.4	31.6	40.1
	Cytoplasm	13.6	13.1	23.2	48.1	64.5	99.4
L.P.	Whole nuclei	201.0	425.1	523.2	608.0	725.2	801.0
	Nucleolar residue	194.2	426.0	714.1	846.2	1109.0	1559.
	PO_4 extract	28.1	85.2	72.6	191.0	287.3	352.2
	NaCl extract	87.4	294.1	472.0	112.1	132.4	111.0
	Cytoplasm	16.4	29.3	60.7	77.9	109.0	204.8

TABLE 25.

Relative specific activities of the RNA fractions in salt extracts of rat liver nuclei, at various time intervals after isotope administration.

Number of Animals		2	2	2	8	4	4
Minutes after Injection		15	30	45	60	120	180
Fraction	Diet	Relative Specific Activity					
Nucleolar Residue	H.P.F.	0.72	0.81	0.98	1.31	1.47	1.74
	H.P.	1.01	1.44	1.62	1.82	2.13	2.17
	L.P.	0.97	1.01	1.36	1.39	1.52	1.94
PO_4 extract	H.P.F.	0.16	0.20	0.22	0.23	0.25	0.44
	H.P.	0.18	0.12	0.18	0.21	0.23	0.39
	L.P.	0.14	0.20	0.14	0.31	0.40	0.44
NaCl extract	H.P.F.	1.84	1.83	1.34	0.22	0.18	0.08
	H.P.	0.75	1.02	1.22	0.18	0.10	0.10
	L.P.	0.44	0.69	0.94	0.18	0.18	0.14
Cytoplasm	H.P.F.	0.05	0.10	0.17	0.21	0.22	0.27
	H.P.	0.12	0.08	0.12	0.18	0.21	0.25
	L.P.	0.08	0.07	0.12	0.13	0.15	0.26

values of relative specific activities of fractions from several experiments are shown in Table 25 .

The results may be considered from two points of view: (1) variation between different subnuclear fractions within a dietary group, and (2) variations between corresponding fractions as a consequence of diet. Both aspects of the problem are discussed concurrently here.

The relative uptakes of ^{14}C-adenine into the RNA of the cytoplasm, nucleus and nuclear subfractions are in general similar for all dietary groups (Table 24; Figs. 7-9), and are compatible with the findings of other workers (see below). There are, however, some striking changes in the labelling pattern due to diet, viz:-

(a) In all dietary groups the nuclear RNA is very much more heavily labelled than cytoplasmic RNA throughout the three-hour period after injection. This is a classic finding in all cells (Davidson et al. 1951; Hurlbert and Potter, 1952; Osawa, Takata and Hotta, 1958; Hotta and Osawa, 1958; Goldstein and Plaut, 1955; and Georgiev and Mant'eva, 1960). However, the uptake of RNA precursors into the nucleus and cytoplasm of L.P. rats is very much greater (about 2-fold) than into either the H.P.F. or H.P. groups. (Table 3 and Figs. 7 - 9). There is little difference in degree of labelling between these latter two groups, although the nuclear labelling in the H.P. group tends to be very slightly higher than that of the H.P.F. group for the first hour after injection, and then rises more slowly for the 1 - 3 hour period, whilst the label in the nuclei of the H.P.F. group continues to rise at a more constant and rapid rate throughout the three hours. There is little difference between the specific activities of the cytoplasmic fractions relative to the nuclear RNA in the three dietary groups (Fig.10).

Fig. 7.

Uptake of ^{14}C-adenine by RNA from the liver cytoplasm (■——■), whole liver nucleus (●——●), "nucleolar residue" (○——○), nuclear phosphate extract (□——□), and nuclear M NaCl extract (×——×) from H.P.F. rats. Specific activities are as counts/min/100μg RNA.

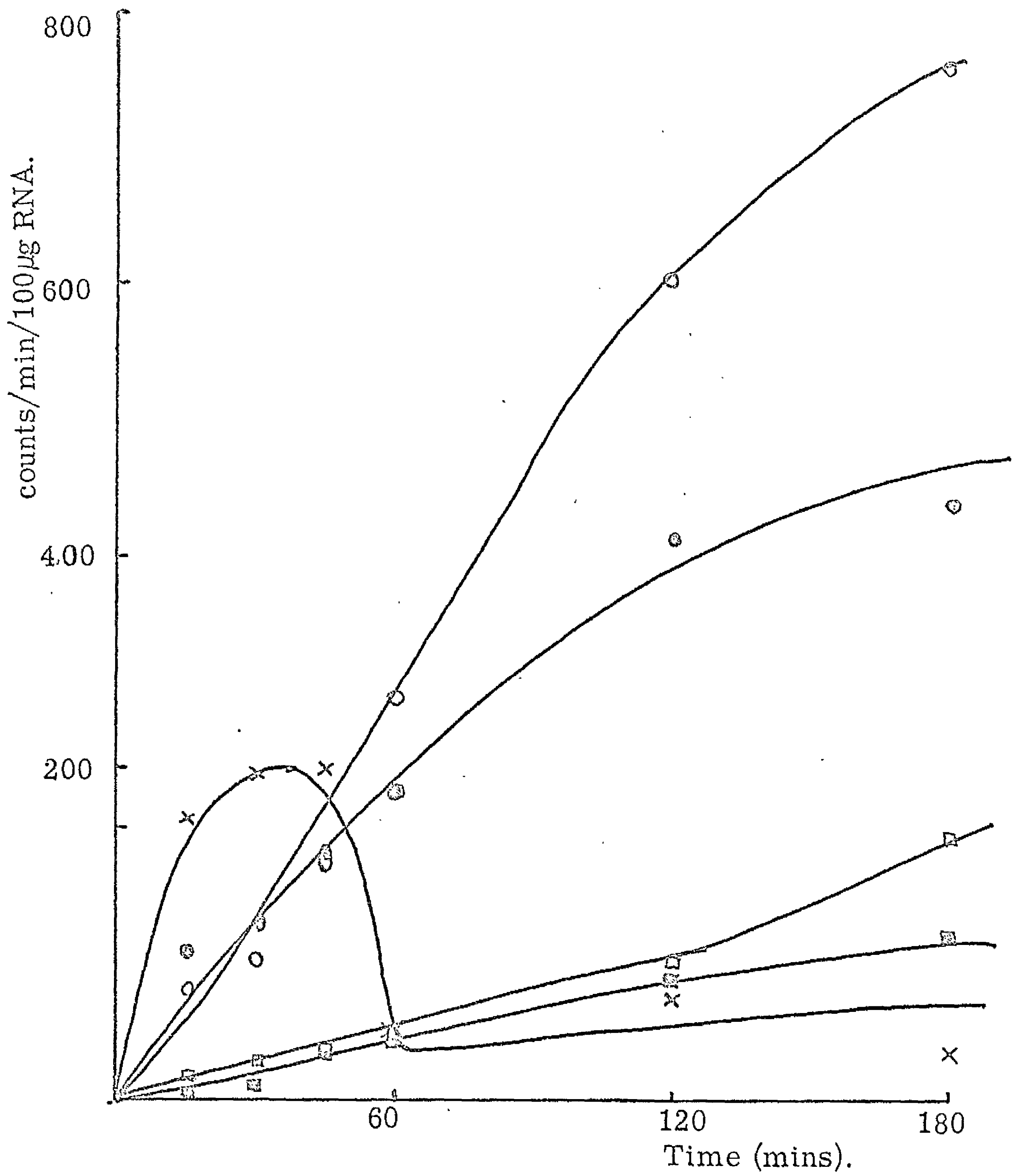


Fig. 7.

Fig. 8.

Uptake of ^{14}C-adenine by RNA from the liver cytoplasm (■——■), whole liver nucleus (●——●), "nucleolar residue" (○——○), nuclear phosphate extract (□——□), and nuclear M NaCl extract (×——×) from H.P. rats.
Specific activities are as counts/min/100μg RNA.

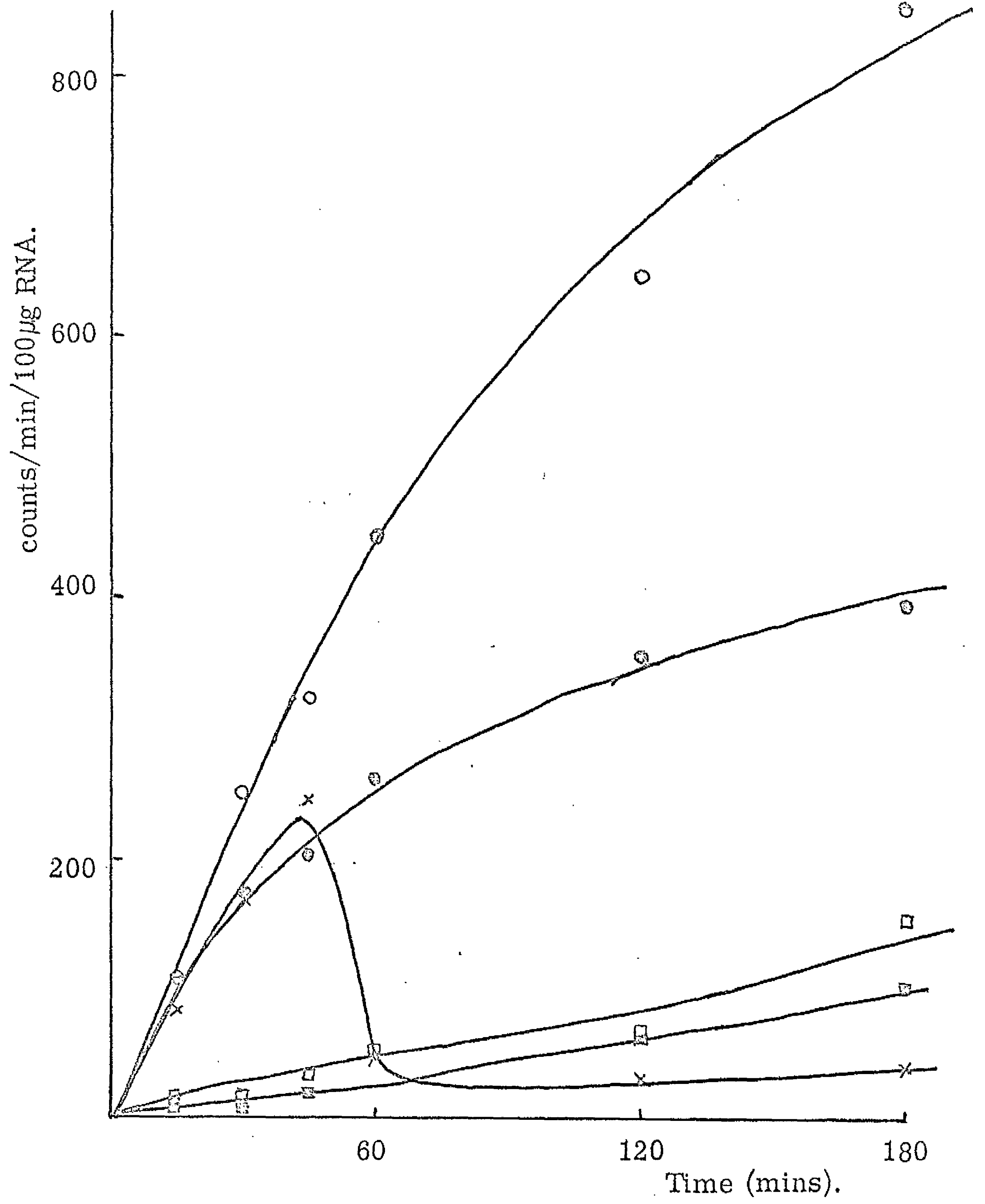


Fig. 8.

Fig. 9.

Uptake of ^{14}C-adenine by RNA from the liver cytoplasm (■——■), whole liver nucleus (●——●), "nucleolar residue" (○——○), nuclear phosphate extract (□——□) and nuclear M NaCl extract, from L.P. rats.
Specific activities are as counts/min/100μg RNA.

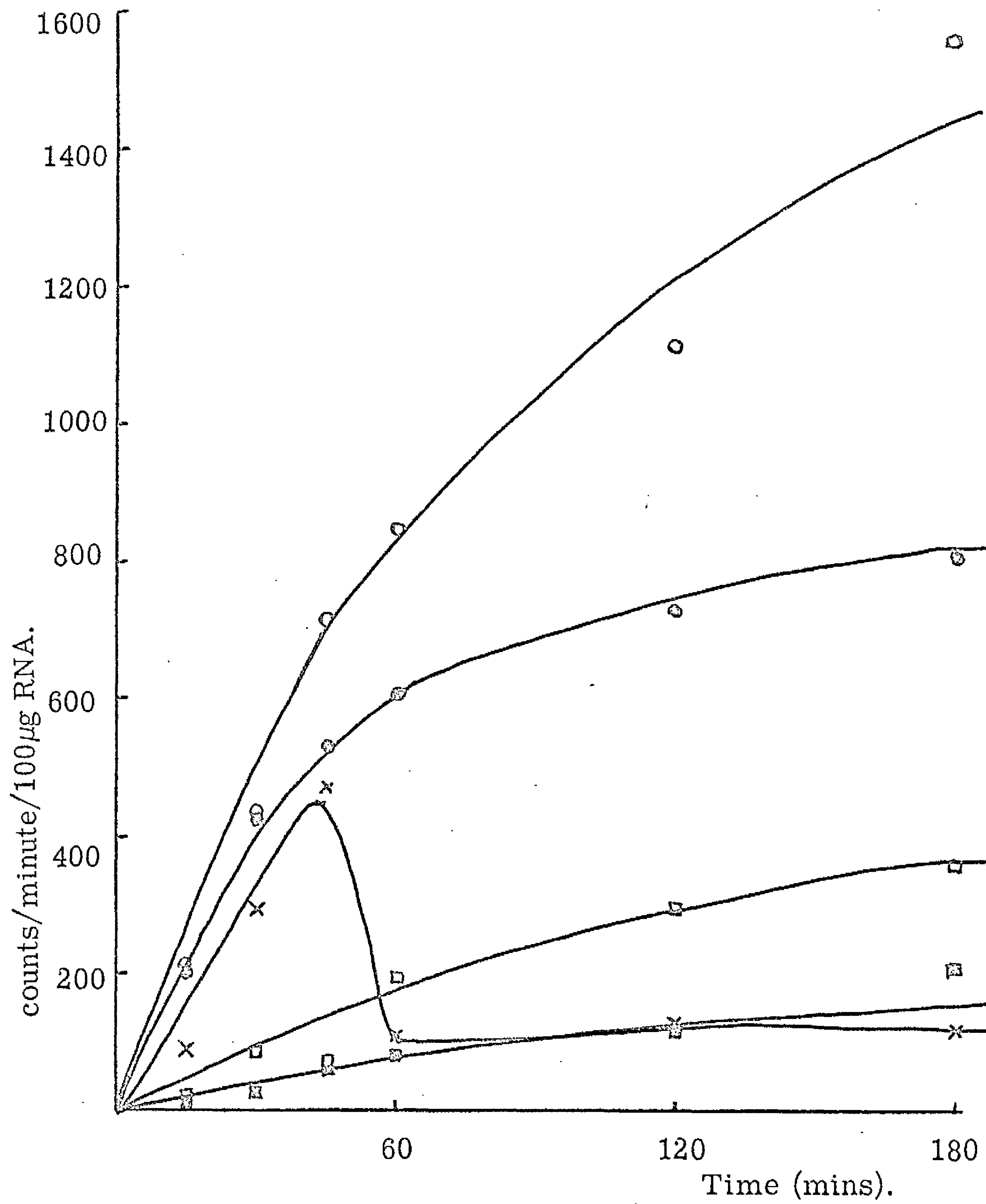


Fig. 9.

The very considerable increase in turnover of whole nuclear RNA of L.P. rats is compatible with the autoradiographic findings of Stenram (1963) in rats and mice, in which the turnover of ^{3}H-cytidine in the nucleus of protein-depleted animals is very much greater than in those fed protein. This coincides with the observations of Munro and Clarke (1959), who showed that the whole liver RNA in protein-depleted rats turns over at a much greater rate than in animals fasting after a diet containing protein. Thus, a very marked effect of protein withdrawal from the diet appears to be a considerable decrease in the stability and consequent rise in turnover of the RNA of the whole nucleus and cytoplasm.

(b) Within the nucleus, the nucleolus is the most active site of ^{14}C-adenine uptake for the H.P. and L.P. groups, at all times, and in the H.P.F. group 1 hour or more after injection (Figs. 7 - 9). In the H.P.F. group at time intervals of less than 1 hour, the RNA of the M NaCl extract is about twice as active as the nucleolus (see Table 24).

The observation of the high activity of the nucleolus is very well documented from both autoradiographic studies (McMaster-Kaye and Taylor, 1958; Sirlin, Kato and Jones, 1961; Perry, Hell and Errera, 1961; Srinivasan et al, 1963), and by chemical studies (Allfrey, Mirsky and Osawa, 1955; Allfrey and Mirsky, 1957; Logan, 1957; Georgiev and Mant'eva, 1960; Rho and Bonner, 1961; Sibatani et al, 1962).

It is obvious from Figs. 7 - 9 and Table 25 that the influence of diet on the metabolism of nucleolar RNA is dramatic. The nucleolar residue of the L.P. group turns over at about twice the rate of the nucleolar residues of either the H.P.F. or the H.P. groups. This is again

Fig. 10.

Uptake of ^{14}C-adenine by the cytoplasmic RNA from livers of H.P.F. (●——●), H.P. (□——□) and L.P. (○——○) rats, expressed as a ratio of the uptake of ^{14}C-adenine by the whole nuclei (i.e. the relative specific activities).

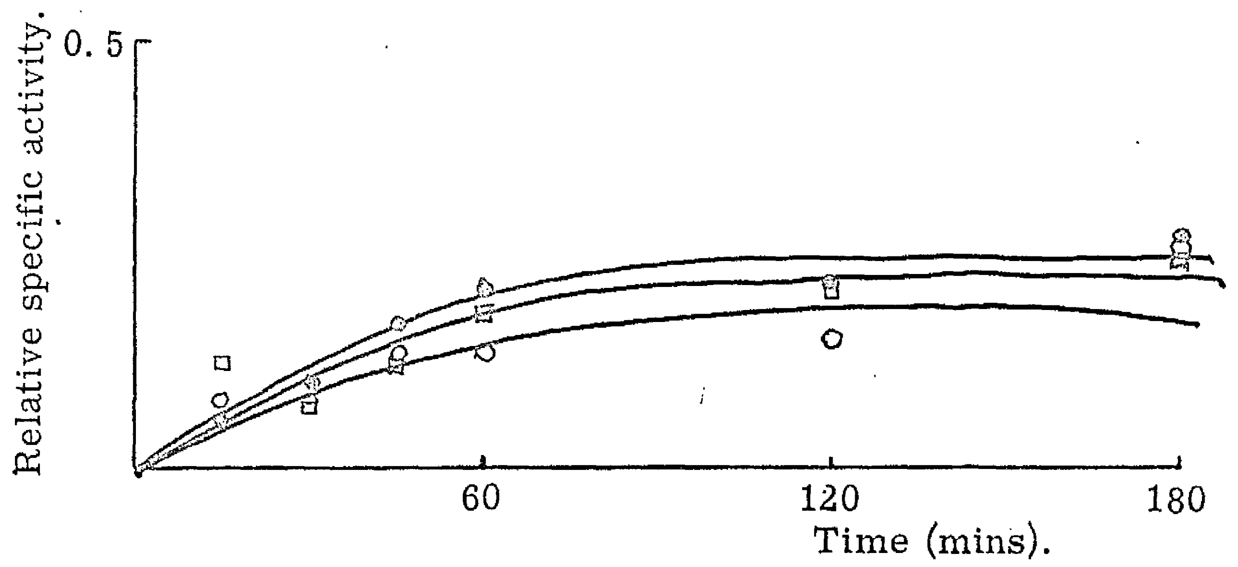


Fig. 10.

Fig. 11.

Uptake of ^{14}C-adenine by the "nucleolar" RNA from liver nuclei of H.P.F. (●——●), H.P. (□——□) and L.P. (○——○) rats, expressed as a ratio of the uptake of ^{14}C-adenine by the whole nuclei (i.e. the relative specific activities).

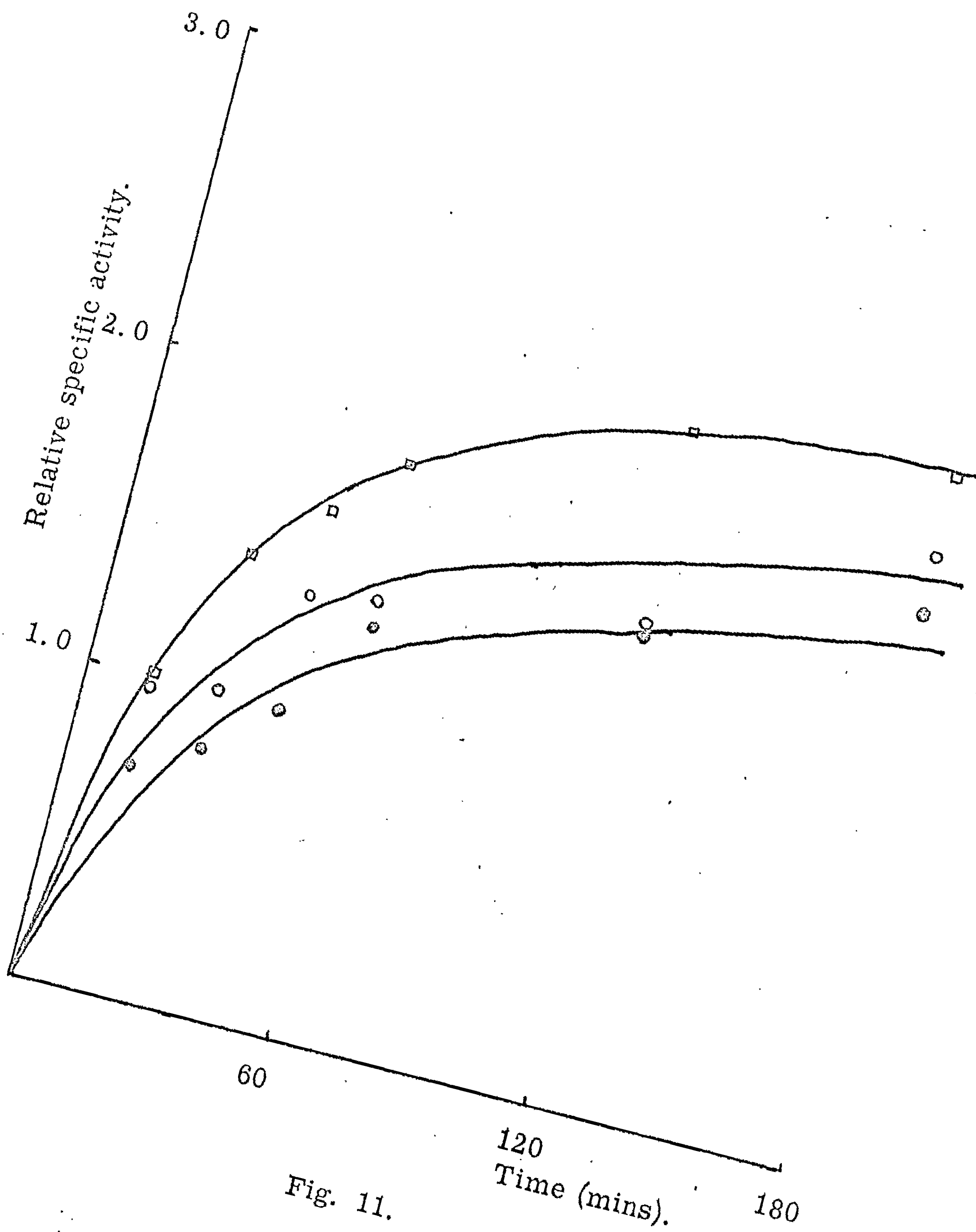


Fig. 11.

consistent with the autoradiographic findings of Stouran (1962) who found that the liver nucleoli of protein-depleted rats and mice were much more heavily labelled than the nucleoli of protein-fed animals. The relative specific activity of the nucleolus with respect to the whole nuclear RNA is, however, greatest in the H.P. group and least in the H.P.F. group. (Table 25, Fig. 11).

(c) The activity of the phosphate fraction in general follows the same pattern in all three dietary groups, and is about twice as active as the cytoplasm after three hours labelling, although only half as active as the whole nuclear RNA. This is in agreement with the findings of other workers (Allfrey, Mirsky and Osawa, 1955; Allfrey and Mirsky, 1957; Logan, 1957; and Osawa, Takata and Hotta, 1958, for calf thymus nuclei; and by Hotta and Osawa (1958) for rat liver). This two-fold difference in specific activity between the phosphate fraction and the cytoplasm is further evidence that the phosphate RNA from rat liver nuclei is not cytoplasmic contamination. Indeed, Hotta and Osawa have shown that their phosphate-soluble RNA fraction of rat liver nuclei has a different base composition from microsomal RNA; starving the rats for 10 days and then refeeding them with protein did not alter the base composition (Hotta and Osawa, 1958).

In our experiments, the phosphate-extractable RNA showed the same response to dietary conditions as the nucleus and nucleolus; that is, a considerable increase in the specific activity of the L.P. group phosphate RNA, which was two or three times as great as the corresponding H.P. and H.P.F. fractions at 30 minutes after injection of the isotope. From Fig. 12, it will be seen that the relative specific activity of the L.P. group is also

Fig. 12.

Uptake of ^{14}C-adenine by the phosphate extract from liver nuclei of H.P.F. (●——●), H.P. (□——□) and L.P. (○——○) rats, expressed as a ratio of the uptake of ^{14}C-adenine by the whole nuclei (i.e. the relative specific activities).

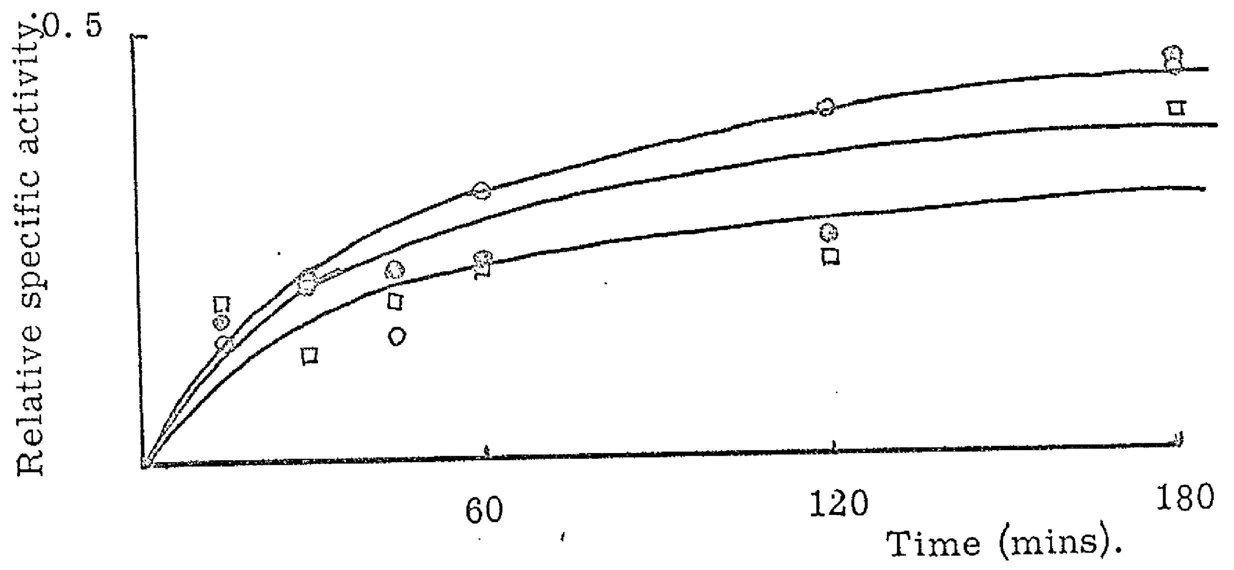


Fig. 12.

Fig. 13.

Uptake of ^{14}C-adenine by the $\underline{M}$ NaCl extract from liver nuclei of H.P.F. (●——●), H.P. (□——□), and L.P. (○——○) rats, expressed as a ratio of the uptake of ^{14}C-adenine by whole nuclei (i.e. the relative specific activities).

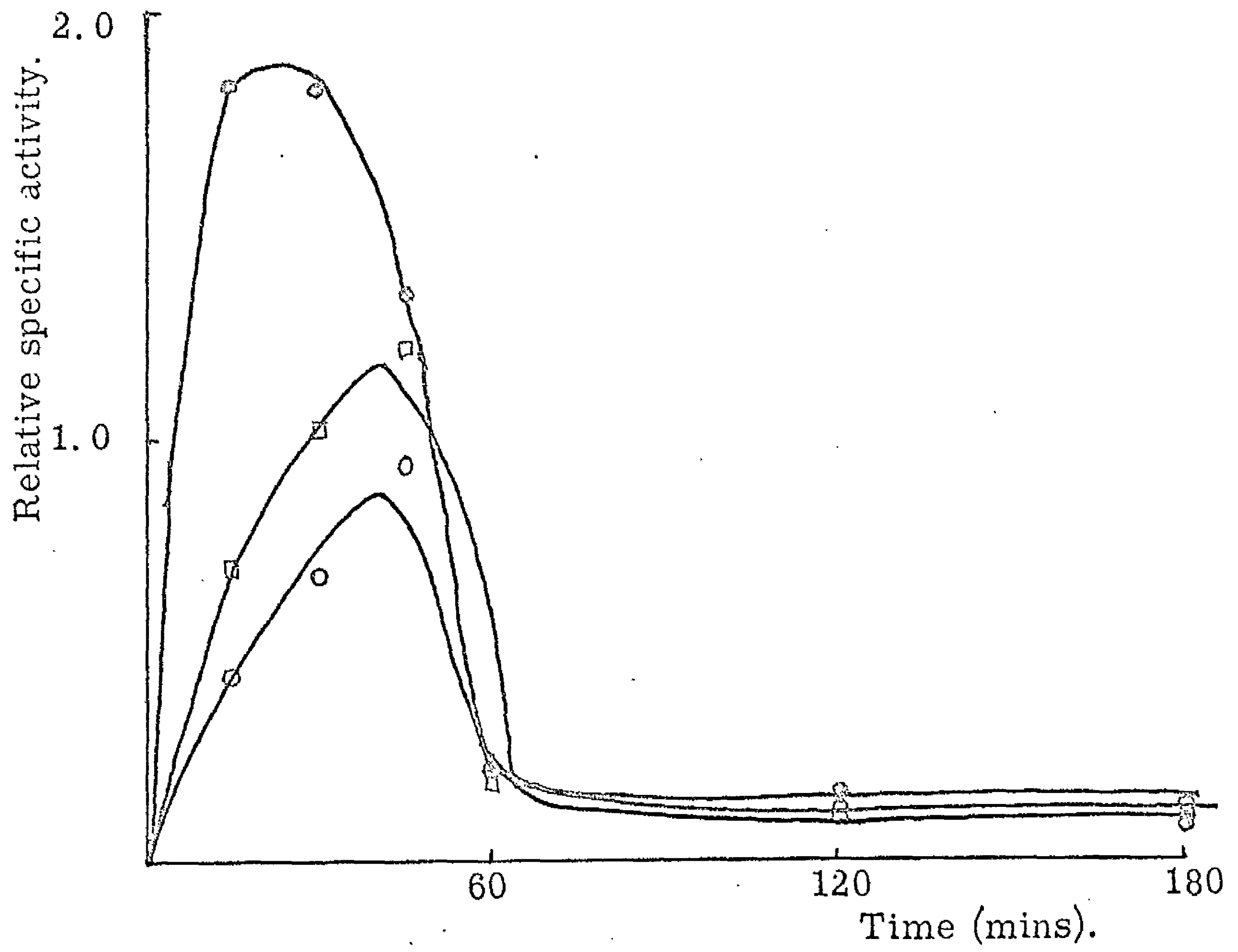


Fig. 13.

greater than that for either the H.P. or the H.P.F. livers, although the differences are not dramatic. The phosphate-soluble RNA is the only fraction of the nucleus whose relative specific activity is greater in the L.P. group than in either the H.P. or the H.P.F. groups, and must therefore contain the part of the nuclear RNA in which the increased rate of turnover due to protein withdrawal represents the greatest departure from normal.

(d) The RNA in the M NaCl fraction is labelled very rapidly, reaching a peak at 45 minutes after injection. At 60 minutes after injection, the activity in this fraction has disappeared, and the activity falls to below that of the cytoplasm, in all dietary groups. The increase in absolute specific activity observed throughout the fractions of the L.P. group is also maintained here (Fig. 9, Table 24). A striking effect of diet on the relative specific activities of this RNA from the three dietary groups is shown in Fig. 13, where the relative specific activity from the H.P.F. group at short time intervals is nearly twice that from the other two groups. This would suggest that the M NaCl RNA of the H.P.F. group is RNA which is synthesised in immediate response to the supply of amino acids available as a result of feeding protein. Our previous results indicate that the actual quantity of RNA in the M NaCl fraction is not significantly affected by diet, although the RNA of the combined phosphate and M NaCl fractions do show a significant decrease in the L.P. group. It is therefore probable that the rapidly-labelled RNA in the M NaCl fraction represents only a small proportion of the total M NaCl extractable RNA, but one which is very sensitive to supplies of amino acids.

Experiments using the Nucleolar Antibody.

During the experiments on salt extraction of rat liver nuclei, a series of tests was carried out using a fluorescent antibody which is found in the sera of patients suffering from a type of lupus erythematosus. Sera from some of these patients contain antinuclear antibodies, one of which is immunologically specific for nucleoli from both mammalian and amphibian somatic cells (Beck, 1963). If nuclei are first incubated with serum containing this antibody, the antibody specifically attaches to the nucleoli. If then the section is treated with fluorescein-tagged rabbit anti-human- γ-globulin, the human-γ-globulin attached to the nucleoli shows up as a fluorescent structure in otherwise unstained nuclei. This technique of staining is known as the "sandwich technique" (Weller and Coons, 1954). (See Fig. 14).

One of the fractions in the salt extraction scheme of Allfrey et al (1955), (and adopted by us), is described as the "nucleolar residue". A series of tests on this residue, and on the nuclear extracts, was made using the antibody staining technique to see if "nucleoli" prepared in this way could be identified in this residue. This was performed by a more complicated variant of the antibody technique, in which the extract was allowed to react with serum containing the antinucleolar antibody; if the extract did contain nucleolar antigenic material, it would remove the antibody. Thus, when the same serum was used after this treatment to identify nucleoli in intact cells, it would no longer do so, because it had lost its own antinucleolar antibody. The results of five antibody tests on rat liver nuclei and nuclear fractions are tabulated in Table 26.

Fig. 14.

Schematic representation of the "sandwich technique" of staining rat liver nucleoli with fluorescein-tagged-rabbit-anti-human-γ-globulin-human-nucleolar antibody complex. (see p. 71 and 72).

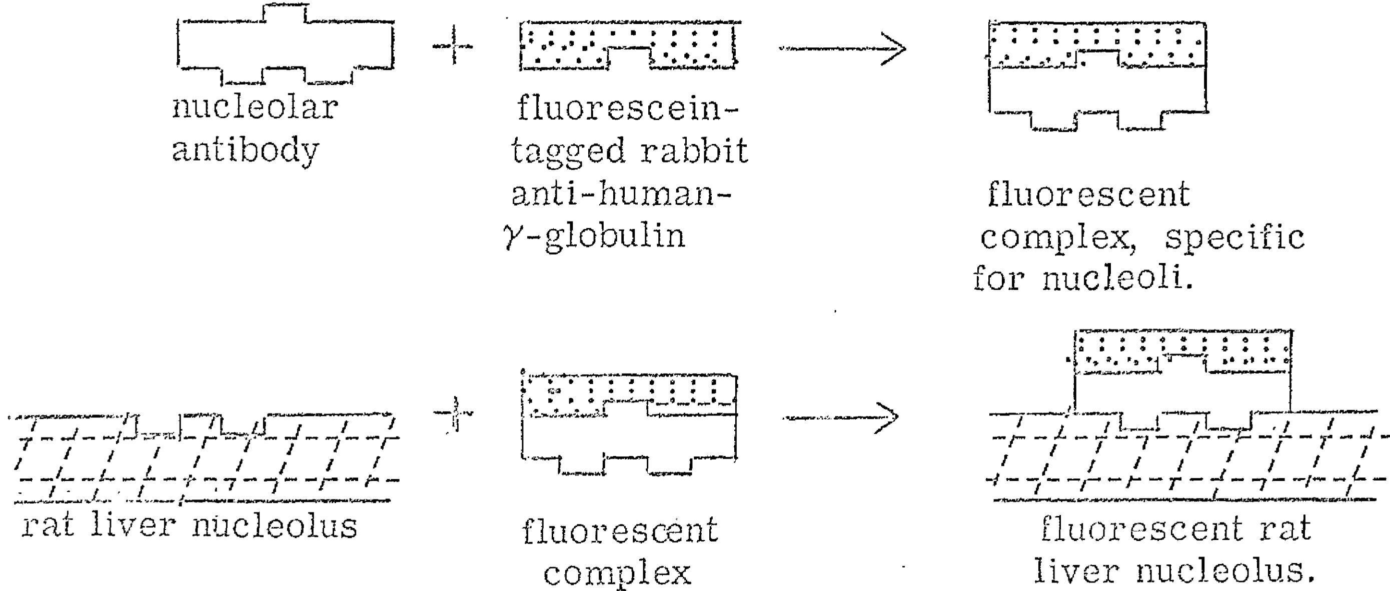


Fig. 14.

TABLE 26.

Table showing location of nucleolar antigen in subfractions of rat liver nuclei.

Expt.No.	Whole nuclei	Nuclear Fractions		
		PO_4 extract	M NaCl extract	Nucleolar residue
1	+	-	+	-
2	+	-	+	±
3	+	-	+	-
4	+	-	+	±
5	+	-	+	-

The results are expressed qualitatively since it is not possible, with this technique, to give a quantitative value to the degree of fluorescence observed. The reaction of nucleoli in the purified but unextracted rat liver nuclei to the antibody was identical in all three dietary groups.

It will be seen from Table 26 that the nucleolar antibody is almost completely extracted into the M NaCl fraction, although it is insoluble in pH 7.1 phosphate buffer. The nucleolar antigen has been recently shown to be RNase-sensitive, although the antibody is not absorbed by preparations of nuclear RNA (Beck, 1963). It is possible that the antigen is a protein (? histone) associated with nucleolar RNA.

The location of the nucleolar antigen in the M NaCl fraction raises the possibility of other nucleolar material, particularly RNA, being released into either the phosphate buffer or the sodium chloride during extraction (Maggio, Palade and Siekevitz, 1963), certainly report that 5-10% nucleolar RNA is soluble in M NaCl. It is possible that the antigen could be associated with the very highly active RNA found in the M NaCl, discussed in more detail later in this work. Whatever the final conclusions on these points, the extraction procedures clearly remove at least some components of intact nucleoli. It therefore follows that extraction with phosphate or M NaCl does not necessarily mean that the substance extracted is non-nucleolar in origin.

Loss of RNA and counts into PCA after salt fractionation of rat liver nuclei.

It was shown earlier in this work (p. 65) that there was a persistent loss of RNA from rat liver nuclei after salt fractionation, and acid precipitation of the fractions. Table 27 shows that there is also a substantial loss (20-30%) in nuclear counts after salt fractionation. This could represent the loss of a very highly active nuclear RNA into 0.2N PCA during acid precipitation, since the supernatants after acid precipitation of the undigested extracts were the only fractions discarded in these experiments. A similar loss of apparently highly active RNA into PCA has been reported by Levy and Lynt (1963) for HeLa cell RNA, and PCA-soluble RNA, chemically different from the RNA precipitated by PCA, has been found by Finamore and Volkin (1961) in amphibian eggs, and by Lu and Finamore (1963) in carp eggs.

In our experiments the amount of unrecovered RNA varied significantly between the three dietary groups, being highest for the protein-depleted animals, and lowest for the protein fed (Table 27). In order to establish whether this loss of RNA was a direct consequence of acid treatment, or whether some degree of sensitisation to acid occurred as a result of preliminary treatment with neutral salt solutions, a series of analagous extractions was carried out on samples of isolated nuclear RNA.

Nuclei were treated with SLS and phenol, and RNA was isolated from both phenol and aqueous phases by the method described in the "Experimental" section (p. 32). Samples of both types of RNA were treated with either 0.1M phosphate buffer pH 7.1 and 0.2N PCA, or M NaCl and PCA, or PCA alone, or left untreated (Table 7, p. 33) in order to reproduce the nuclear treatment on the isolated RNA; PCA precipitates and untreated nuclear RNA were digested in 0.3M KOH for 1 hour

TABLE 27

Table showing counts/100 μg DNA-P for whole nuclei and nuclear sub-fractions, and unrecovered counts, 1 hour after intraperitoneal injection of 5 μc ^{14}C-Adenine sulphate.

Dietary group	Whole nuclei	Counts/100 μg DNA-P in whole nuclei				Counts recovered as % whole nuclear counts	Unrecovered counts / 100 μg DNA-P.	Unrecovered RNA/100 μg DNA-P.	Sp. activity unrecovered RNA (c.p.m./100 μg RNA) (A) *	Sp. activity whole nuclear RNA (c.p.m./100μg RNA See Table 24) (B)	Sp. activity (A) / Sp. activity (B)
		Nuclear subfractions									
		PO_4 fraction	M NaCl fraction	Nucleolar residue	Counts recovered						
H.P.F.	60.5	3.5	2.3	38.2	43.0	71%	17.5	0.3	5,800	227	25.6
H.P.	67.6	3.6	3.2	49.2	56.0	83%	11.6	1.5	785	262	3
L.P.	173	10.0	6.0	112	128	74%	45	1.7	2,650	608	4.3

at 37°C to hydrolyse the RNA, and the alkaline digests were then acidified and the O.D. at 260mμ was read to determine the RNA content. Examination of the spectra of acid-precipitated RNA, and PCA supernatants from both phases showed that, whilst the acid-precipitated RNA was free from protein contamination in both aqueous and phenol RNA, the PCA supernatants in both cases were often considerably contaminated with peptide material. For instance, about 10% of the absorbancy at 260mμ of the PCA supernatant from the M NaCl-treated phenol phase was due to peptide contamination, as calculated by the method of Fleck and Munro (1962) for rat liver (see Fig. 15). Although this contamination means that the apparent recovery of "PCA-soluble RNA" would be increased, the interest in these experiments was primarily focused on the finding of a fraction in the PCA supernatant which was highly radioactive after labelling with ^{14}C-adenine, and it was considered that the errors introduced by increased absorption at 260mμ due to peptide contamination were of secondary importance to this objective.

Tables 28, 29 and 30 show the effect of PCA on nuclear RNA which has been treated with either neutral phosphate buffer or M NaCl followed by PCA, or with PCA alone, and Table 31 summarises these three Tables. The specific activities of the RNA are given as counts/minute/100μg RNA. The apparently high recoveries of RNA in the PCA supernatants are probably due to peptide contamination as noted above.

Tables 28 to 30 show that the counts released into the PCA supernatant from the highly radioactive RNA of the phenol phase are only a small proportion (2-8%) of the total counts, the M NaCl fraction tending to have slightly higher but less reproducible values (Table 29) than the others (Tables 29 and 30). The proportion of counts appearing in the PCA supernatant after treating phenol phase RNA with

Fig. 15.

U.V. absorption spectra for various KOH digests of PCA precipitates and supernatants of nuclear RNA, extracted with phenol, or of salt-treated nuclear RNA.

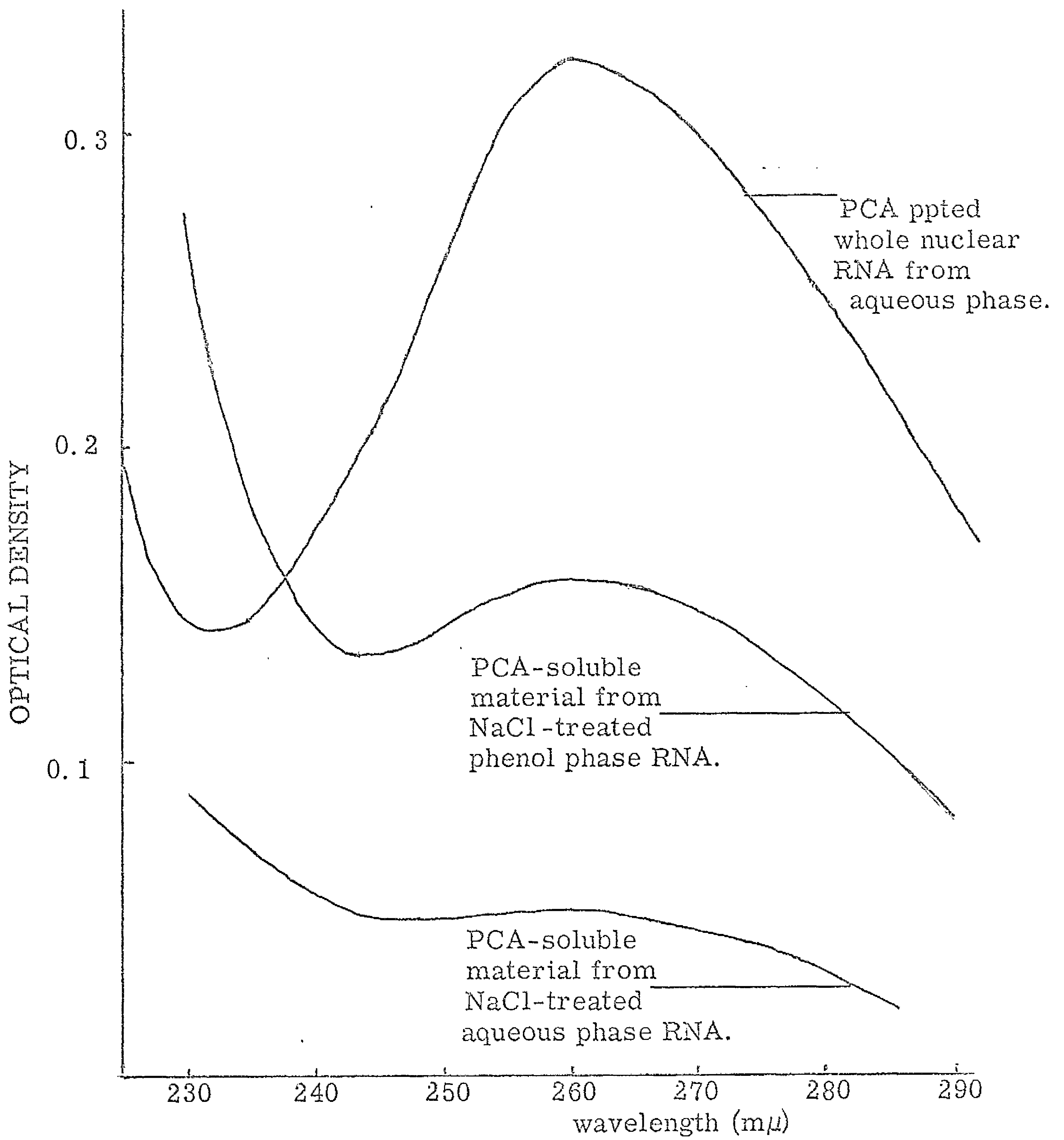


Fig. 15.

either of the salt extractants followed by PCA, or with PCA alone, does not vary with diet.

The proportion of counts lost from the aqueous phase RNA into the PCA precipitant is about three times that lost from the phenol phase RNA, irrespective of whether the aqueous phase has been treated with neutral salt solutions or not (Tables 28 to 30), although the absolute amounts of acid-soluble material, calculated from the absorbancy at 260mμ, are similar to that for the phenol phase RNA. The proportion of counts and U.V. absorbing material lost into the PCA supernatant from aqueous phase RNA treated with salt solutions, followed by PCA precipitation does not differ significantly from that released after acid precipitation alone (Tables 28 to 30). In addition, there is no discernable variation in the amounts lost into the PCA between the three dietary groups; indeed, the results cover a wide range of values in all the dietary groups, indicating a non-specific breakdown of the RNA. The results of this series of experiments are summarised below:

1. Phenol phase RNA.

(i) The proportion of counts released from the phenol phase RNA into the PCA supernatant after acid precipitation is very small, and is not significantly affected by pretreating the RNA with salt solutions, although there is a tendency for more counts to be released after treatment with M NaCl. Diet does not affect the release of counts into the PCA supernatants.

(ii) The release of U.V. absorbing material into the PCA supernatants is unaffected by pretreatment with salt solutions or by diet.

2. Aqueous phase RNA.

(i) The proportion of counts appearing in the PCA supernatant is higher (up to 48% of the total counts) than

that for the phenol phase RNA, but the values are very widely scattered (0-48%). There is no obvious alteration in the radioactivity released into the PCA after pretreatment with salt solutions, or due to dietary effect.

(ii) The amounts of acid-soluble U.V. absorbing material again vary widely between experiments, but are not significantly different from the values obtained with phenol phase RNA.

It would therefore appear that the labelled nuclear RNA released into the aqueous phase during isolation is possibly more sensitive to 0.2N PCA than the phenol phase RNA, but this sensitivity is non-specific and may well be due to the presence of RNA degradation products. These experiments have failed to show a highly active RNA consistently released into the PCA supernatant. Notably, they have shown no relation of results to diets fed.

TABLE 28.

Table showing specific activities, and proportion of counts and U.V. absorbing material lost into the PCA supernatant, after treating phenol and aqueous phase nuclear RNA from dieted rats with 0.1M phosphate buffer pH 7.1 followed by 0.2N PCA.

Diet	Specific activity (c.p.m/100µg RNA)				% Soluble in PCA			
	Phenol phase RNA		Aqueous phase RNA		Phenol phase		Aqueous phase	
	PCA insol.	PCA sol.	PCA insol.	PCA sol.	µg	counts	µg	counts
H.P.F.	1290	463	112	124	8	3	8	10
	2425	417	397	430	12	3	7	13
H.P.	1040	243	-	-	7	2	-	-
	885	259	268	474	7	2	10	15
	935	236	161	171	11	4	45	30
	2020	107	133	77	33	2	38	18
L.P.	1485	1040	163	400	10	2	6	2
	-	115	142	92	31	2	31	19

TABLE 29.

Table showing specific activities, and proportion of counts and U.V. absorbing material lost into the PCA supernatant, after treating phenol and aqueous phase nuclear RNA from dieted rats with M NaCl followed by 0.2N PCA.

Diet	Specific activity (c.p.m/100µg RNA)				% Soluble in PCA			
	Phenol phase RNA		Aqueous phase RNA		Phenol phase		Aqueous phase	
	PCA insol.	PCA sol.	PCA insol.	PCA sol.	µg.	counts	µg.	counts
H.P.F.	1260	420	123	510	17	6	8	24
	2250	485	381	830	33	10	26	48
H.P.	827	272	101	0	20	8	11	0
	694	154	253	352	17	3	15	17
	2470	113	151	92	43	2	42	24
	715	530	140	151	14	8	24	22
L.P.	1180	1040	122	400	12	11	8	19
	2350	104	175	187	41	3	39	49

TABLE 30.

Table showing specific activities, and proportion of counts and U.V. absorbing material lost into the PCA supernatant, after treating phenol and aqueous phase nuclear RNA from dieted rats with 0.2N PCA.

Diet	Specific activity (c.p.m/100µg RNA)				% Soluble in PCA			
	Phenol phase RNA		Aqueous phase RNA		Phenol phase		Aqueous phase	
	PCA insol.	PCA sol.	PCA insol.	PCA sol.	µg.	counts	µg.	counts
H.P.F.	935	188	120	200	11	2	3	7
	1820	331	379	243	14	3	15	8
H.P.	710	233	110	0	10	3	22	0
	606	636	256	250	16	1	10	8
	2340	54	118	109	33	1	35	24
	655	212	142	261	11	4	39	26
L.P.	1335	594	134	445	5	2	3	10
	1680	96	151	35	34	2	35	12

TABLE 31.

Table showing means of values given in Tables 28 to 30.

Extract	Diet	Specific activity (c.p.m/100µg RNA)				% Soluble in PCA			
		Phenol phase RNA		Aqueous phase RNA		Phenol phase		Aqueous phase	
		PCA insol.	PCA sol.	PCA insol.	PCA sol.	µg.	counts	µg.	counts
PO_4 and PCA	H.P.F.	1858	440	255	277	10	4	26	20
	H.P.	1223	236	213	299	15	3	26	20
	L.P.	1485	578	153	246	20	2	19	11
NaCl and PCA	H.P.F.	1755	453	252	570	25	8	17	36
	H.P.	1177	268	162	149	24	6	23	16
	L.P.	1765	572	149	294	27	7	24	34
PCA	H.P.F.	1378	260	250	222	13	3	9	8
	H.P.	1078	141	157	155	18	3	26	15
	L.P.	1508	345	143	240	20	2	19	11

TABLE 32.

Table showing distribution of RNA and radioactivity between aqueous and phenol phases after treating nuclei with various concentrations of sodium lauryl sulphate.

SLS conc'n.	Phase	Total in sample	µg RNAP in sample	µg DNAP in sample	DNAP/RNAP in sample	% counts total counts in aqueous and phenol phases	% RNA RNA in aqueous and phenol phases
0.05%	Aqueous	40	7.0	1.1	0.16	5	22
"	Phenol	720	24.2	32.6	1.35	95	78
0.1%	Aqueous	80	16.7	1.3	0.08	10	45
	Phenol	730	21.5	22.6	1.05	90	55
0.2%	Aqueous	220	22.5	1.5	0.07	40	77
	Phenol	330	6.7	19.6	2.90	60	23
0.5%	Aqueous	365	23.1	25.1	1.09	55	77
	Phenol	290	7.0	1.5	0.21	45	23
1.0%	Aqueous	415	36.7	26.9	0.73	73	87
	Phenol	160	5.3	0	0	27	13

Sedimentation Analyses of rat liver RNA.

Recently developed techniques of separating undenatured RNA from both bacterial and mammalian sources on sucrose density gradients have been very successful in elucidating the cellular origins of the RNA components, and their relative metabolic activities (e.g. Bolton et al, 1960; Hiatt, 1962; Scherrer, Latham and Darnell, 1963). Our own studies, reported earlier, have shown that the salt extractable RNA components of the rat liver nucleus have different metabolic properties which are very significantly affected by diet, but we still had very little knowledge of the nuclear site from which these fractions originated. We therefore used sucrose density gradients in attempts to separate the components of nuclear, cytoplasmic, and whole liver RNA from all three dietary groups. It was hoped that information gained on molecular size and metabolic activity of the RNA components would give some indication of their origins and functions.

In later experiments, the number of components of RNA isolated from the nucleus, cytoplasm, and whole liver was determined using the Schlieren optical system of the Spinco Model E Ultracentrifuge; from these experiments it was possible to calculate approximate sedimentation constants for the components observed, and their relative proportions in the original RNA sample.

Separation of rat liver RNA on sucrose density gradients.

1. Nuclear RNA.

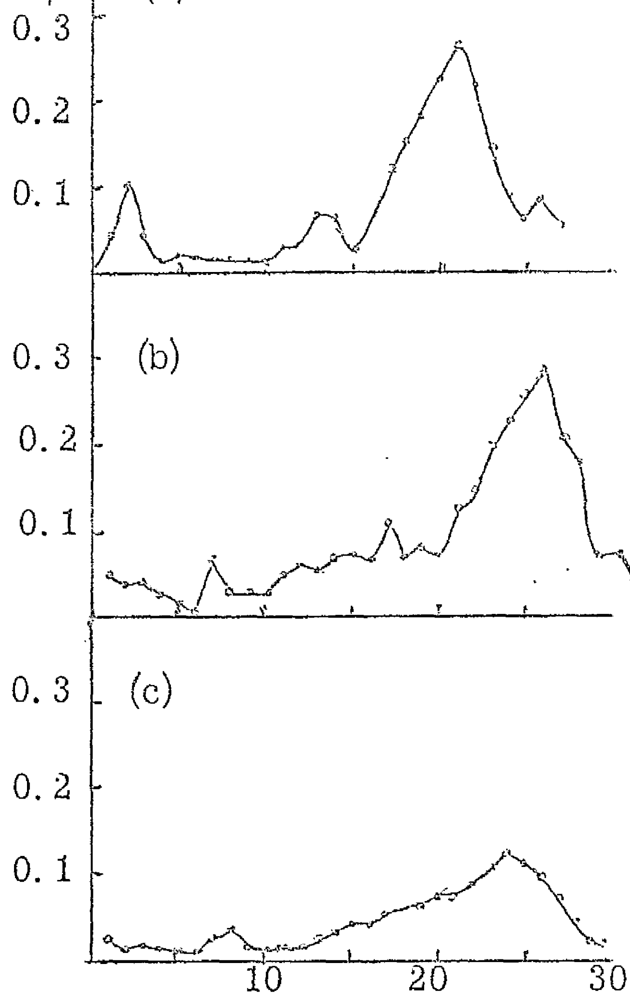
Density gradients analyses were first performed on RNA isolated by the method of Reiner et al (1963) from lipid-extracted nuclei by treating them with 0.2% sodium lauryl sulphate and phenol. RNA solutions at a concentration corresponding to 10 O.D. units at 260mμ were layered on to a linear gradient of 3-20% sucrose buffered with 0.01M tris at

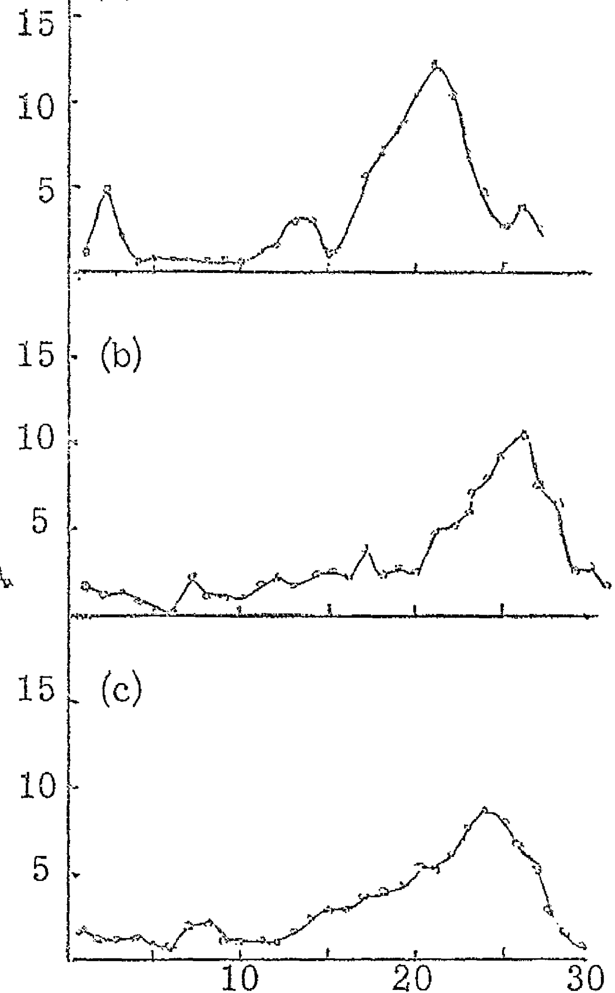
Fig. 16.

Profile of rat liver nuclear RNA after 11 hours centrifugation on a 5-20% sucrose gradient. The RNA was prepared by the method of Reiner *et al* (1963).
(a) H.P.F. group, (b) H.P. group, (c) L.P. group.

Fig. 17.
The results obtained from Fig. 16 presented as O.D. %. (a) H.P.F. group, (b) H.P. group, (c) L.P. group.

Fig. 18.
The results of Reiner *et al* (1963) for comparison. (a) Fresh nuclei, (b) "Aged" nuclei.

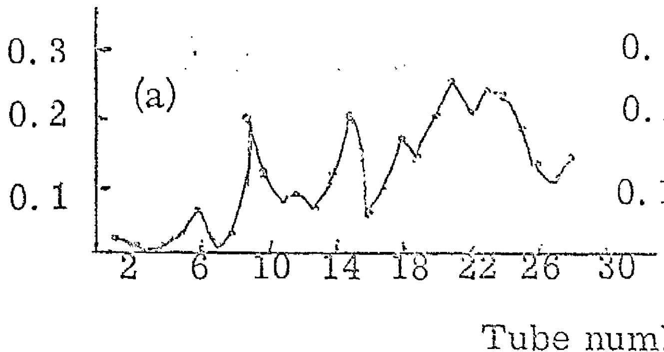


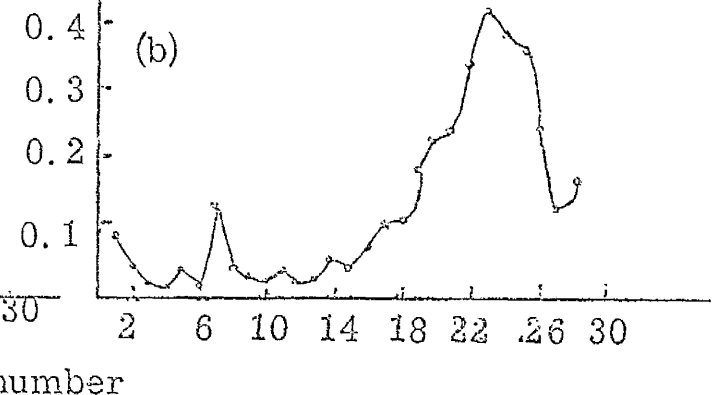


Tube number

Fig. 16.

Fig. 17.





Tube number

Fig. 18

* $\text{O. D.\%} = \dfrac{\text{O. D of individual fraction}}{\text{summated O. D.}}\%$

pH 7.3, and spun overnight at 23,000 r.p.m. in the SW39 head of the Spinco Model L Ultracentrifuge. In one experiment, the rats were injected with 5µc ^{14}C-adenine sulphate 1 hour before killing, but the counts recovered were too low to be significant.

The U.V. profiles obtained from samples of RNA prepared by this method are shown in Fig. 16, for all three dietary groups. The RNA profiles published by Reiner et al (1963) for RNA prepared from both "fresh" and "aged" nuclei are also shown in Fig. 18. The profiles obtained both by Reiner et al and by us for nuclear RNA suggest that rat liver nuclear RNA is a very heterogeneous mixture of molecular sizes, most of which are in the range 13-4S. Fig. 17 shows that only about 10% of the RNA recovered is greater than about 13S, although the profile obtained by Reiner et al (1963) for RNA from "fresh" nuclei shows that about 30% is composed of RNA of molecular size greater than 13S. Our nuclei, although "fresh", gave a similar picture to their "aged".

It seemed probable that the RNA prepared by this method was degraded to some extent, possibly as a result of RNase activity during the prolonged extraction of the nuclei with SLS buffer at 37°C. Indeed, observation showed that the effectiveness of SLS for rupturing the nuclei and releasing the nuclear RNA was established immediately on contact with the nuclei: clumps which formed on treating the nuclear pellet with weak (0.05%) SLS solutions did not disperse even after prolonged extraction. A preliminary experiment was therefore run to test the efficiency of various concentrations of SLS in releasing nuclear RNA.

Male albino rats, fasted overnight, were injected with 5µc ^{14}C-adenine, killed 1 hour later, and the liver

nuclei extracted in 2.2M sucrose. After rinsing the nuclear pellet in ice-cold distilled water, the nuclei were suspended in 10.5ml acetate buffer, 1ml aliquots of the nuclear suspension were pipetted into each of 10 tubes, and SLS was added to the final concentrations shown in Table 32. The nuclear suspensions were shaken vigorously while the SLS was being added, and immediately after addition of the SLS an equal volume of 90% phenol was added. The mixture was shaken for an hour at $4^{o}C$, the phases were separated by spinning at 30,000g for 30 minutes, and the upper aqueous phase was withdrawn. Both phenol and aqueous phases were washed once with aliquots of the opposing phase, and the washings were pooled with the original phases.

RNA in the aqueous phase was estimated by treating the aqueous phase with an equal volume of 0.4N PCA, containing 3mg/ml ^{12}C-adenine as quencher, washing the precipitate twice with 0.2N PCA, and digesting the precipitate for an hour in 0.3N KOH at $37^{o}C$ by the method of Fleck and Munro (1962). The digest was acidified to 0.2N PCA to precipitate DNA and protein; the acidified supernatants containing the RNA digestion products were made up to 2.5ml, and 0.5ml aliquots were pipetted out on to stainless steel planchets and counted for 500 counts. The remaining solutions were made up to 10ml and the U.V. absorption was read at 260mμ.

The phenol phase was washed once with buffer containing cold adenine and the phenol was dissolved in 3 volumes of ice-cold ethanol-ether 3:1, and discarded. The precipitate was washed three times with ice-cold ether to remove all traces of U.V. absorbing phenol, and three times with ice-cold 0.2N PCA, the first wash containing 3mg/ml ^{12}C-adenine as quencher. The precipitate was then digested for an hour in 0.3N KOH at $37^{o}C$, and the DNA and protein

were precipitated as before. 0.5ml aliquots of the supernatant were plated out for counting, and the U.V. absorbancy of the remaining ribonucleotide solution was determined at 260mμ, as for the aqueous phase. The acid precipitates for both phases were dissolved in 0.3N KOH and the DNA estimated by the method of Ceriotti (1952). The results are shown in Table 32 and are the mean of duplicates.

Table 32 shows that concentrations of 0.2% or less of SLS release less than 50% of the counts into the aqueous phase, although at 0.2% SLS concentration nearly 80% of the RNA is released. It is not until the concentration of SLS is high enough to release a significant amount of DNA as well (at 0.5%) that the highly labelled fraction of the RNA is also solubilised. We therefore used 1% SLS in all following experiments, despite the resulting high DNA contamination in the RNA preparation, since we considered it more important to be able to extract the high counting RNA from the nuclei than to have preparations of nuclear RNA free from DNA: this was removed by DNase under the conditions described in the Methods Section. Since the previous experiments with sucrose density gradients had indicated that the use of ^{14}C-adenine was likely to prove expensive, ^{32}P-orthophosphate was used as the radioactive precursor of RNA, since this isotope could be conveniently used in much larger quantities.

Separation of radioactive nuclear RNA.

Nuclear RNA was isolated in the presence of bentonite, 8-hydroxyquinoline and naphthalene 1:5-disulphonate (NDS) using 1% SLS, by the procedure described on p. 47, from the liver nuclei of dieted rats which had been injected with 200-700μc ^{32}P-orthophosphate 1 hour before killing. The actual profiles obtained, after density gradient centrifugation, for both U.V. absorbancy and radioactivity for all three dietary groups are shown in Figs. 19 and 20 .

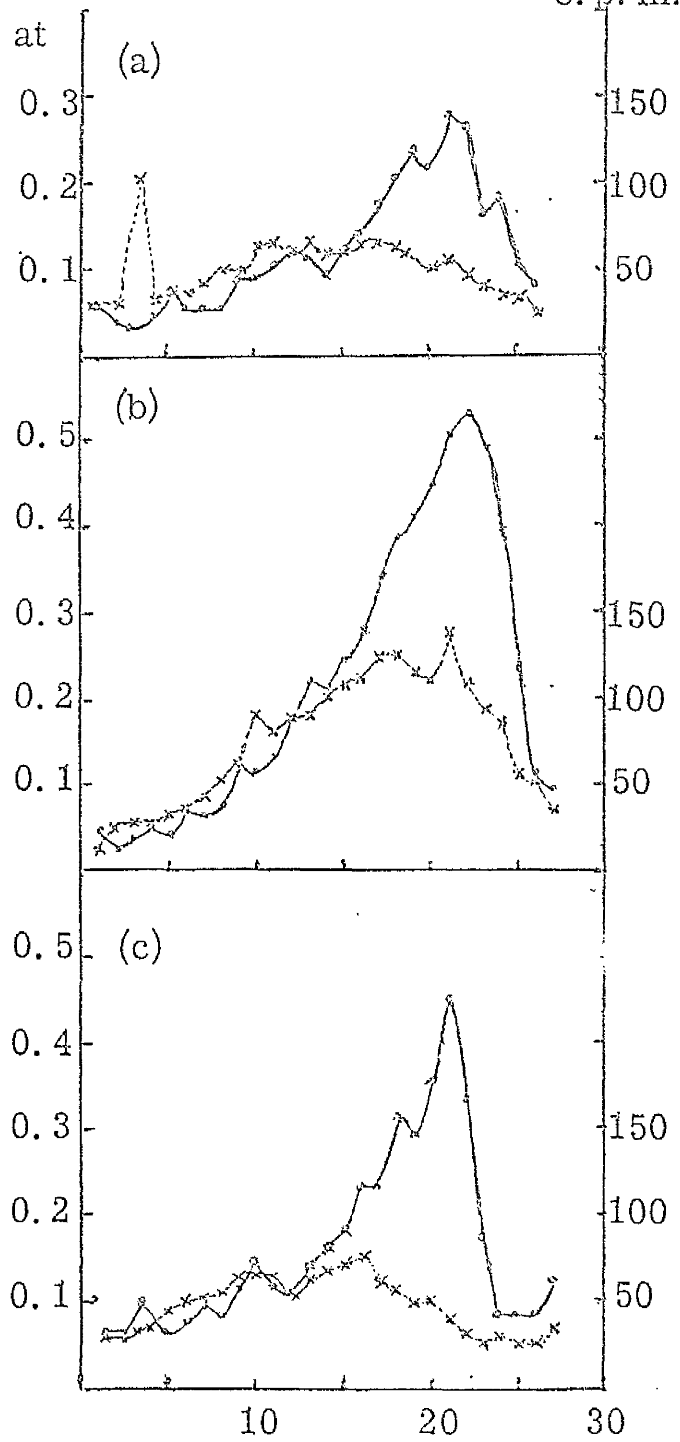
The diagrams shown in Fig.20 were arrived at by summating the values obtained for optical density or radioactivity in all fractions, and then expressing the values for the individual fractions as a percentage of the total O.D. or radioactivity recovered. By expressing the result in this way, it was possible to obtain a direct comparison of the relative amounts of U.V. absorbing material and radioactivity in each fraction, and to eliminate the variation in radioactivity levels due to differences in the level of the original dose given. It also gave a measure of specific activity.

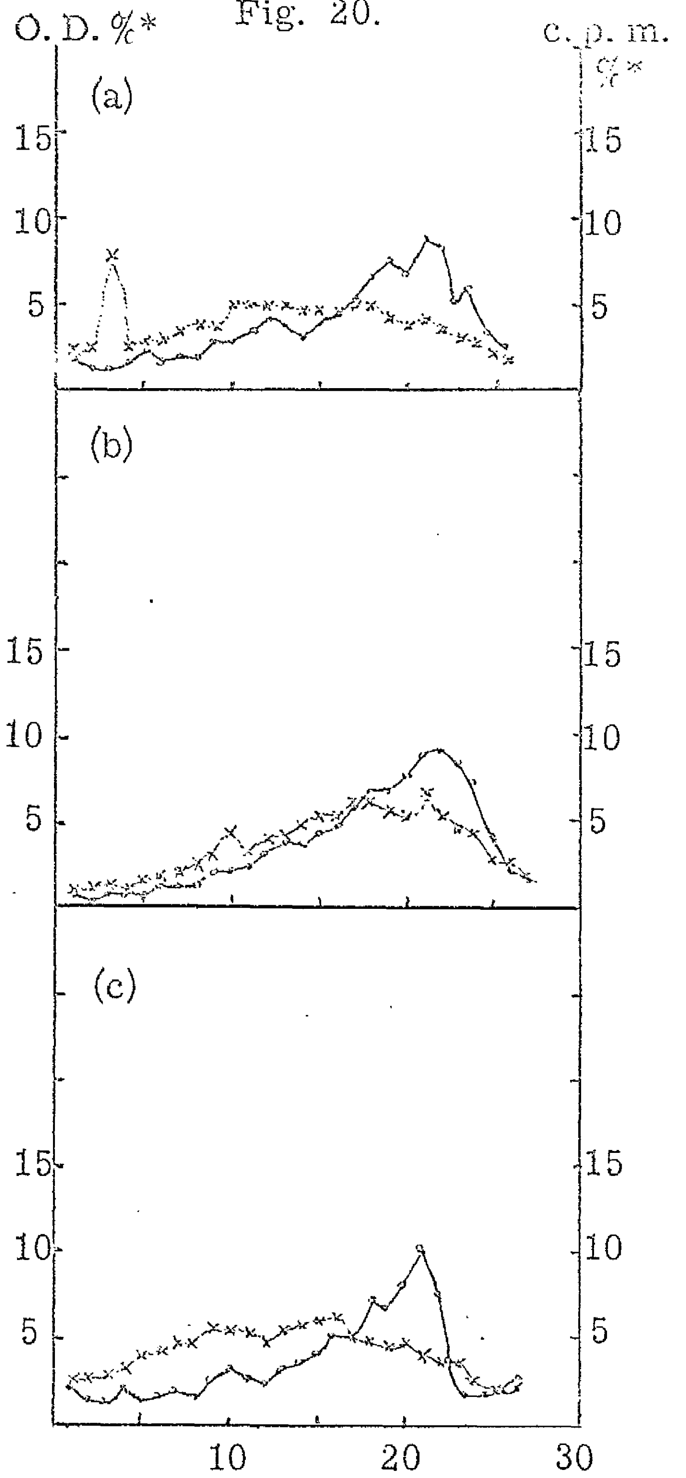
The profiles illustrated in Fig. 19 show that the nuclear RNA from all three dietary types again separates into a heterogeneous mixture of molecular sizes, mostly 4 to 16S. This result was surprising since it suggested that degradation of the RNA had occurred, despite the addition of the RNase inhibitors bentonite, DNS, and 8-hydroxyquinoline to the nuclear suspension, and the strict temperature controls observed throughout the isolation of the nuclei and nuclear RNA. It was therefore considered that either degradation by some other means had occurred (e.g. some hydrolysis by a contaminating enzyme during DNase treatment), or that the profiles obtained were a correct representation of the distribution or RNA molecular size in nuclear RNA. If one assumes the latter to be correct, then several points emerge from the O.D. and radioactivity profiles illustrated in Figs. 19 and 20 .

(i) The optical density patterns for all three dietary groups are very similar: no major shift in the distribution of molecular size has occurred with diet. There is, however, less heavy RNA (i.e. O.D. in tubes 1-10) in the H.P. group than in either the H.P.F. or L.P. groups (Fig. 20).

Fig. 19.
Profiles of optiwal density and radioactivity obtained for rat liver nuclear RNA isolated in the presence of bentonite, NDS and 8 hydroxy quinoline. Rats were injected with 100-150uc ^{32}P-orthophosphate and killed 1 hour later. (a) H.P.F. group, (b) H.P. group, (c) L.P. group.

Fig. 20.
Results obtained from Fig. 16 are presented as O.D. % and c.p.m. %. (a) H.P.F. group, (b) H.P. group, (c) L.P. group.





Tube number

—o—o— = O.D.
-x-x-x-x- = c.p.m.

* O.D.% = $\frac{\text{O.D. of individual fraction}}{\text{summated O.D.}}$ %

* c.p.m.% = $\frac{\text{c.p.m. of individual fraction}}{\text{summated c.p.m.}}$ %

(ii) There are some differences in the radioactivity patterns obtained for the three dietary groups. Both H.P.F. and H.P. groups are similar (Fig. 19), except that one fraction (tube 3) in the H.P.F. group is very heavily labelled. Out of three sucrose density gradient separations of nuclear RNA from the H.P.F. group, two had this peak. The interfacial RNA/DNA complex of Kidson et al (1963) also showed a heavily labelled fraction in this region with fed animals. If this peak is a correct finding, the possibility arises that it corresponds to the heavily labelled RNA appearing in the M NaCl fraction in H.P.F. animals 45 minutes after injection (p. 70). The distribution of the percentage of radioactivity through the gradient from the L.P. group shows that the specific activity of the heavy RNA (tubes 3-16) is higher in this group than for the other two, with the exception of the very active peak in tube 3 of the H.P.F. group (compare Fig.13, p. 70). The specific activity of the light RNA is, if anything, lower in the L.P. group than in either the H.P.F. or H.P. groups. The increased specific activity in tubes 3-16 suggests that the nuclear ribosomal RNA, and other nuclear species with an S value greater than about 14S, turns over more rapidly in the L.P. group than in the H.P.F. or H.P. groups, and would agree with our earlier findings of increased specific activity of the nuclear RNA of L.P. rats.

(iii) It is interesting to observe that the lightest RNA in nuclei is about 4S, but there is very little activity associated with this area (Fig. 20). In contrast, Figs. 24-26 for whole liver and cytoplasm show considerable activity in the 4S peak: this is discussed in more detail later. The absence of radioactive material at the top of the gradient is evidence in favour of the nuclear RNA preparation being undegraded: if it were degraded one would

predict that a higher proportion of the counts would appear in the terminal tubes, as in the profiles obtained for RNase treated RNA (Kidson, Kirby and Ralph, 1963; Hill, Miller-Faures and Errera, 1964).

A survey of the literature made at this point showed that the patterns obtained by other workers of the components of nuclear RNA separated on a density gradient were various. Hiatt (1962) and Jewell and Osnes (1963) have both obtained three components of rat liver nuclear RNA separated on a sucrose density gradient. However, Sporn and Dingman (1963) have pointed out that Hiatt's nuclei were significantly contaminated with cytoplasm. Indeed, our survey of the methods used for isolating nuclei (p. 53) would indicate that the RNA/DNA ratio of the nuclei used by Hiatt, and by Jewell and Osnes (1963) would be about 1.0, instead of the ratio 0.25 - 0.27 for pure nuclei. That is, about 65% to 75% of the RNA in their preparations would be cytoplasmic in origin. The profiles obtained by Harris (1963) for HeLa cell nuclei isolated using Tween 80 contained 28S and 16S components, but again, we were unable to prepare pure nuclei by this method, and the above criticism may still apply. Certainly 28S and 16S are values ascribed by Scherrer, Latham and Darnell (1963) and by Tamaoki and Mueller (1962) to the cytoplasmic ribosomal components of HeLa cell RNA. On the other hand, profiles of rat kidney nuclear RNA obtained by Revel et al (1963) are similar to ours in that the RNA separates into a heterogeneous mixture, with a large proportion of the radioactivity and optical density appearing in the top half of the gradient. Also, Kidson et al (1963), show that the very active RNA/DNA complex of rat liver, appearing in the phenol-water interphase during extraction with phenol, is also a heterogeneous mixture of relatively small molecules, but

since this preparation comprises both RNA and DNA, the profile obtained is not representative of pure RNA. In addition, Georgiev et al (1963) obtained a similar pattern for Ehrlich ascites cell nuclear RNA released after treatment with phenol at 65°C, and corresponding to the highly active "DNA-like RNA". Sporn and Dingman (1963) have given convincing evidence that the RNA from pure rat liver nuclei has three components of 33S, 19S and 6S, with an increasingly large proportion sedimenting with the 6S peak as the magnesium concentration increases. At the level of 1mM Mg^{2+}, used by us, they showed that a large proportion would be in the 6S region. In order to test this hypothesis, we therefore prepared nuclear RNA both in the presence and absence of Mg^{2+}, and compared the RNA profiles. In the sample which was prepared in the absence of Mg^{2+}, 0.01M EDTA, pH 7.3, was added. The usual precautions against RNase activity were observed throughout. The alteration in pH was not thought to be significant, since it has been established that liver RNA separated at pH 5.25 or pH 7.3 does not show any significant differences in sedimentation pattern (Scherrer, Latham and Darnell, 1963).

The sedimentation patterns obtained for both samples are shown in Fig. 21; there appears to be no difference in the sedimentation rate of the components. In our hands, therefore, the presence or absence of magnesium ions has no effect on the separation of rat liver nuclear RNA. This coincides with the findings of Scherrer et al (1963) for whole cell RNA from HeLa cells.

It has been suggested by Barlow et al (1963) that cane sugar sucrose preparations (e.g. B.D.H. AnalaR), contain RNase. If this is true it would explain the differences in the patterns obtained for nuclear RNA separated on a sucrose

Fig. 21.
O.D. profile of liver nuclear RNA from H.P. rats, after 11 hours centrifugation on a 5-20% sucrose gradient. (a) Centrifugation carried out in presence of 0.001M Mg^{2+} ; (b) RNA prepared in presence of 0.01M EDTA.

Fig. 22.
O.D. profile of liver nuclear RNA from H.P. rats, after 11 hours centrifugation on a 5-20% sucrose gradient that was pretreated with bentonite.

Fig. 23.
(a) O.D. profile of liver nuclear RNA from H.P. rats, after 11 hours centrifugation on a 5-20% gradient. RNA prepared in absence of Mg^{2+}, and presence of 0.05M NaCl.
(b) As (a), but RNA prepared in presence of 0.5M NaCl.

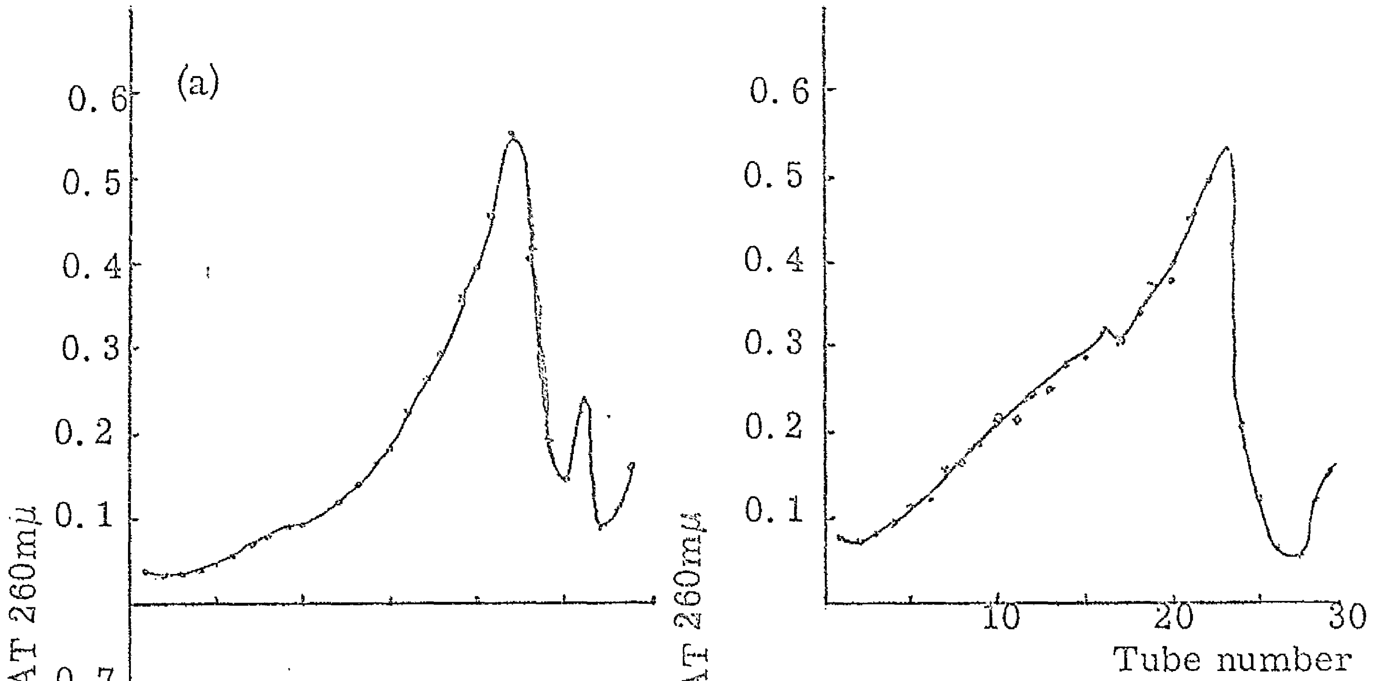


Fig. 22.

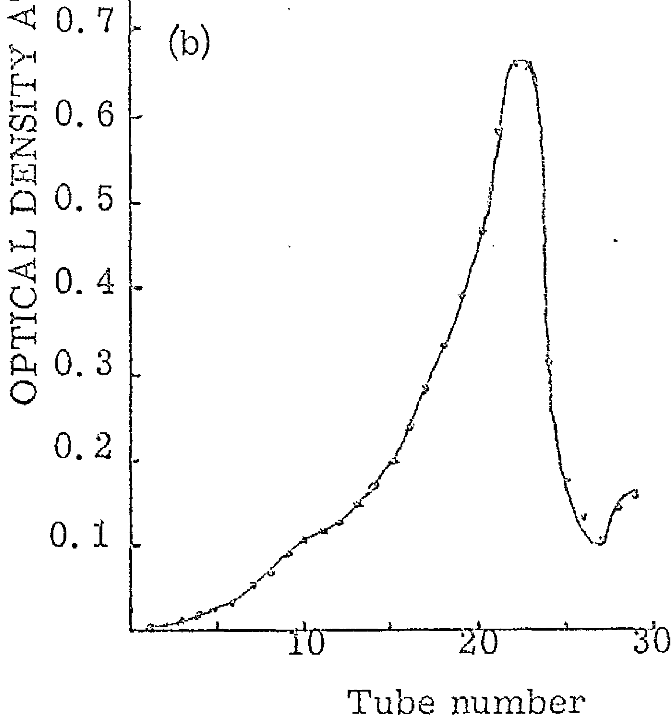


Fig. 21.

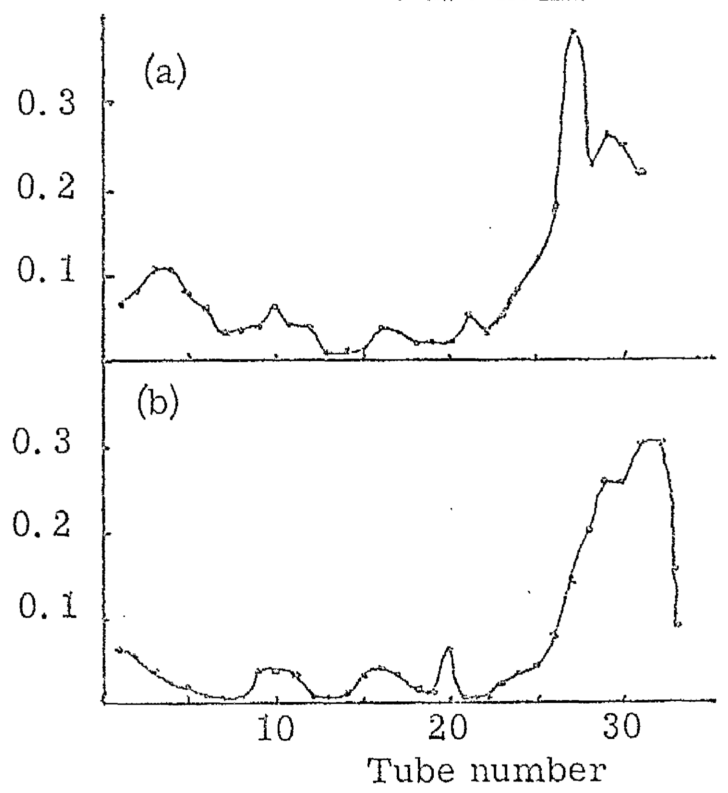


Fig. 23

density gradient, and those obtained using the Schlieren optics of the Spinco Model E Ultracentrifuge (see p.92 and e.g. Fig. 31). A sample of nuclear RNA from H.P. rats was therefore separated on a gradient of which the component sucrose solutions (5% and 20%) had been pre-treated with bentonite. The profile obtained for RNA separated on this gradient was similar to those obtained previously (Fig.22).

A possibility that intermolecular binding between the RNA molecules was interfering with the degree of separation obtained on the gradients was investigated by preparing a sample of nuclear RNA from H.P. rats in acetate buffer containing 0.5M NaCl. This high salt concentration should prevent intermolecular binding between RNA species. In this experiment, the sucrose solutions were pre-treated with bentonite, and buffered with acetate buffer containing 0.5M NaCl. The U.V. profile obtained is shown in Fig.23a, from which it is clear that this ionic strength in the medium has no effect on the separation of nuclear RNA.

The results from these experiments are open to the uncertainty that the RNA is degraded. However, it is possible that the profiles obtained are correct. If this is true, then the conclusions to be drawn, outlined on pages 81 and 82, are that (i) nuclear RNA mostly contains components of molecular size 4-16S; (ii) for all dietary groups, the heavy RNA has the highest specific activity, but the L.P. group has the highest specific activity of the three dietary groups; the 4S peak in all groups has the lowest activity; (iii) there is a heavily labelled peak in the H.P.F. group which sediments faster than 28S, and which may correspond to the highly labelled RNA found in the <u>M</u> NaCl extract of H.P.F. nuclei.

2. <u>Whole liver and cytoplasmic RNA.</u>

In addition to nuclear RNA, samples of whole liver and

cytoplasmic RNA from dieted rats were also separated on a sucrose density gradient. It was hoped that possible differences in the patterns obtained from these two samples might provide further information on the distribution of molecular size in nuclear RNA. Both whole liver and cytoplasmic RNA were prepared from dieted rats which had been injected with 100 - 700μc ^{32}P-orthophosphate 1 hour before killing. RNA was isolated from both homogenates by the procedure described for nuclear RNA, similar precautions against RNase activity being observed throughout, and DNase treatment of both samples being identical to that employed during the isolation of nuclear RNA.

U.V. and radioactivity profiles obtained for whole cells and cytoplasmic RNA are shown in Figs. 24 to 27, for all three dietary groups. The RNA samples in all cases separated into three major components, which is in agreement with the findings of other workers for both mammalian and bacterial RNA (e.g. Hiatt, 1962, for rat liver and E. Coli RNA; Scherrer, Latham and Darnell, 1963, for HeLa cell RNA; and Harris et al, 1963, for HeLa cell RNA). The three major peaks are assumed to correspond to the 29S, 18S and 4S RNA components in rat liver found by Gierer, (1958). However, comparison of the three groups indicate that there are some significant changes in the profile of rat liver cell RNA with diet, and particularly after protein withdrawal.

a) The U.V. profile obtained for both whole cell and cytoplasmic RNA from L.P. rats indicates the presence of a fourth component, greater than 30S. The fact that the percentage of this component is higher in cytoplasmic RNA than it is in whole cell RNA would indicate that it is not present in the nucleus (Fig. 27). An additional peak of 36-43S, constituting 6% of the total RNA sample, was also present in whole cell RNA from L.P. rats when a sample was analysed

Fig. 24.

Profiles of O.D. and radioactivity obtained for whole rat liver homogenate after centrifuging for 11 hours on a 5-20% sucrose gradient. (a) was obtained from the gradient illustrated in Fig. 3(a), whilst (b) and (c) were separated on a 3(b) gradient. (a) H.P.F. group, (b) H.P. group, (c) L.P. group.

Fig. 25.

O.D. and radioactivity obtained in Fig. 24 expressed as O.D. % and c.p.m. %.

Optical density at 260mμ

c. p. m.

(a)

0. 3 0. 2 0. 1

150 100 50

(b)

0. 6 0. 5 0. 4 0. 3 0. 2 0. 1

300 250 200 150 100 50

(c)

0. 3 0. 2 0. 1

150 100 50

10 20 30

Tube number

Fig. 24.

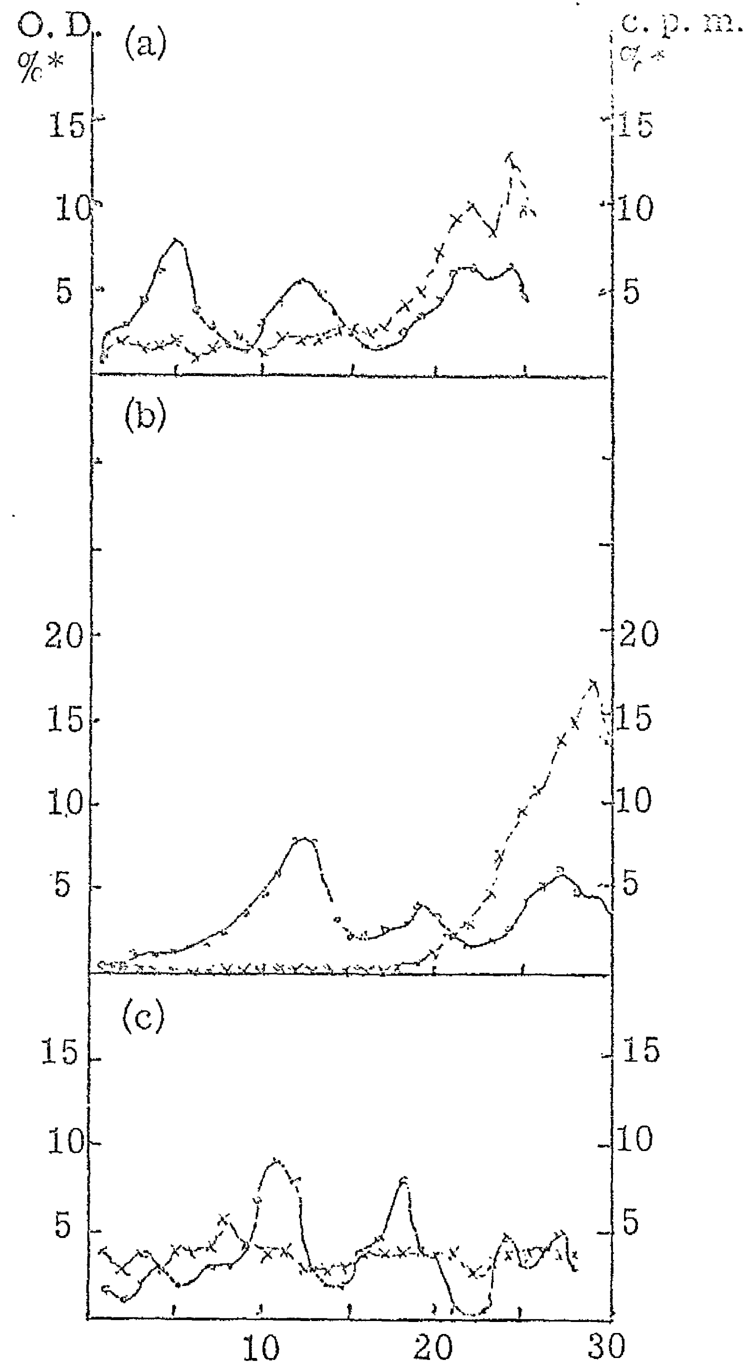


Fig. 25.

$$*O.D.\% = \frac{\text{O.D. of individual fraction}}{\text{summated O.D.}}\%$$

$$*c.p.m.\% = \frac{\text{c.p.m. of individual fraction}}{\text{summated c.p.m.}}\%$$

Fig. 26.
O.D. and radioactivity profiles obtained for whole rat liver homogenate after centrifuging for 11 hours on a 5-20% secrose gradient. Both (a) and (b) were obtained from gradient type 3(a), and (c) was separated on a gradient type 3(b). This would explain the shift in positioning of the peaks found in (c). (a) H.P.F. group, (b) H.P. group, (c) L.P. group.

Fig. 27.
O.D. and radioactivity obtained in Fig. 26 expressed as O.D.% and c.p.m.%.

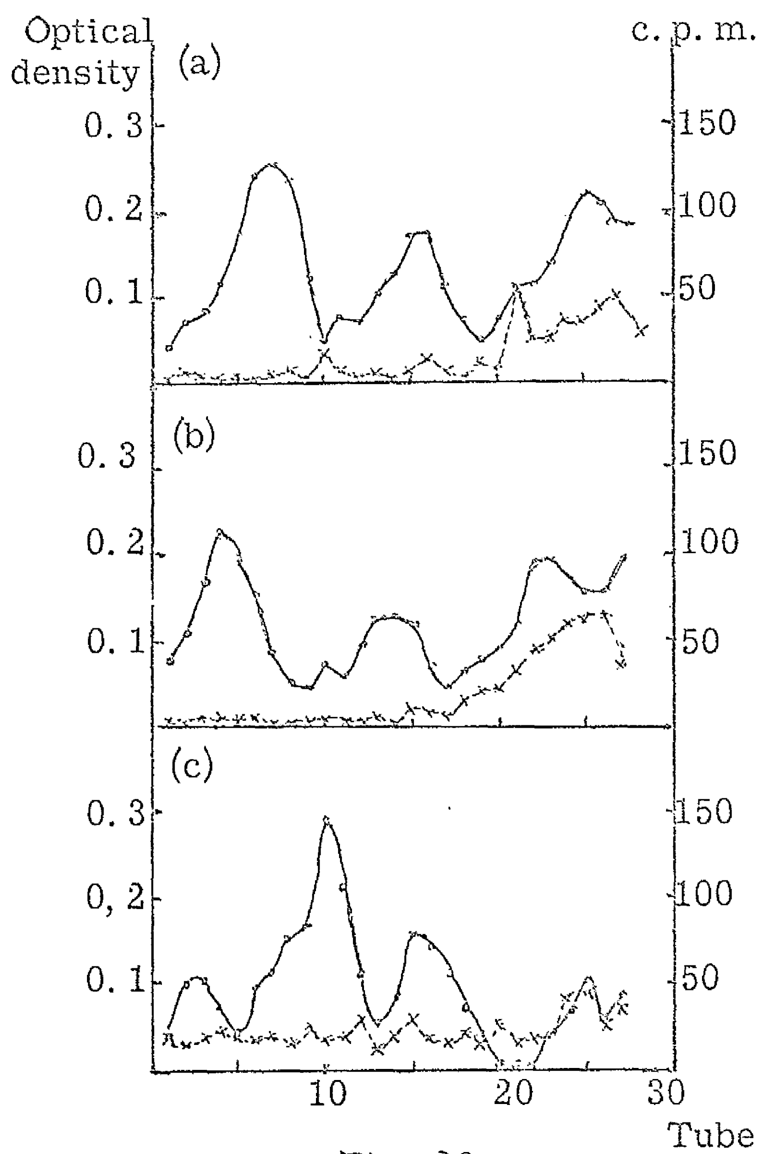
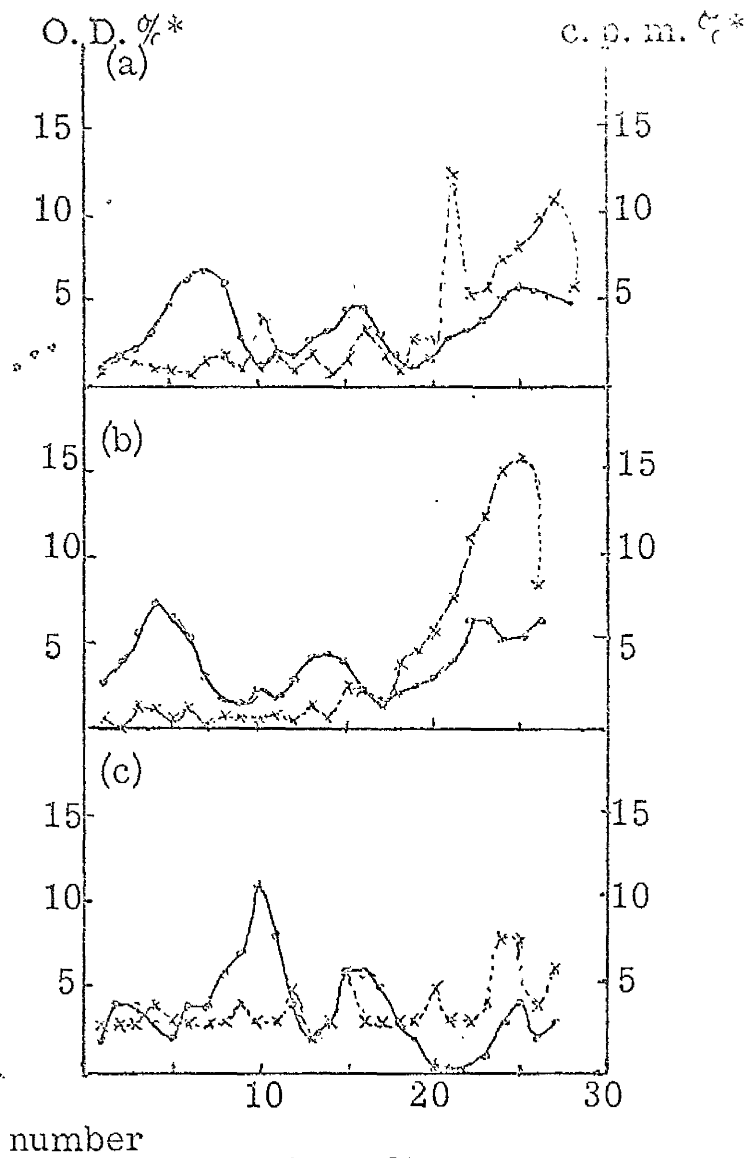


Fig. 26.



Tube number

Fig. 27.

$$* \quad \text{O.D.\%} = \frac{\text{O.D. of individual fraction}}{\text{summated O.D. of all fractions}} \%$$

$$* \quad \text{c.p.m.\%} = \frac{\text{c.p.m. of individual fraction}}{\text{summated c.p.m.}} \%$$

in the analytical ultracentrifuge (Fig.30 p. 90): this peak was present in much smaller proportions in the RNA of the H.P.F. group and absent in RNA from the H.P. group. It would therefore seem that withdrawal of protein from the diet results in a large increase of the heavy component (≥ 36S) of cytoplasmic RNA of the liver. The specific activity of this peak was higher than the specific activity of corresponding regions in either the H.P.F. or H.P. group. It is interesting to note that an RNA species of molecular weight greater than 28S has also been found by Kubinski _et al_ (1963) in animal cells growing in culture under unfavourable nutritional conditions. They report that this RNA is very highly labelled with ^{32}P-orthophosphate, but is more stable than messenger RNA.

b) Although the relative proportion of 29S:18S RNA in the three dietary groups is approximately the same, there is a marked decrease in the amount of 4S RNA (Figs. 25 and 27) or s-RNA, in the L.P. rats, and the specific activity is greatly reduced. This decrease in O.D. and counts in the 4S RNA region in L.P. rats must be a response by the liver cell to the continued absence of amino-acids: it is not surprising that the mechanism for transferring amino acids to the ribosomes is kept to a minimum when there are no amino acids to maintain it. Conversely, the large amount of s-RNA in the livers of animals maintained on a high protein diet would be required for transporting the high concentration of amino acids to the ribosomes.

Further comparison of Fig. 25 a and b and Fig. 27 a and b, shows that the specific activity of the s-RNA in the H.P. group is much higher than that in the H.P.F. group. If one assumes that the majority of $^{32}PO_4$ uptake in this region is due to pCpCpA end group turnover, then it follows that

the presence of a rich supply of amino acids inhibits pyrophosphorolysis of the terminal triplet. This would be an advantageous situation in the liver cells of animals fed protein, as it would result in the s-RNA molecules being able to recycle more rapidly between incoming amino acids and the ribosomes, since the amino acyl group is condensed on to the pCpCpA end group (Lipmann et al, 1959; Leahy et al, 1960).

Determination of sedimentation coefficients of rat liver RNA. The Schlieren optical system of the Spinco Model E analytical ultracentrifuge was used to determine (a) the number of components in the RNA samples and (b) the approximate sedimentation coefficients of the components. Samples of both whole liver RNA, and nuclear RNA were centrifuged at 44,770 r.p.m. at 20°C, and exposures were taken at 0 minutes and every 8 minutes thereafter. At the concentrations of RNA used (about 15mg/ml) in these experiments, the rates of sedimentation of the components are retarded, and low S values are obtained. The U.V. optical system was therefore also used to check the S values of some of the components.

Sedimentation coefficients (S values) were calculated using the equation

$$w^2 s = \frac{d \log_e r}{dt} \qquad (I)$$

where w = angular velocity (radians/sec)

r = distance of the boundary from the centre of rotation (cm.)

t = time (sec.)

s = sedimentation coefficient.

From equation (I)

$$s = \frac{d \log_e r}{dt} \cdot \frac{1}{w^2} \text{ secs.}$$

Therefore, at 44,770 r.p.m.

$$s = \frac{d \log_{10} r}{dt} \times 17.47 \times 10^{-10} \text{ secs.}$$

The value of $\frac{d \log_{10} r}{dt}$ was computed from a plot of $\log_{10} r$ vs. t, and the results were expressed in Svedberg (S) units, where a sedimentation coefficient of 10^{-13} secs. is one Svedberg (S) unit. It was also possible to make a rough estimation of the proportion of each component in a sample by calculating the

approximate area occupied by each peak in the Schlieren patterns obtained. These values are by no means accurate; they serve merely to give an indication of the proportions present.

1. Whole liver RNA.

(a) From the Schlieren diagrams obtained (Figs. 28, 29 and 30) it will be seen that, in the whole cell RNA of both H.P.F. and L.P. groups, there is a small proportion (about 1% in the H.P.F. group and about 5% in the L.P. group) of a heavy component greater than 30S. Table 33 shows that this is about 36S in the H.P.F. group (U.V. correction of Schlieren value), and about 43S in the L.P. group. U.V. analysis of the RNA from the H.P. group also showed that there is a heavy (32S) component in this sample, although this component was not apparent in the Schlieren pattern obtained from this group. Thus, in liver cells of rats maintained on a diet containing protein there is a very small amount (? 1%) of an RNA component sedimenting at 32-36S; in L.P. rats this proportion is increased to about 5%, and the material sediments at 43S. The 32-36S and 43S components may or may not be identical RNA species in the three groups.

(b) From Figs. 28, 29 and 30 it is seen that there is a major component of 23-28S (U.V. data suggest 28S) in whole cell RNA from all three dietary groups. This represents about 40-50% of the RNA in the sample. This component probably corresponds to the 28S ribosomal RNA component obtained by Staehelin et al (1964) from rat liver RNA, and by Scherrer, Latham and Darnell (1963), and Harris et al (1963) from HeLa cell RNA.

(c) In addition, there is a smaller amount (about 10-20% of RNA sedimenting at about 17-19S (U.V. correction, Table 33); Figs. 28, 29 and 30 show that this component is also present

32 24 16 8 mins.

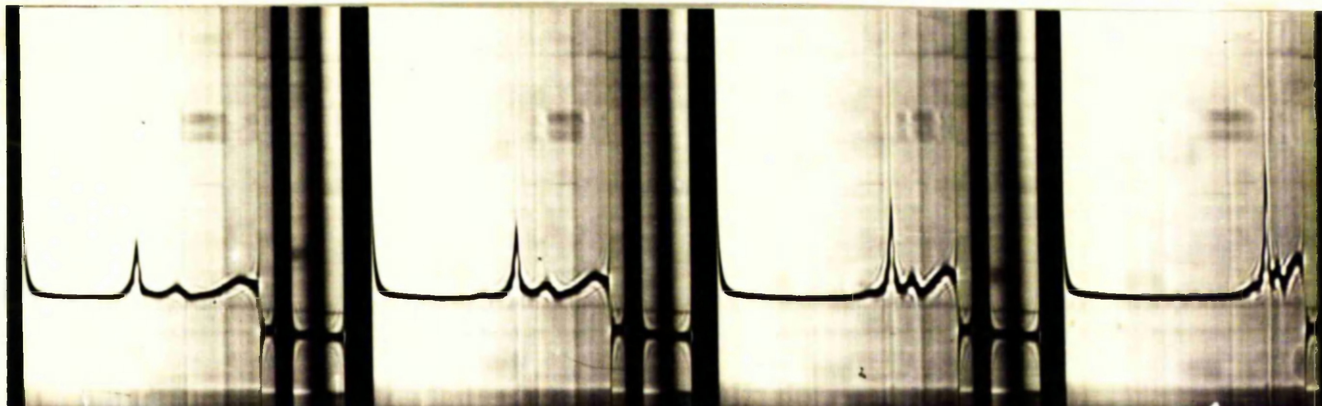


Fig. 28. Schlieren pattern of RNA from whole liver homogenate of H. P. F. rats. Conditions described in the text.

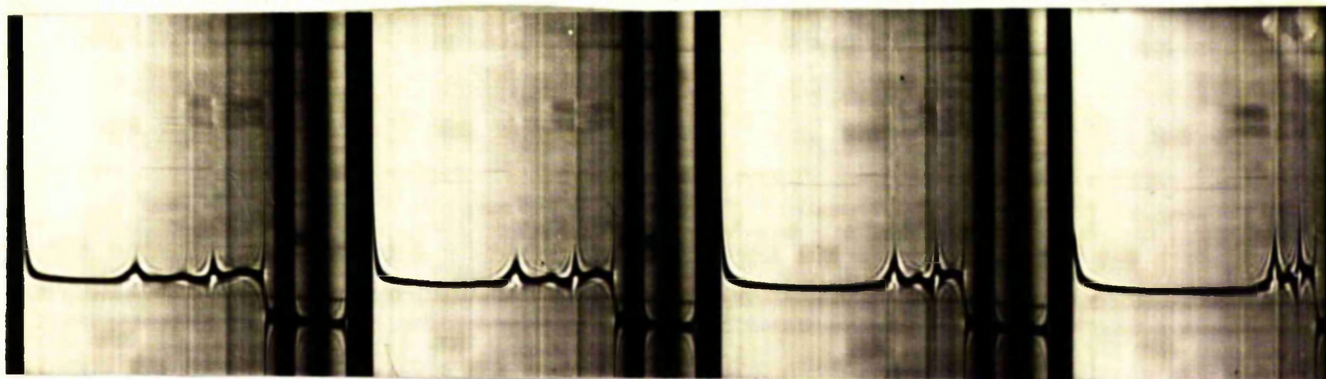


Fig. 29. Schlieren pattern of RNA from whole liver homogenate of H. P. rats. Conditions described in the text.

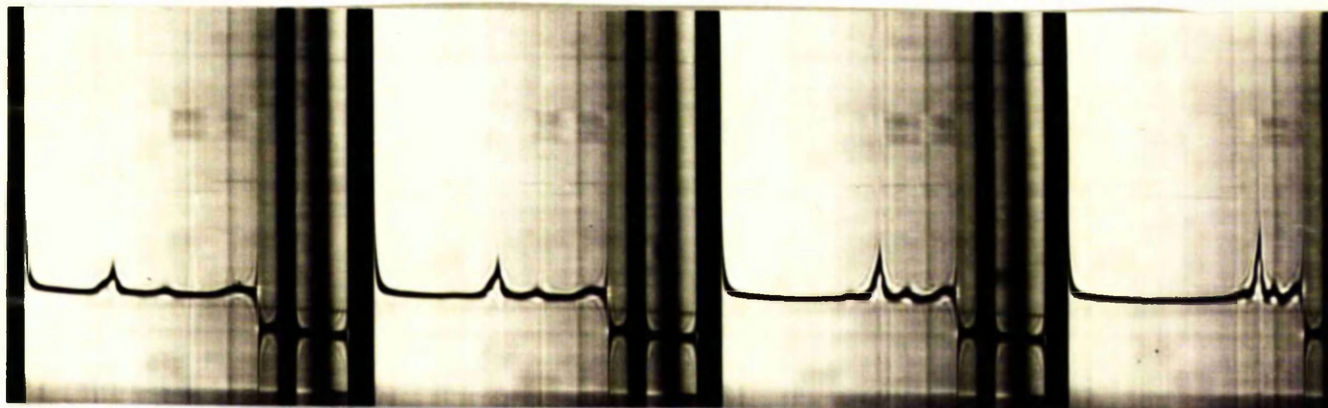


Fig. 30. Schlieren pattern of RNA from whole liver homogenate of L. P. rats. Conditions described in the text.

TABLE 33

Table showing sedimentation coefficients of RNA components from whole liver homogenates of dieted rats. The results are calculated from data obtained by Schlieren and U.V. analysis, and are expressed in Svedberg units.

Dietary group	Analytical Method	Peak Number				Figure No.
		1	2	3	4	
H.P.F.	Schlieren	31	23	15	4	28
	x U.V.	36	-	16	-	
	x (%)	0.1	41%	10%	53%	
H.P.	Schlieren	-	24	15 (10)	5	29
	U.V.	32	20-30	19	-	
	x (%)	-	35%	11%(24%)	30%	
	xx Correct for DNA	-	47%	15%	39%	
L.P.	Schlieren	36	28	17	5	30
	U.V.	43	29	18		
	x (%)	6	54	14	25	

x Area occupied by individual peak as % area occupied by all peaks.

xx Some DNA was present in this specimen, and sedimented at 10S in the Schlieren pattern. This component has been deleted from the calculation.

in the cell RNA of all three dietary groups. An RNA component of 18S was found by Staehelin *et al* (1964) in rat liver, and a value of 16S has been quoted by Tamaoki and Mueller (1962), and by Harris *et al* (1963) and Scherrer, Latham and Darnell (1963) for HeLa cell RNA. Both 28S and 16-19S components originate from the 50S and 30S sub-ribosomal particles of cytoplasmic ribosomes (see Spirin, 1963). Liver RNA from the N.P. group also contains about 20-30% of a component sedimenting at 10S (Fig.29 , Table 33). To our knowledge, this material has not been reported by other workers, and it is possible that it represents DNA contamination, since a similar pattern was obtained by us from RNA which had not been treated with DNase.

(d) The slowest component appearing in the Schlieren patterns of whole liver cell RNA sediments at about 4-5S (Table 33); it was not separable on the U.V. densitometric traces, however. A comparison of the dietary groups shows that about 40-50% of the RNA of the H.P.F. and H.P. groups sediments at this speed, but the proportion is decreased to about 25% in the L.P. group. This decrease in the amount of 4S RNA in the L.P. group agrees with our earlier observations from sucrose density gradients (p. 87 ; Fig. 25).

The main conclusions to be drawn are, therefore, that (i) there is a gain in the proportion of a very heavy (35S+) RNA component in the liver RNA of L.P. rats, and (ii) the proportion of 4S RNA decreases in this group. Both these observations agree with our earlier findings.

2. Nuclear RNA.

Both Schlieren and U.V. optical analyses were performed on nuclear RNA; the Schlieren patterns, however, were obtained immediately after isolation of the RNA, whilst the U.V. runs were made at a later date. It was subsequently found to be

32 24 16 8 mins.

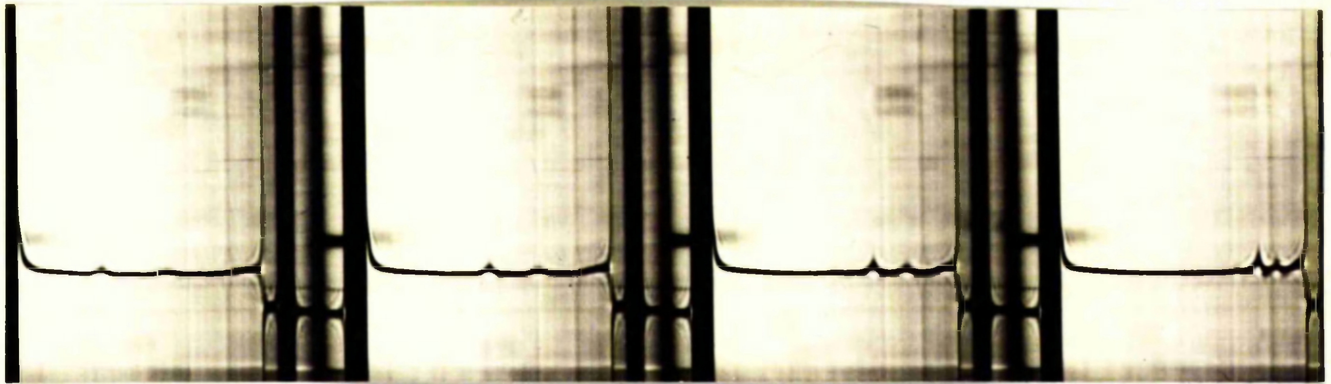


Fig. 31. Schlieren pattern of nuclear RNA from livers of H. P. F. rats. Conditions described in the text.

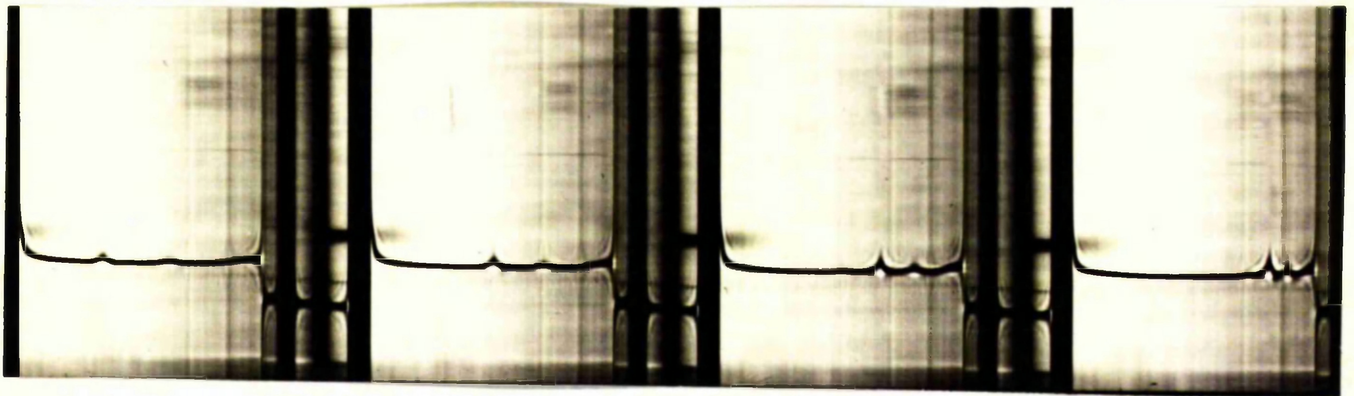


Fig. 32. Schlieren pattern of nuclear RNA from livers of H. P. rats. Conditions described in the text.

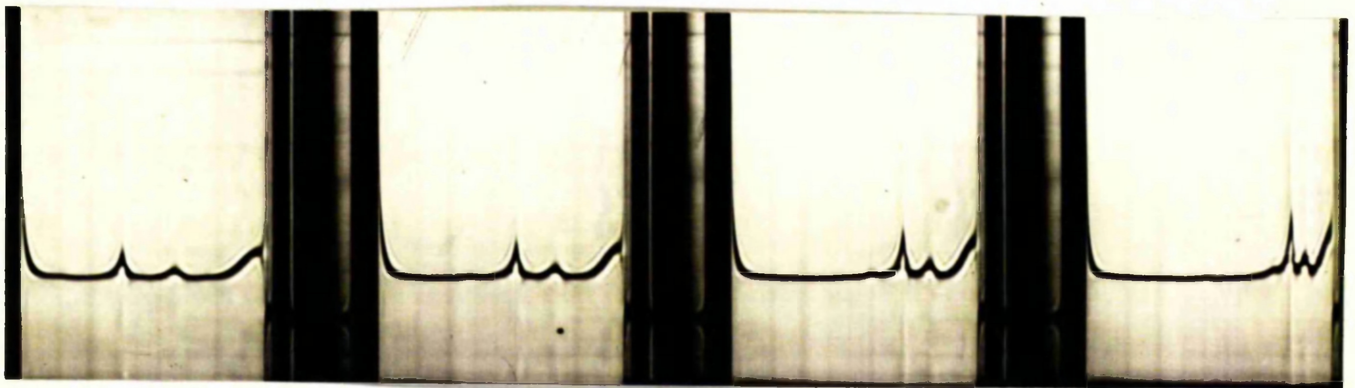


Fig. 33. Schlieren pattern of nuclear RNA from livers of L. P. rats. Conditions described in the text.

TABLE 34

Table showing sedimentation coefficients of RNA components of nuclei from livers of dieted rats. Results are from Schlieren data only, and are expressed as Svedberg units.

Dietary group		Peak Number				Figure No.
		1	2	3	4	
H.P.P.	S value x(%)	40S (c.1%)	30 (30%)	18 (21%)	5 (56%)	31
H.P.	S value x(%)	43 (c.1%)	30 (30%)	17 (21%)	7 (49%)	32
L.P.	S value x(%)	33 (3%)	28 (29%)	18 (10%)	4 (58%)	33

x % of total amount in sample.

impossible to calculate S values from the U.V. densitometric traces obtained, since the nuclear RNA appeared to have become degraded or dissociated into fragments of RNA with S values ranging from 3-28S. Thus nuclear RNA was labile whilst stored at -20°C, in the presence of RNase inhibitors, whereas these conditions did not seem to affect whole cell RNA, even after 3 months.

However, it was possible to calculate S values from the Schlieren patterns obtained, and at the lower concentrations used with these samples (about 5mg/ml), the calculated values are probably fairly close to the true ones. The results for the three dietary groups are given in Table 34 from which the following points emerge:

(a) Both Figs. 31, 32 and 33 and Table 34 show that the nuclear RNA from all dietary groups contains a small amount of material sedimenting more rapidly than 30S. Thus, the nuclear RNA of the H.P.F. and H.P. groups contains about 1% of 40-43S RNA, whilst the nuclear RNA of the L.P. group contains about 3% of a 33S component. Since the concentration of the sample from the L.P. group was higher than that of the other two, the value of 33S for this component may possibly be too low. The differences in the proportions of these components in the three dietary groups are almost certainly insignificant. A component sedimenting faster than 40S has also been observed in rat liver nuclear RNA by Sporn and Dingman (1963), but they found that this comprised 19% of the total nuclear RNA. Values of both 45S and 33S have been quoted by Girard, Penman and Darnell (1964), and by Tamaoki and Mueller (1963) for two of the RNA components of HeLa cell nuclei, although neither of these groups has isolated these components directly from pure nuclei.

(b) Fig.31-33 and Table 34 show that the nuclear RNA of all three dietary types contains a component sedimenting at

28-30S, which represents about 20-30% of the total RNA sample. The proportion of this component does not vary with diet, but the proportion of 28S RNA in nuclear RNA is only 50% of the proportion of the corresponding RNA in whole liver RNA. These differences in proportion were also observed by Sporn and Dingman (1963), who ascribed a value of 33S for the second heaviest component of rat liver. Values of 28S have been also quoted by Harris et al (1963) for a component of nuclear RNA from HeLa cells.

(c) Figs.31-33, and Table 34 show that rat liver nuclear RNA contains a 17-18S component comprising 10-20% of the nuclear RNA. The amount is least in the L.P. group, but this variation is probably insignificant. Our figures are in close agreement with those of Sporn and Dingman (1963) for rat liver nuclei; these authors also found a 19S component which constituted 20% of the total nuclear RNA. The 18S RNA has also been reported by Harris et al (1963) to be in HeLa cell nuclear RNA, and by Hovel et al (1965) to be in rat kidney nuclear RNA, although the samples in the latter investigation were separated on a serum albumin column.

(d) In our experiments, the major component of rat liver nuclear RNA is a component sedimenting between 4 and 7S, representing 40-60% of the total nuclear RNA. Again, the proportions are not greatly affected by diet. Confirmation of the existence of this component in rat liver nuclei again comes from the work of Sporn and Dingman (1963) who found a component of average 6S (range 2-10S) in rat liver nuclei, but this comprised only 19% of the total RNA sample. Harris et al (1963), however, did not find a light component in HeLa cell nuclei.

The conclusions to be drawn from these experiments are that rat liver nuclear RNA contains three major components of

28-30S, 17-18S, and 4-7S, the latter representing 50% of the total nuclear RNA. Diet does not appear to affect the proportions of these components except possibly the 18S peak. Nuclei also contain a small amount of RNA sedimenting at, or faster than 33S. This component may or may not alter significantly with diet.

SECTION IV : STUDIES ON THE METABOLIC RELATIONSHIP OF THE NUCLEAR MEMBRANE AND CYTOMEMBRANES.

Studies on the metabolic relationship of the nuclear membrane and cytomembranes.

These experiments were performed as a collaborative study with Mr. T. Hallinan and Mr. T. Duffy some time after the bulk of the studies already described. They are here presented as a complete and independent subsection. Mr. Hallinan used his technique for isolating the submicrosomal fractions, and Mr. Duffy devised a method of separating the lecithin and sphingomyelin components of the phospholipid extracts, by thin layer chromatography. The following account includes an introductory paragraph summarising briefly the literature pertaining to the subject, together with the rationale behind these studies, as well as a detailed account of the methods used and results obtained. Further discussion of the findings and their relevance is included in the general Discussion in Section V.

Introduction.

It has already been mentioned in the general Introduction to this Thesis that the most popular views on the relationship between the nuclear membrane and cytomembranes regard the cytomembranes as arising from the nuclear membrane (Gay, 1955; Swift, 1956; Merriam, 1959; Gay, 1960; Robhun, 1961; Kessel, 1963), and suggestions have been made that the nuclear membrane is, in fact, a specialised region of the cytomembranes (see Mirsky and Osawa, 1961). Alternatively, it has been suggested by Porter and Machado (1960) that the cytomembranes re-organise themselves after mitotic division to form the nuclear membrane; i.e., the nuclear membrane arises from the cytomembranes.

The cytomembranes themselves appear to be made up of granular and agranular reticulum (Palade and Siekevitz, 1956). Both types of reticulum are double layered membranes, made

of lipoprotein material, but the surface of the granular reticulum is studded with ribosomes (Palade and Siekevitz, 1956). The relationship of the agranular and granular reticula to the nuclear membrane has been studied by various authors (e.g. Fawcett, 1955; Robhun, 1961; Kessel, 1963), and from their work it would appear that it is the agranular reticulum which arises from the nuclear membrane, and subsequently becomes granular on acquisition of ribosomes from the cytoplasm. Metabolic experiments, therefore, would be expected to show a precursor-product relationship existing between these three structures thus:
Nuclear membrane ⟶ agranular reticulum ⟶ granular reticulum. This relationship has never been tested by a biochemical approach, and the concepts outlined above are based entirely on evidence from observations by electron microscopy, using fixed tissues. Many of the published electronmicrographs (see Gay, 1955, and others, quoted above) support the precursor-product relationship admirably, but it is difficult to be entirely uncritical of conclusions drawn from a static two-dimensional picture of a dynamic three-dimensional system. Thus, conclusions drawn from electron microscopy remain open to the criticism that the relationship observed between cytological structures may or may not be representative of those actually existing in the living cell.

Dietary considerations, however, suggest that some relationship may exist between the metabolic state of the nucleus and of the cytomembranes (see Munro, 1964). Thus it appears that the size of the nucleolus varies with protein intake, since, when the endoplasmic reticulum diminishes during protein deficiency, the nucleolus enlarges. This would be readily explicable if the cytomembranes arise from the nuclear membranes under the regulation of the nucleolus.

It was therefore of particular interest to us to try and correlate the turnover of structural phospholipid components of the nucleus with that of the cytomembranes under various dietary conditions. This was achieved using ^{14}C-choline as a radioactive precursor of the phospholipid components of both nuclear and cytomembranes, and measuring the uptake of label by the total phospholipid, and also by the lecithin and sphingomyelin fractions, of the nucleus and of the agranular and granular reticula at different time intervals. Lecithin and sphingomyelin were selected for this study since, as far as is known, the function of these components is a purely structural one (Fletcher and Sanadi, 1961). It was considered that the uptake of isotope into the nuclear phospholipids would be representative of the turnover of the nuclear membrane, since it has been shown (Gurr et al 1963) that the nuclear phospholipid is almost totally accounted for by the nuclear membrane. The agranular and granular reticula were isolated as smooth-surfaced vesicles (SSV) and rough-surfaced vesicles (RSV) respectively (see Hallinan, 1964). These vesicles arise during homogenisation, when portions of the membranes are pinched off by shearing forces.

Studies made included (i) experiments on the general metabolic relationship of the three subcellular fractions in animals fasting overnight; and (ii) experiments on the regeneration of endoplasmic reticulum by starved rats which had been fed protein, following the observation with the electron microscope made by Fawcett (1955) that feeding protein (but not carbohydrate) to starved rats leads to a regeneration of, first, agranular membrane, and, then, granular membrane.

General Methods.

1. Administration of Isotope and Time of Killing.

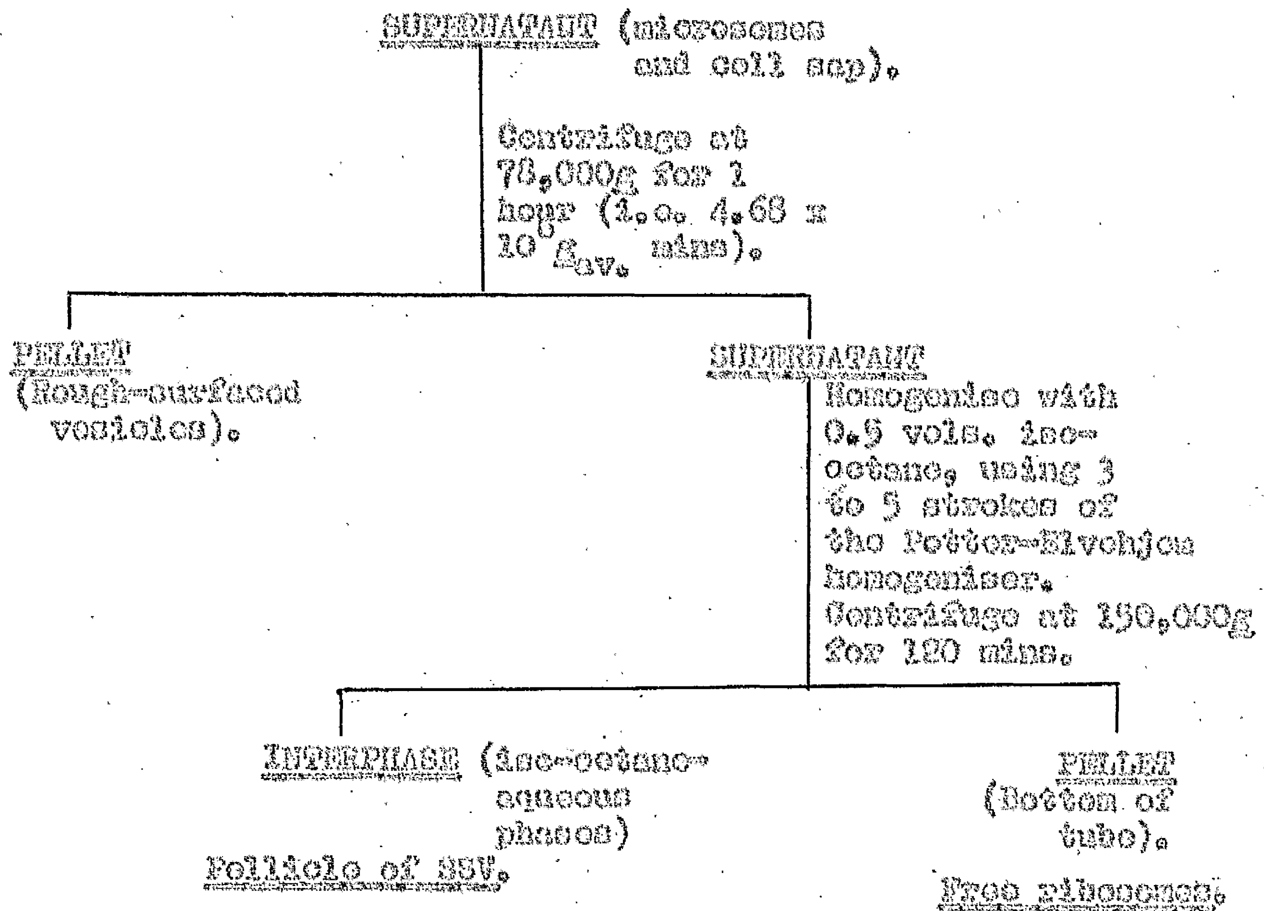
Rats which had been either previously fasted overnight, or kept on diets described later in the text, were injected intraperitoneally with 6.8μc ^{14}C-methylcholine. In the case of rats which had been fed casein, the injection was given two hours after the meal had been consumed. The rats were killed at time intervals of 20, 40 and 60 minutes after injection, although in one experiment, these intervals were altered to 10, 20 and 30 minutes after isotope administration.

2. Isolation of subcellular fractions.

(i) Nuclei were isolated by the method described on p. 29, rinsed in ice-cold distilled water, and sedimented as a pellet by centrifuging at 1,500g for 15 minutes.

(ii) Sub microsomal fractions (RSV and SSV) were isolated by the method of Hallinan (1964). Rat livers were rapidly excised, rinsed in ice-cold distilled water, blotted, and weighed. They were then chopped finely with scissors, homogenised in 4 volumes of 30% sucrose and centrifuged at 18,000g for 20 minutes to remove particles larger than microsomes. The RSV were then centrifuged down at 78,000g for 1 hour, and the supernatant containing the SSV and free ribosomes was emulsified with 0.5 volumes of iso-octane (AnalaR grade) in a Potter-Elvehjem homogeniser (usually 3-4 strokes of the pestle were sufficient). The emulsion was centrifuged at 150,000g for 120 minutes, so that the free ribosomes were precipitated as a tightly packed pellet at the bottom of the tube, whilst the SSV sedimented centripetally to form a pellicle at the interphase of the iso-octane and aqueous phases. The separation is shown schematically in the flow sheet (Fig. 34).

Fig. 34. Flow sheet summarising method used to fractionate sub-microsomal components (SSV and RSV) from free ribosomes.



3. Extraction of phospholipids from the subcellular fractions.

Phospholipids were extracted from the subcellular fractions by a modification of the method of Folch et al, (1957). These modifications were found necessary in order to remove the counts due to contaminating free radiocholine which was extracted together with the phospholipids. A more detailed account of the rationale and testing of the extraction procedures used is given by Duffy (1964).

The method of extraction finally elaborated was as follows; The pellets of nuclei and RSV, and the pellicle of SSV were each homogenised in 3 to 5 volumes of distilled water, and aliquots (0.5ml) of these homogenates were extracted with 20 volumes (10ml) of chloroform:methanol 2:1 in 15ml stoppered centrifuge tubes. The extraction mixture was centrifuged to precipitate the residue, and the supernatants were quantitatively transferred to 30ml stoppered centrifuge tubes. The residues were re-extracted with a further 10ml of $CHCl_3$: CH_3OH, and the washings added to the initial extracts. The crude extracts were washed by vigorously shaking them with a volume of 0.73% aqueous NaCl solution such that the ratio of the volumes of $CHCl_3$:CH_3OH:NaCl solution was 8:4:3. The solutions were then centrifuged for 5 minutes at 1,000 r.p.m. and the upper layer removed and discarded. Five ml of "pure solvents upper phase" (Folch et al, 1957), were added to each tube, and the tubes vigorously shaken. The tubes were centrifuged at 1,000 r.p.m. for 5 minutes and the upper phase again removed. This process was repeated three times.

In later experiments, where larger amounts of material were dealt with, the extraction of the pellet with $CHCl_3$:CH_3OH was limited to one, it being assumed that this non-quantitative recovery of lipids was not selective.

4. Estimation of phosphorus.

It was found that the published methods for the estimation of

choline-containing phospholipids were very unsatisfactory (see Duffy, 1964, for a detailed analysis); lecithin and sphingomyelin were therefore estimated by measuring the phosphorus content of the isolated compounds. Phosphorus was estimated by the method of Allen (1940), but the volumes of the reagents used were scaled down by a factor of 5 ("1/5th Allen").

Aliquots of the solutions, containing 5-30µg organic phosphorus, were digested at about 195°C in a sandbath with 0.4ml 72% PCA for 2 hours, or until the mixture cleared. This converted the organic phosphorus to inorganic phosphorus. The tubes containing the digestion mixture were then removed from the heat, allowed to cool, and 4ml of water was added down the side of the tube. Freshly prepared 1% amidol (0.4ml) was then added, followed by 0.2ml 8.3% ammonium molybdate. The reagents were mixed thoroughly, and the blue colour was allowed to develop for 15 minutes from the time of mixing. The extinction coefficients of the solutions were then read at 725 mµ against a reagent blank. The extinction coefficient of the sample was compared against a standard curve prepared for the range 5-30µg of phosphorus.

5. Separation of lipids by thin layer chromatography.

Lecithin and sphingomyelin were separated by thin layer chromatography on silica gel plates. (See Truter, 1963, for general techniques). The silica gel plates were prepared by the method of Skipski, Paterson and Barclay (1962). The initial slurry of silica gel was prepared by mixing 30g. silica gel G with 60ml 0.01M Na_2CO_3. Plates were prepared from this by using a "spreader" set at 250µ thickness; by this method, five plates could be prepared from the initial slurry. The plates were left for 20 minutes at room temperature, and were then activated by heating in an oven at

approximately 95°C for 1 hour, after which they were allowed to cool in a desiccator, over silica gel. Plates which were not used on the day of preparation were reactivated immediately before use; plates were used within 7 days, or discarded. The solvent used for chromatography was a mixture of $CHCl_3$, CH_3OH, acetic acid, and 0.01M Na_2Co_3 in the proportions 50:25:8:4, respectively. Before chromatographic runs were made, the solvent tank was lined to a depth of 5-6mm with filter paper soaked in solvent, solvent was also layered on the bottom to a depth of 5-6mm, and the tank was allowed to equilibrate for at least 45 minutes before use.

Solutions of phospholipids for separation were concentrated by evaporating the samples in a water bath at 37°C, and blowing a stream of nitrogen through the solution. This procedure also removed any water present, and thus prevented micelle formation. Evaporation was continued to a final concentration of 10-20mg/ml, and 100µl of the samples were then applied using the "line technique" (see Truter, 1963). The charged plates were placed in the chromatography tank, the system was made airtight, and chromatography was allowed to proceed until the solvent from had reached a predetermined line (about 3cms. from the end). The plates were then removed, and the components located by spraying the plate with a bismuth nitrate-potassium iodide spray (Dragendorff 1953).

6. Elution of the choline-containing phospholipids.

The spots corresponding to lecithin and sphingomyelin on the plate were mapped out immediately after spraying, before the dye colour faded. The spots were removed, and the resulting phospholipid-containing silica gel powder was collected on a glossy surface and transferred to a centrifuge tube. The phospholipid was eluted by shaking the powder with 2-5ml $CHCl_3$:CH_3OH 2:1, the suspension was centrifuged, and the supernatant was transferred to a 10ml graduated, stoppered

centrifuge tube. The powder was extracted a further three times, and the four extractions were pooled. Aliquots of the eluate were pipetted on to lens paper discs in stainless steel planchets, in amounts less than 0.3mg, to avoid self absorption, and the radioactivity was counted for 200 counts. Parallel samples of the lecithin and sphingomyelin solutions were also digested, and the phosphorus estimated as described above. Specific activity was computed from activity and phosphorus content.

Experimental and Results.

1. Turnover of the total phospholipids, and of lecithin and sphingomyelin, in liver cell fractions of rats maintained on a stock diet.

In the first series of experiments, rats were maintained on a stock diet of rat pellet and water; they were fasted for 18 hours before they were killed. The uptake of ^{14}C-choline into the phospholipids of the nuclei, smooth-surfaced vesicles (SSV) and rough-surfaced vesicles (RSV) at 20, 40 and 60 minutes after injection is shown in Fig.35 . The results are expressed as counts/minute/mg-phospholipid, and are corrected to a dose of 6.8μc ^{14}C-choline given to a 130g. rat.

Fig.35 demonstrates that the most rapid uptake of ^{14}C-choline label occurs in the phospholipids of the SSV, at all times up to 60 minutes after injection of the isotope. The specific activity of the SSV is closely paralleled by that of the RSV, at a slightly lower level of activity. The lowest activity occurs in the phospholipids of the nucleus, at all time intervals studied.

The uptake of ^{14}C-choline into the lecithin and sphingomyelin components of these fractions was studied in a similar experiment, using rats under identical dietary conditions. The results, shown in Fig.36 , indicate that, again, the highest activity is in the lecithin and sphingomyelin of the SSV. A comparison of the uptake of isotope into the lecithin and sphingomyelin of the nucleus, however, shows that the activities of both of these components are higher at 40 minutes after injection than are those of the RSV. At 20 and 60 minutes, the activities of both nuclear lecithin and sphingomyelin are lower than those of the RSV. The differences in the pattern of incorporation of ^{14}C-choline by the total phospholipids, which are still

Fig. 35.

Incorporation of ^{14}C-methylcholine into the phospholipids of nuclei, smooth-surfaced vesicles (SSV) and rough-surfaced vesicles (RSV) from the livers of rats fed a stock diet and then killed after an 18 hour fast. Rats were injected with 6.8μc ^{14}C-methylcholine before killing.

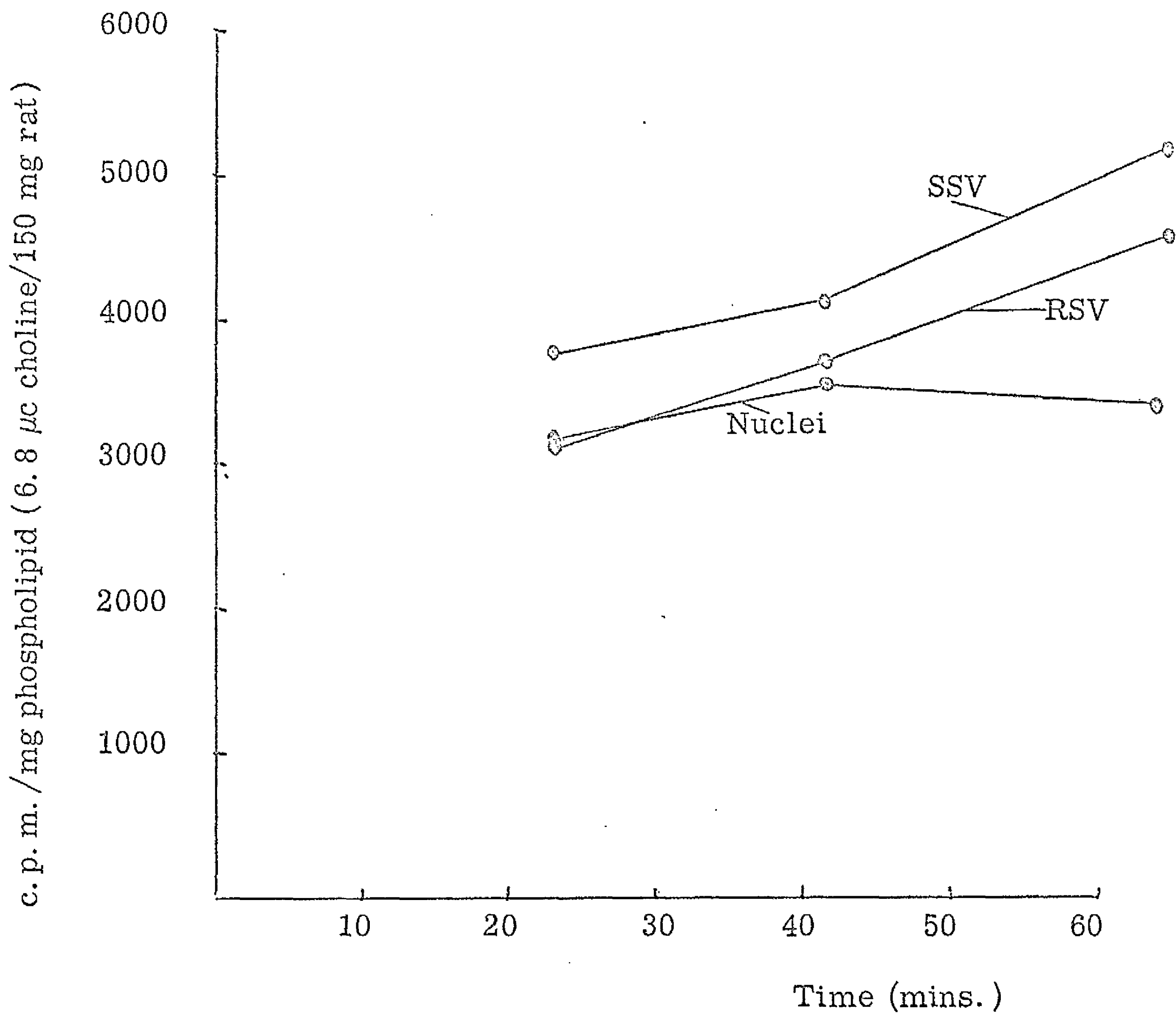


Fig. 35

Fig. 36.

Incorporation of ^{14}C-methylcholine into the lecithin and sphingomyelin of nuclei, smooth-surfaced vesicles (SSV) and rough-surfaced vesicles (RSV) from the livers of rats fed a stock diet and then killed after an 18 hour fast. Rats were injected with 6.8μc ^{14}C-methylcholine before killing.

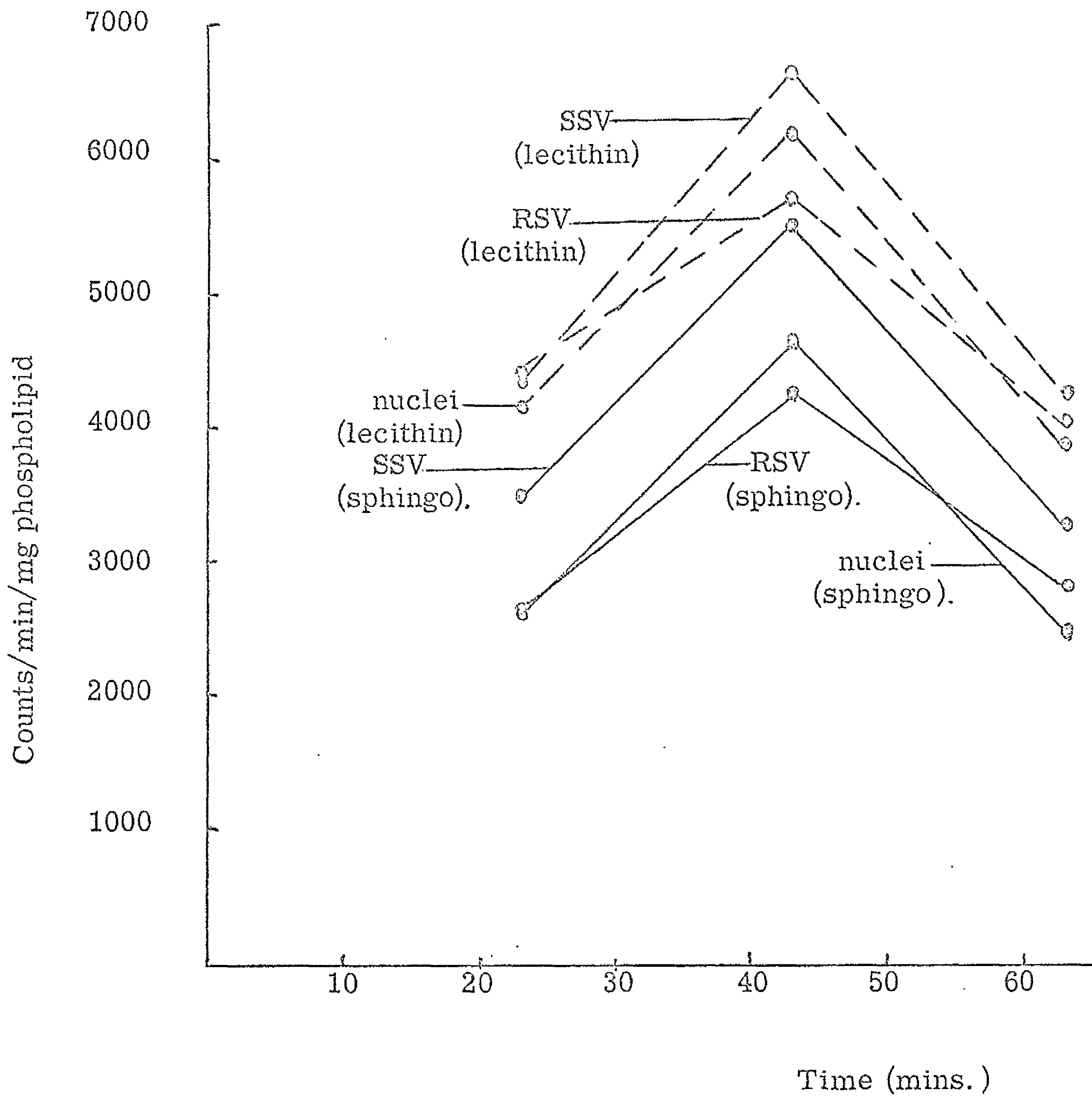


Fig. 36

increasing after 60 minutes (Fig.36), and by the lecithin and sphingomyelin, which peak at 40 minutes (Fig.36) are difficult to explain. Another curious feature is the difference in level of activity of sphingomyelin and lecithin within the same membrane fractions (Fig.36). This divergence would be explained if the sphingomyelin pool was larger than the lecithin pool, so that the radiocholine precursor of sphingomyelin would be more dilute, resulting in a lower incorporation of radiocholine. To this end, it may be significant that the activities of both lecithin and sphingomyelin are parallel over a period of time, even though that for lecithin is higher. Alternatively, the possibility of these, presumably structural, components turning over at different rates within the membrane is unconvincing, but it is possible that the turnover of lecithin does not represent the turnover of the membrane alone. It is clear from both Figs.35 and 36, however, that in fasting animals on a stock diet, the uptake of choline into the phospholipid components of the nucleus, SSV and RSV does not give any indication of a precursor-product relationship between these cell fractions, as one would expect if the electronmicrographic evidence, discussed on p.96 , were correct.

2. Turnover of the total phospholipid, and of lecithin and sphingomyelin, in liver cell fractions of rats deprived of protein.

Rats were either kept on a protein-free diet, or were starved for four days. Both starved and protein-depleted rats were fed 2g. casein 2 hours before administration of isotope; in one experiment, however, the protein-depleted rats were killed in the fasting state (L.P. group). Thus, in the starved, or protein-depleted rats which had been fed protein, there should be a very active synthesis of endoplasmic reticulum, whilst the animals which had not been fed protein would serve as "controls".

Fig. 37.

Incorporation of ^{14}C-methylcholine into the phospholipids of nuclei RSV and SSV from the livers of rats which had been maintained on a protein-free diet for 4 days, but had been fed 2g. casein 2 hours before killing. Rats were injected with 6.8μc ^{14}C-methylcholine before killing.

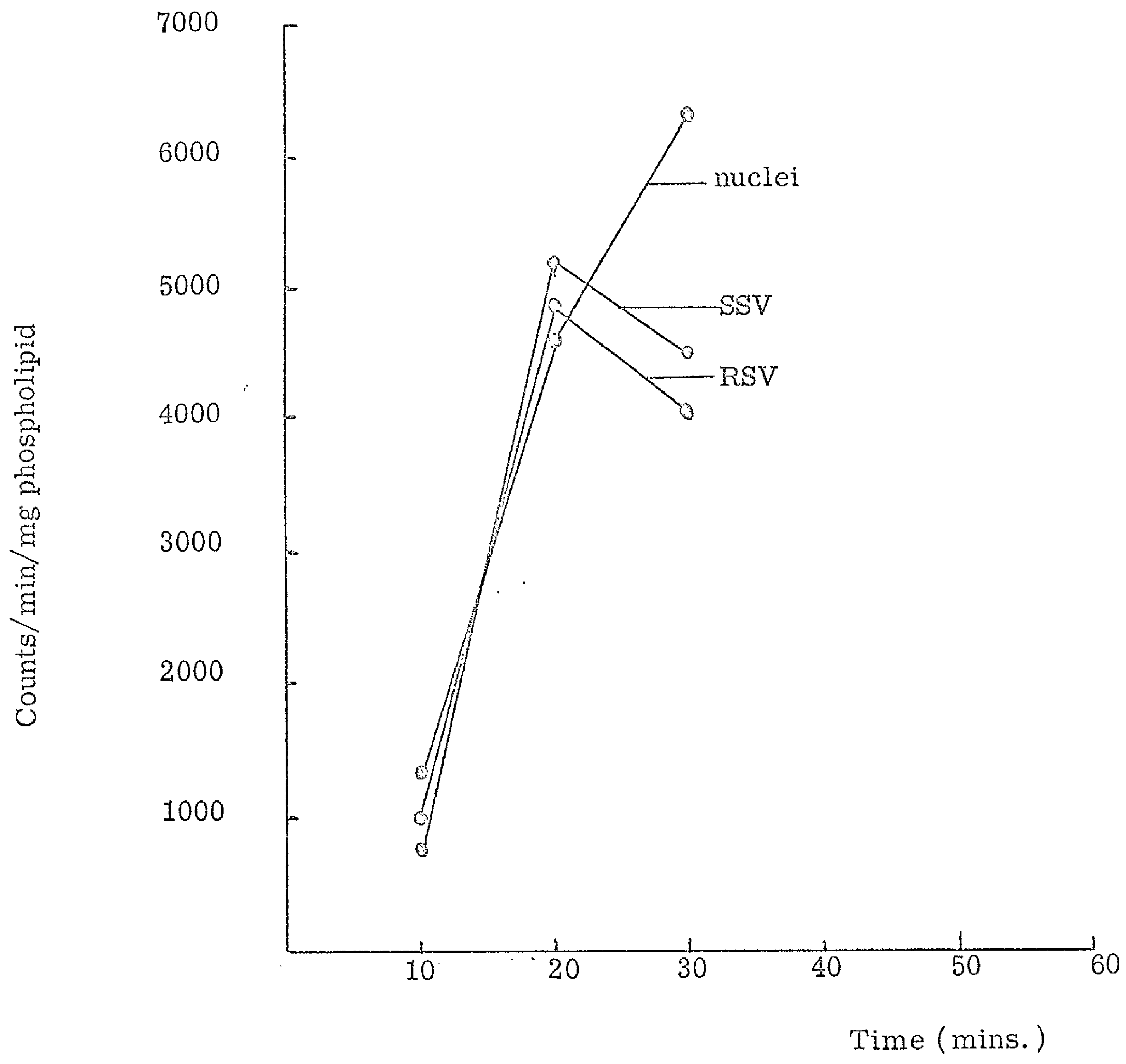


Fig. 37.

Fig. 37 shows the incorporation of ^{14}C-choline into the total phospholipids of L.P. rats fed protein 2 hours before injection, and killed at 10, 20 and 30 minute intervals after injection. It will be seen that, once again, the activities of the SSV and RSV are closely parallel, those of the SSV being higher than those of the RSV at 20 and 30 minutes. The activity of the nuclear phospholipids, however, is lowest at the 20 minute interval, but is above those of the SSV and RSV at 10 minutes, and appreciably higher at 30 minutes after injection. A comparison of the uptake of isotope into the lecithin and sphingomyelin components (Fig.38) shows that this pattern still exists, but the time intervals are different. Thus, at the earliest time interval (20 minutes), the activity of both the lecithin and sphingomyelin of the nuclei is higher than that of either the SSV or RSV, and similarly at 60 minutes. At 40 minutes after injection, however, the activity of the SSV is highest.

Fig 39 shows the uptake of ^{14}C-choline into the lecithin and sphingomyelin of the subcellular fractions of L.P. fasting rats. The levels of activity are very much more irregular, but again, incorporation of isotope into the lecithin moiety of the RSV and into that of the SSV are parallel. This is not the case with the sphingomyelin component, however. In this instance, the activity of the RSV is much the lowest, although it is still rising at 60 minutes. From a consideration of the data in Fig.39 it is possible that the SSV gives rise to the RSV. The phospholipids of the nucleus, however, appear to undergo independent synthesis.

The discrepancy between the activities of the lecithin and sphingomyelin which were observed in Fig.36 are much more noticeable under these dietary conditions, of low protein feeding, both in fasting and in fed rats (Fig.39, and 37). As previously discussed, it is possible that these differences

Fig. 38.

Incorporation of ^{14}C-methylcholine into the lecithin and sphingomyelin of nuclei, RSV and SSV from the livers of rats which had been starved for 4 days, and then fed 2g. casein 2 hours before killing. Rats were injected with 6.8μc ^{14}C-methylcholine before killing.

Fig. 38.

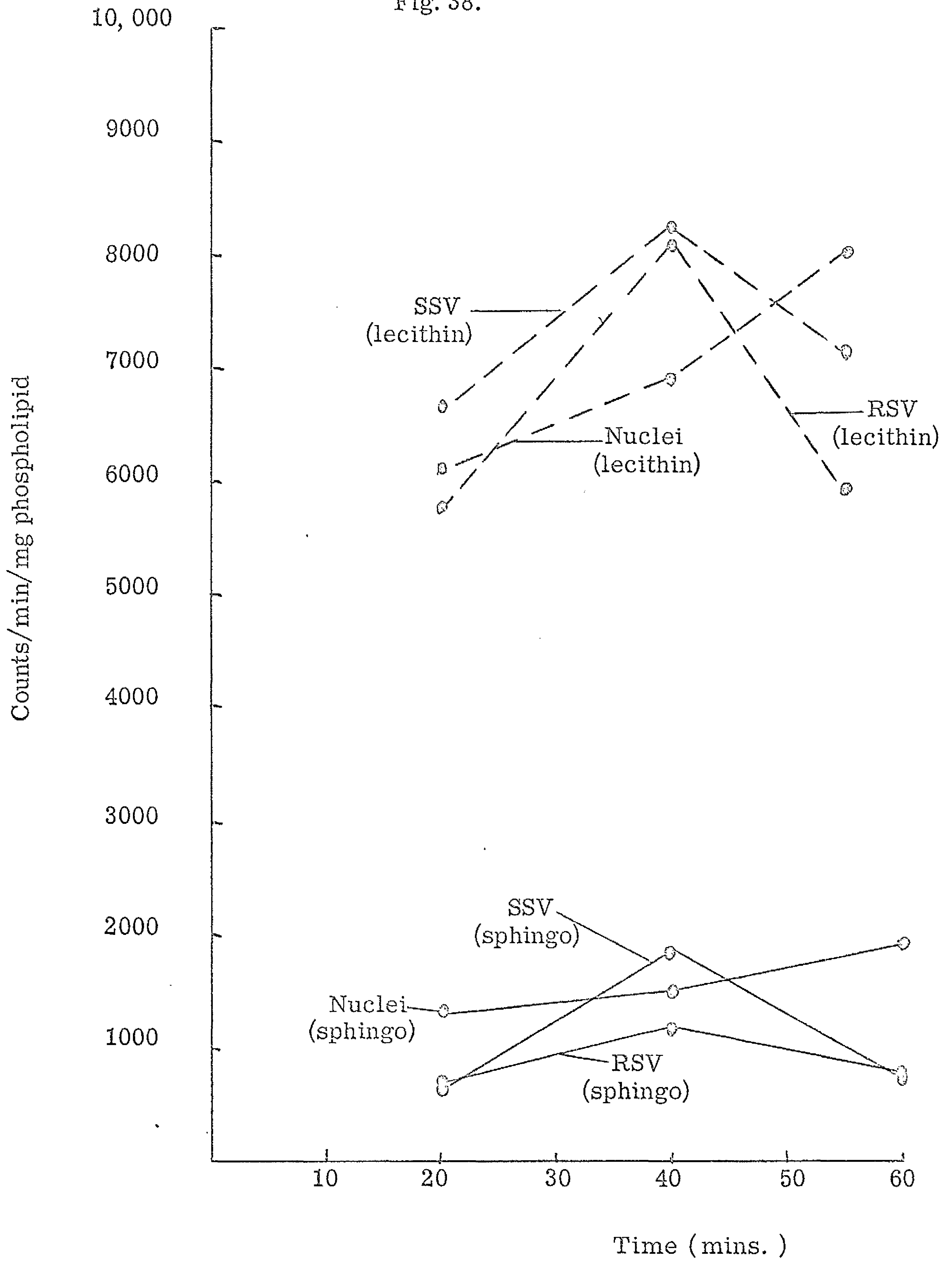
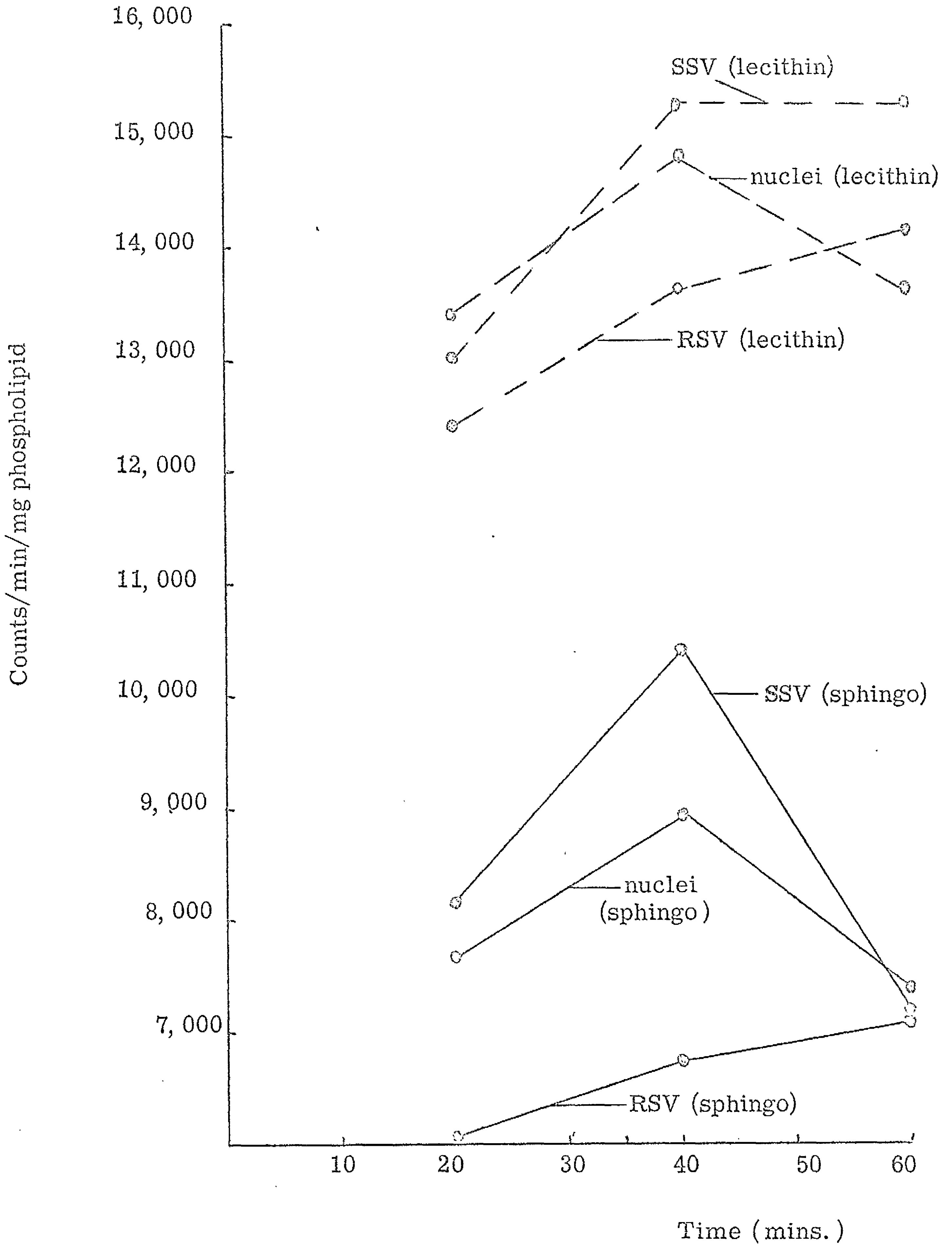


Fig. 32.

Incorporation of ^{14}C-methylcholine into the sphingomyelin and lecithin of nuclei, RSV and SSV from the livers of rats maintained on a protein-free diet for 4 days, and fasted 18 hours before killing. Rats were injected with 6.0uc ^{14}C-methylcholine before killing.

Fig. 39.



in levels of uptake of radiocholine may represent variations in pool size. If these discrepancies are due to the varying amounts of "cold" precursor in the cell, it seems, from Fig. 39 as if the greatest variation in pool size exists in L.P. fasted rats. Under these conditions, the endoplasmic reticulum is breaking down (Fawcett, 1955; Munro, 1964), and therefore the possibility of a variable amount of free phospholipid does not seem surprising. After a meal of protein, however, there is an immediate increase in the amount of nitrogenous material available, and again, this may well lead to a flux in the size of the precursor pools.

One fact which does seem quite clear from these studies is that the nuclear phospholipids turn over independently of those in the cytomembranes, and, in both instances in which protein was fed just prior to ^{14}C-choline injection (Figs. 36 and 37), uptake of the isotope is enhanced in the nuclear phospholipids relative to the phospholipids in the other fractions at the longer time intervals.

SECTION V : DISCUSSION.

DISCUSSION.

The object of this investigation, which has been outlined in the Introduction, was to study the response of the nucleus to variations in the amount of dietary protein. We were particularly concerned with the changes which occur during protein depletion. This interest was prompted by two observations. First, as we have already mentioned, (p.20), there is a gross reduction in the amount of endoplasmic reticulum in the liver cell after 4 days' starvation (Fawcett, 1955; Bernhard and Rouiller, 1956), and secondly, there is a considerable increase in nucleolar volume and RNA content under these conditions (Stenram, 1953, 1958a, b). These observations, coupled with the possibility that the nucleolus participates in the formation of the nuclear membrane, and that the nuclear membrane is the precursor of the cytomembranes, suggested that the nucleolus may exert a regulatory control over the formation of endoplasmic reticulum. Thus, particular attention has been paid, during the course of this work, to the function that the nucleolus might have in regulating the response of the liver cell to a deficiency in the supply of amino-acids coming to the liver cell.

At the start of this work, some evidence existed for the regulatory factor being an RNA species in the nucleus, which was subsequently transferred to the endoplasmic reticulum (Munro, 1964). Thus, Goswami et al (1962), in this laboratory, prepared from pyro-phosphate-treated microsomes an RNA which had a nucleotide composition similar to mixed "nuclear RNA". This RNA comprised 30% of the total microsomal RNA. Goswami et al suggested that this species, which was localised in the endoplasmic reticulum, might originate in the nucleus, and be involved in controlling the

response of the endoplasmic reticulum to protein withdrawal. Moreover, it had already been shown by Munro and Clark (1959), that the rudimentary endoplasmic reticulum of hepatoma cells, in protein deprived rats, does not develop into the extensive structure which is found in normal cells, after feeding protein. Also, Stenram (1958a) has found that the large nucleoli in rat hepatoma cells are similarly unresponsive to variations in the amount of protein in the diet. It has therefore been suggested (Munro, 1964), that the disorganised endoplasmic reticulum of the liver tumour cells does not contain this regulatory "membrane-RNA", and therefore does not respond to variations in dietary protein. The regulatory pathway proposed is outlined schematically in Fig. 40.

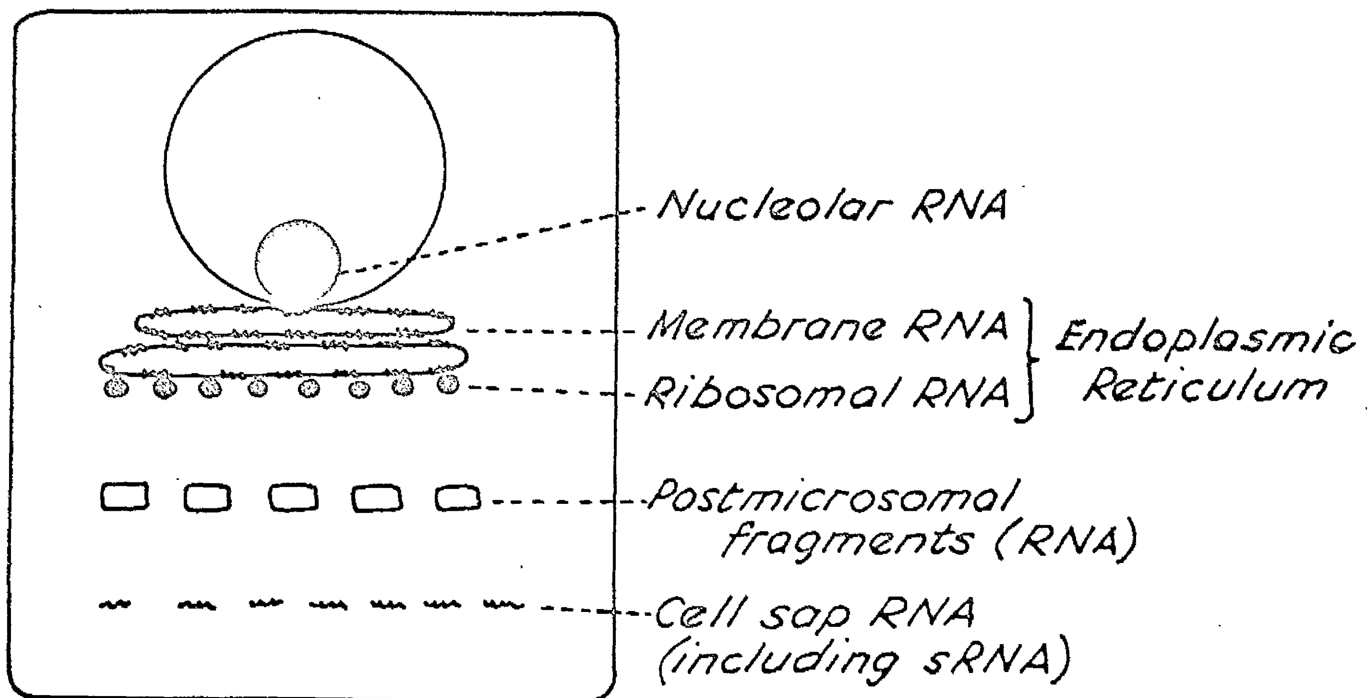
Several facts have emerged during the course of this work which may throw some light on the hypothesis represented by Fig. 40, although some aspects are still open to question. These are discussed below.

Origin of "membrane-RNA" and of the endoplasmic reticulum. The membrane bound RNA species of Goswami *et al* (1962) has been studied by Hallinan (1964), and his results are quoted here with his permission. Using pigeon pancreas and pigeon liver microsomes, Hallinan has shown that the RNA remaining with the membrane after treatment of the microsomes with pyrophosphate does not yield an RNA species with a nucleotide composition comparable to nuclear RNA, but rather, it is a degraded fraction of ribosomal RNA which remains. The similarity of the nucleotide composition of this RNA fraction in rat liver to that of whole nuclear RNA appears to be purely fortuitous. It may also be worth mentioning that the term "nuclear RNA" is almost certainly meaningless in this context, since nuclear RNA has been shown by many

Fig. 40.

Schematic representation of effect of protein intake on RNA metabolism in the liver cell. (From Munro, 1964).

Effect of protein intake on Ribonucleic acid metabolism in the liver cell.



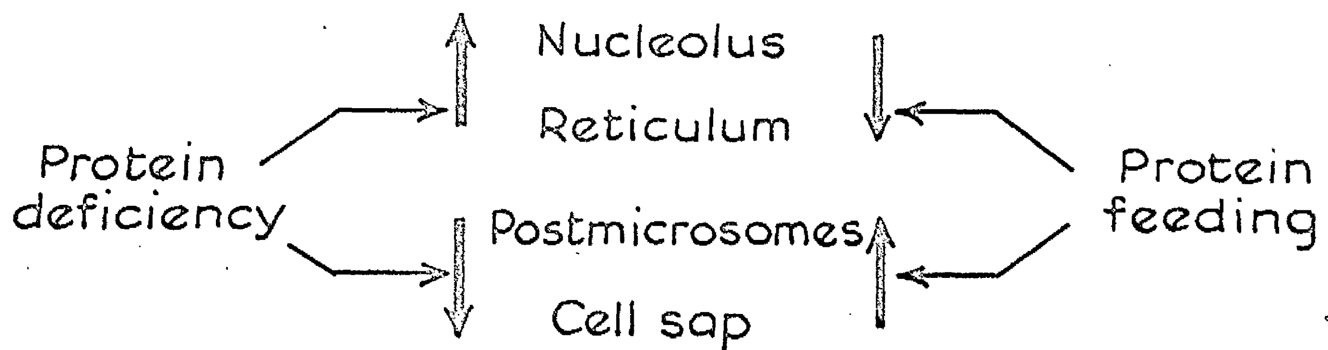


FIG. 40.

workers to comprise several different fractions (e.g. Logan and Davidson, 1957; Sporn and Dingman, 1963), and the presence of a species of nuclear RNA having a base composition representative of them all is unlikely. Thus, although a relationship between nucleolar RNA and the endoplasmic reticulum may exist, it is unlikely to operate through the "membrane-RNA" of Goswami et al (1962).

The origin of the endoplasmic reticulum itself is uncertain, but our experiments with ^{14}C-choline (p.103) would almost certainly suggest that the nuclear membrane is not the immediate, direct precursor of either the granular or agranular reticulum. Support for this finding comes from the work of Davis (1963) who found that in regenerating liver the nuclear and cytoplasmic membranes form at different phases of mitosis. There is also some chemical evidence in the literature to suggest that the nuclear membrane is not the structural precursor of the cytomembranes. Thus, it has been shown by Biezonski et al (1963) and by Gurr et al (1963) that the proportions of lipid components of the nucleus are very different to those of the microsomes, and if we assume that most of the lipid of the nucleus is concentrated in the nuclear membrane (See Gurr et al 1963), then this is strong evidence that the nuclear membrane and cytomembrane are structurally different. In addition, it seems, from the work of Merriam (1961) that the nuclear membrane has a higher protein/lipid ratio than the cytomembranes: this is supported by the observations of the different behaviour of the two membranes when treated with surfactants (Palmer et al, 1961). The evidence, therefore, suggests that the nuclear membrane and the cytomembranes are synthesised independently, rather than one being a precursor of the other, and it would appear that any regulatory control which the nucleolus exerts over

the endoplasmic reticulum probably does not include the nuclear membrane itself as a structural intermediate.

General response of nuclear and cytoplasmic RNA to protein feeding and protein withdrawal.

From the work reported in this thesis, and from the studies of others, it is apparent that RNA of both the nucleus and cytoplasm is sensitive to a deficiency in dietary protein, although for reasons outlined above, it is unlikely the response of the nucleus affects that in the cytoplasm through portions of the nuclear membrane peeling off to become the endoplasmic reticulum. In our experiments, the effects of protein withdrawal on nuclear RNA, and on sub-fractions thereof, are both quantitative and qualitative in nature, and from studies on the speed of sedimentation, and number of components of nuclear and cytoplasmic RNA, it appears that the physical size of RNA may also be affected. The significance of these changes is discussed below.

1. Response of the RNA of nuclear fractions to withdrawal of dietary protein.

Figs. 41 and 42 summarise in graphical form the effects of 4 days' protein deprivation on cytoplasmic and nuclear RNA, and on "salt extracts" of nuclear RNA. This picture is a synthesis of published data and our findings. It is seen from these graphs that the considerable decrease in the amount of cytoplasmic RNA, observed by Munro *et al* (1953), is coincident with a similar, but less drastic decrease in the amount of nuclear RNA, since after 4 days' protein-free diet the liver cell as a whole has lost about 20% of its total RNA (shown by Wikramanayake *et al*, 1953, to be mostly derived from the microsomal fraction), whereas the nucleus has lost only 5%. The loss of nuclear RNA is almost entirely accounted for by a reduction of RNA in the combined phosphate and *M* NaCl fractions, although there is also an

Fig. 41.

Summary of effects of protein deprivation on the amount of RNA in the liver cell, and in the liver nucleus and subnuclear fractions.
x from Munro et al (1963).

Fig. 42.

Summary of effects of protein deprivation on the incorporation of radioactive RNA precursors (42a) $^{32}PO_4$; 42 b-e, ^{14}C-adenine) into RNA of the liver cell and of the liver nucleus and subnuclear fractions.
x * from Munro et al (1963).

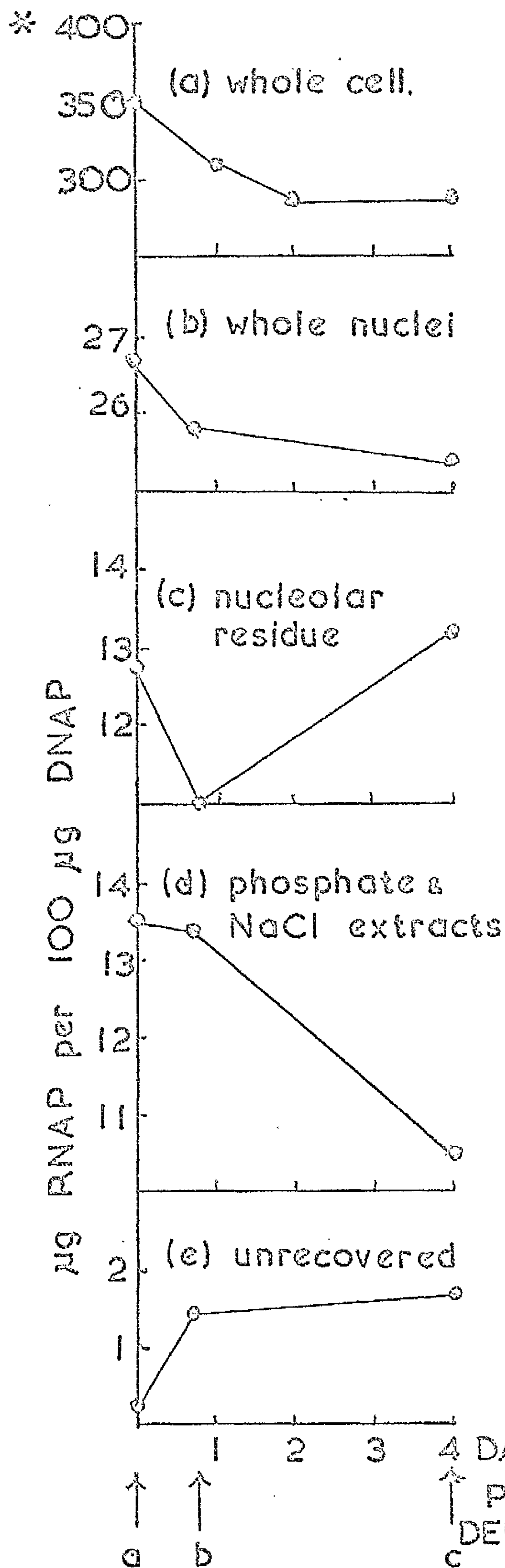


Fig. 41.

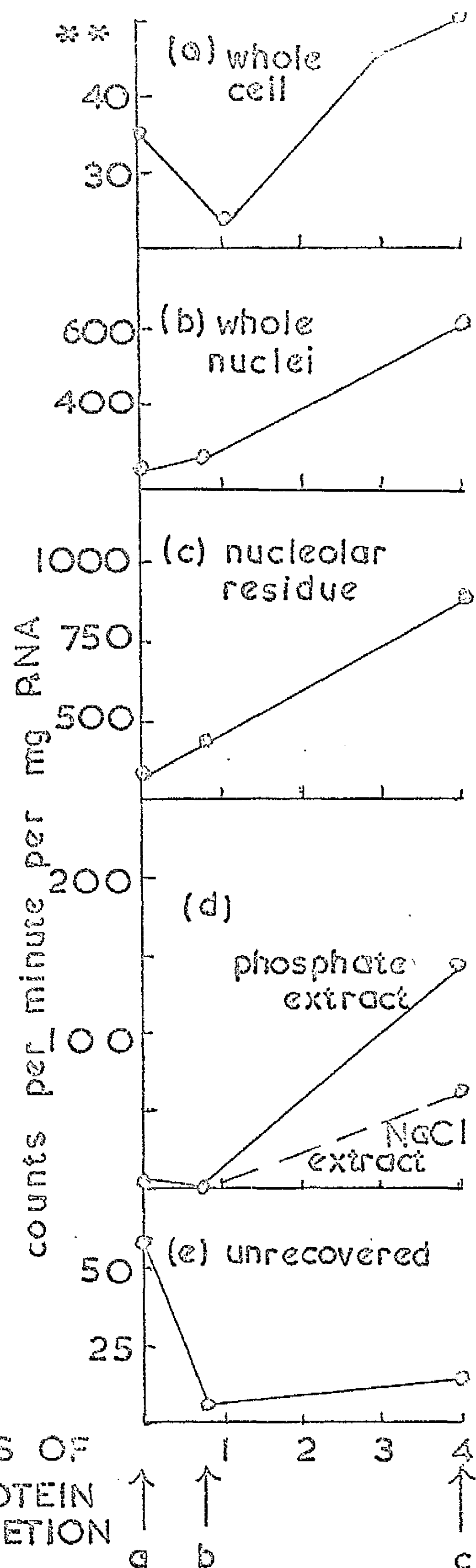


Fig. 42.

increase in the small amount of RNA which is not recovered in the sum of the nuclear fractions. The significance of this loss of RNA from these combined fractions during protein deprivation becomes apparent after a consideration of the nuclear origins of the RNA extracted. Thus, it has been suggested by Allfrey, Mirsky and Osawa (1955; 1957), that the phosphate fraction represents the protein-synthesising ribonucleoprotein particles of the nucleus (i.e. the nuclear ribosomes). The origin of the M NaCl fraction, however, is obscure, but it is possible that it contains RNA from two sources, since the quantitative response of this fraction to protein withdrawal is parallel with that observed in the phosphate fraction, although the metabolic activities of the two fractions are very different; the phosphate fraction shows a fairly low, but steadily increasing activity after injection of ^{14}C-adenine, whilst the M NaCl extract becomes highly labelled within a short time after isotope administration (see p. 70, Fig. 7). We therefore suggest that there are probably two species of RNA in the M NaCl extract. (i) The majority of the RNA in this extract represents a fraction of the nuclear ribosomes which is perhaps more strongly bound to structural protein than the fraction extracted by the phosphate buffer, and which is therefore extracted only after treatment with solutions of a relatively high ionic strength, and (ii) there is a much smaller amount of an RNA species which is rapidly labelled at short time intervals, but loses its label within an hour after isotope administration. If these conclusions are correct, then the majority of the RNA measured in the combined phosphate and molar sodium chloride extracts represents RNA of the nuclear ribosomes: the decrease in the amount of RNA observed in these fractions would then parallel

the decrease in the RNA from the cytoplasmic microsomal fraction after protein deprivation, observed by Wikramanayake *et al* (1953). As well as the similarities in the quantitative response to protein deprivation shown by whole liver (microsomal) RNA, and RNA of the nuclear phosphate and *M* NaCl extracts, there is also some similarity between their metabolic response to withdrawal of dietary protein. Thus, Fig.42 shows that the rate of incorporation of ^{32}P into whole liver RNA (corrected for the activity of the liver inorganic phosphate pool) decreases after one day on a protein-free diet, but after 4 days without protein it is increased to a level 1½ times that of the original rate (data of Munro *et al*, 1953). The interpretation of Munro *et al* (1953) of these findings is that, at first there is an increase in the amount of unlabelled nucleotides, due to RNA breakdown products after 24 hours of protein-deprivation, resulting in a fall in RNA activity; this is followed by a decrease in the available nucleotide pool, resulting in the subsequent increase in RNA activity. It will be observed from Fig. 42 that the activities of the separate phosphate and *M* NaCl fractions, at one hour after isotope administration, follow a similar pattern. (At intervals before one hour, the *M* NaCl extract is much more highly labelled). The specific activities of the nuclear fractions, however, have not been corrected for pool size or activity, but it is possible that the trends which they share with the incorporation pattern of whole liver RNA during protein deprivation may correspond to a similar state of flux in the size of the pool of nucleotides within the nucleus, over four days' withdrawal of protein from the diet.

From these considerations of the behaviour of the phosphate and molar sodium chloride fractions of the nuclear

RNA, and the microsomal RNA (which represents 80% of the whole liver RNA) it is very tempting to suggest that the RNA of the phosphate extract, and the bulk of the RNA of the molar sodium chloride extract, represent nuclear ribosomal RNA, and the variations observed in the RNA of these fractions during protein withdrawal represent the response of the nuclear ribosomes to protein deprivation. If this is correct, then we can conclude that these dietary conditions induce similar responses in both nuclear ribosomes and cytoplasmic microsomes.

Fig. 13 and Table 24 show that the rapidly labelled fraction of the RNA extracted into the M NaCl is very sensitive to diet, since its "relative specific activity" in the H.P.F. group is twice that in the H.P. and L.P. groups between 15 and 45 minutes after isotope administration. It is suggested that this RNA is rapidly synthesised in response to a supply of amino-acids coming into the cell. The fate of this rapidly labelled RNA is not known, but it is possible that it represents newly synthesised transfer RNA which is still bound to the chromatin, and which is therefore not removed by the phosphate buffer. Certainly, separation on a sucrose density gradient of whole liver and cytoplasmic RNA from H.P.F. rats indicates that there is an increased proportion of 4S RNA in this group; the above suggestion is compatible with this observation.

Fig. 41 shows that, in contrast to the decrease in RNA of the phosphate and molar sodium chloride fractions of the nucleus, the RNA of the "nucleolar residue" is increased after four days' protein deprivation. Before considering the significance of this observation, it is first necessary to discuss briefly the nature of the "nucleolar" residue.

The term "nucleolar residue" was coined by Allfrey, Mirsky and Osawa (1957) and by Logan (1957) for a

corresponding fraction of calf thymus nuclei: Mirsky and Osawa (1961) imply that it is made up of "nucleolus-like" particles. This is certainly not true of the "nucleolar residue" of rat liver nuclei; indeed, our experiments with the Beck specific nucleolar antibody show that at least some of the material of the nucleolus is extracted by M NaCl (p. 71), and this extractable nucleolus-specific material has been shown by Beck (1963) to be RNase-sensitive. However, it is likely that the RNA of the "nucleolar residue" represents the bulk of the nucleolar RNA, since the specific activity of this fraction is the highest of the three fractions over a considerable period of time (p. 68 Fig. 7), and it is well known (e.g. Ficq, 1955; Woods and Taylor, 1959), that the concentration of label is highest in the nucleolus during incubation with labelled RNA precursors. On the other hand, the "nucleolar residue" almost certainly contains RNA from sites other than the nucleolus itself; under our conditions of extraction (p. 31), RNA of the nuclear membrane and chromatin are very probably included in this fraction.

The observed increase in the RNA of this residue during protein depletion may therefore be due to an increase in the amount of RNA from any or all of these nuclear sites, but since nucleolar RNA probably represents the bulk of this fraction, it will be assumed that the observed increase in RNA is due to an increase in the amount of nucleolar RNA during protein withdrawal. This assumption would agree with the findings of Stenram (1958a, b) by a histological technique, and from the considerations already discussed on p. 107 , it seems probable that this increase in nucleolar RNA is due to a build-up within the nucleolus of RNA which, under conditions of adequate dietary protein, is transferred to the cytoplasm. This thesis is particularly attractive

when one recalls the evidence suggesting that cytoplasmic ribosomes originate in the nucleolus (Birnstiel, Chipcase and Hyde, 1963, and see Introduction p.15). If this theory is correct, then it seems that, during withdrawal of dietary protein, there is an increased breakdown of ribosomal RNA in the cytoplasm (Wikramanayake et al, 1953; Munro, 1964), whilst new ribosomes are withheld within the nucleolus. This would explain the decrease in microsomal RNA observed by Wikramanayake et al (1953), and the increase in nucleolar RNA observed by Stenram (1958a, b), and by us.

Fig. 42 shows that the activity of "nucleolar" RNA increases linearly over a four-day period of protein deprivation. An increased activity in the nucleolar RNA of protein-deprived rats has also been observed by Stenram (1962, 1963) using autoradiographic techniques. In neither our studies, nor in those of Stenram, however, were any measurements made of the available free nucleotide pool, and consequently a discussion of the significance of this increased activity of nucleolar RNA is complicated by the lack of data on pool size.

Three reasonable alternatives may be proposed with regard to the size of the nucleolar nucleotide pool, which may well be in free equilibrium with that of the nucleus. 1) It is possible that the nucleolar nucleotide pool size decreases more or less linearly over the period during which dietary protein is withdrawn, or 2) it may be that the actual rate of turnover of nucleolar RNA increases over 4 days of protein free diet, whereas the size of the nucleolar nucleotide pool remains constant; or 3) the nucleolar RNA precursor pool may show the same variations in size which have been shown to occur in the corresponding cytoplasmic pool (Munro et al, 1953; Clark et al, 1957).

If the first possibility is correct, then the increased incorporation of labelled precursor into the RNA of the nucleolar residue is explained by the precursor pool becoming more highly labelled, although the rate of turnover of nucleolar RNA remains constant throughout the four days of protein deficiency. If the second alternative is valid, then the increased nucleolar activity might be explained by a build-up of very highly labelled RNA (possibly the rapidly labelled ribosomal RNA precursor described by Girard et al, 1964) over the period of protein deprivation; alternatively, part of the RNA of both the cytoplasm and the nucleolus may become "unstable" during withdrawal of dietary protein (cf. Munro, 1964). The third suggestion, that is, that the variations in the sizes of both nuclear and cytoplasmic precursor pools are similar over the period of protein deprivation, is attractive. It is possible that the increased activity of the RNA of the nucleolar residue may be due to an accumulation of cytoplasmic ribosomal RNA precursors within the nucleolus, whilst the completed ribosomes have been transferred to the cytoplasm. If this situation existed, it would explain (a) the decreased RNA content of the nucleolus, and (b) the increased activity of the nucleolar RNA after 18 hours of protein withdrawal. In this case, the synthesis of the highly labelled RNA would have to be rapid enough for a considerable amount of labelled precursor to be incorporated even in the presence of a large unlabelled precursor pool, due to initial breakdown of RNA. After 4 days' protein deprivation, however, it is postulated that the nucleotide pool would be considerably reduced, and the turnover of nucleolar RNA consequently increased. This apparent increase might be due entirely to reduced pool size, as in (1), or the absolute turnover of nucleolar RNA may also increase, as in (2). As we have pointed out before,

however, there are no data on nucleolar or nuclear pool size to confirm or contradict any of these possibilities, and they therefore remain an open question. It would be profitable to isolate the liver nucleoli from the three dietary groups and study their RNA turnover under *in vitro* conditions; attempts to do this during our investigations, however, ended in failure.

The significance of the variation in the amounts of "unrecovered" or "labile" RNA with diet, has already been implied (p.65). A more mundane reason for this variation is that there is less protein in the nuclear extracts of the H.P. and L.P. groups than there is in those of the H.P.F. groups, since the two former groups have not received protein for some time: the amount of RNA precipitated by "adsorption" on to precipitated protein may therefore be less in the H.P. and L.P. groups, resulting in a lower recovery. We have no relevant data to test this hypothesis, however.

2. Separation of nuclear, cytoplasmic, and whole liver RNA by sucrose density gradient centrifugation, and in the analytical ultracentrifuge.

(a) Nuclear RNA.

The patterns obtained after separating nuclear RNA in the analytical ultracentrifuge were quite different from those obtained by centrifugation on a sucrose density gradient. We infer from these results that the longer time required for separation by density gradient centrifugation was probably sufficient to degrade the nuclear RNA, which has been shown by Harris *et al* (1963) to be particularly labile. Our findings, namely that nuclear RNA cannot withstand conditions of storage that have no effect on whole liver RNA, would agree with this. Harris (1963) has suggested that, within the nucleus, there is a pyrophosphorylase-like enzyme

which destroys rapidly-labelled RNA, and which is not inhibited by the usual RNase inhibitors used by us; he suggests the use of acridine dyes as inhibitors for this enzyme.

The Schlieren profiles obtained after analytical centrifugation show that nuclear RNA of rat liver has a minimum of four components: (i) about 1-3% in the 33-43S range; (ii) about 30% in the 28-30S range; (iii) 10-20% in the 17-18S range; and (iv) about 50% in the 4-7S range. Without suitable metabolic data, it is difficult to assess the significance of these peaks, but it is clear that the proportions of the lighter components, at least, do not alter during protein deprival. The similarity in proportions of the components heavier than 30S, and of the ribosomal components throughout protein withdrawal would indicate that a build-up of heavy rapidly labelled RNA within the nucleolus does not take place under these dietary conditions, and the observed increased activity of RNA in the nucleolar residue may thus be due to reduced pool size, or an elevated degree of "instability" of the RNA, as suggested by Munro (1964) for cytoplasmic RNA. It is possible, however, that the high proportion of 4S RNA in the nuclear samples represents degraded heavier RNA (see e.g. Harris et al, 1963), and the values obtained may therefore be partly artefactual, although the clear cut Schleiron pictures would tend to refute this.

(b) Whole liver and cytoplasmic RNA.

Both sucrose density gradient profiles and Schlieren and U.V. analyses show that withdrawal of dietary protein leads to an increase in the amount of 35S+ RNA in the cytoplasm. The proportion of the total label appearing in this heavy component is higher than in corresponding regions for the H.P.F. and H.P. groups, but the fraction is not particularly

heavily labelled. Kubinski *et al* (1963) have also found in animal cells growing in culture under unfavourable nutritional conditions an RNA component of sedimentation coefficient greater than 28S, which was heavily labelled with ^{32}P-orthophosphate, but was more stable than messenger RNA. The 36-43S fraction in rat liver may correspond to this fraction described by Kubinski *et al*, and the lower rate of labelling in our experiments may well be due to differences in the amount of isotope used (17uc/ml containing 5 x 10^6 cells in their experiments, 200uc/rat in ours).

The lighter RNA components also show some response to diet, in that the proportion of 4S RNA in the L.P. group is about half of that in the H.P.F. and H.P. groups. The significance of this decrease has already been discussed in the Results section, from which it can be inferred that one of the effects of feeding a meal of protein to fasted rats is the stimulation of synthesis of transfer RNA (sRNA); this newly-synthesised sRNA has been postulated to be the rapidly labelled RNA found in the *M* NaCl extract.

Further work on this topic is obviously required before the complexities of the system can be worked out. *In vitro* studies on isolated nuclei would probably give some indication of the gross effects of diet on the nucleus, and isolation of the nucleoli, possibly by the method outlined on p.46 , with subsequent *in vitro* studies could prove valuable. It is also necessary to obtain some idea of the size of the nuclear RNA precursor pool during protein deprivation; this might be achieved using nuclei isolated in organic solvents, but in all events would be difficult. The use of sucrose density gradients for studying nuclear RNA proved unsatisfactory, in our experience, and a repetition of these experiments using the RNase inhibitors suggested by Harris (1963) may well yield more interesting results.

SUMMARY.

SUMMARY

1. We have succeeded in isolating nuclei from rat livers, in a reasonable yield, and virtually free from cytoplasmic contamination. Electronmicrographs of the preparations show their purity, and also show that the isolated nuclei are surrounded by a double membrane.
2. Nuclei, isolated from livers of dieted rats, were fractionated by a salt fractionaton scheme into three fractions. Using a specific antibody, it was found that some of the nucleolar material is extracted into the M NaCl fraction. It was shown that there is a decrease in the amount of extractable RNA (which we have postulated is the nuclear ribosomal RNA) during protein depletion, although the RNA of the nucleolar residue is increased under these conditions.
3. Studies on the incorporation of ^{14}C-adenine into the RNA of these fractions showed that the incorporation of labelled precursor was much higher into RNA of the protein-depleted rats than into either the group fasted after a normal diet or those just fed protein. There was a rapid uptake of label into RNA of the M NaCl of the protein-fed group, however, and it is suggested that this represents synthesis of transfer RNA in response to the incoming supply of amino-acids.
4. Some attention was focused on the variable amount of nuclear RNA and radioactivity recovered after salt fractionation. There was a statistically significant increase in the amount of "unrecovered" RNA after 4 days' protein deprivation, i.e. RNA present in whole nuclei which could not be accounted for in individual nuclear fractions. It is possible that this represents an increase during protein deprivation of a fraction of highly

labelled, acid-soluble or acid-labile RNA. No evidence was presented to support this hypothesis.

5. Attempts to isolate nucleoli were unsuccessful, but a method which might prove useful is outlined, based on our experiences.

6. RNA was labelled *in vivo* with ^{32}P-orthophosphate and was subsequently isolated from whole liver, cytoplasm and nuclei of dieted rats by a method using phenol and sodium lauryl sulphate. Samples of each RNA species, obtained from all dietary groups were separated by centrifugation on sucrose density gradients. Most of the nuclear RNA was found to sediment within the range 16-4S, whereas the cytoplasmic and whole liver RNA separated as 4 discrete components. A comparison of the nuclear RNA profiles of the three dietary types showed little difference between the groups, but in the whole liver and cytoplasmic RNA of the group fed a protein-free diet there was evidence of an accumulation of heavy (greater than 28S) RNA, whereas the 4S peak in this group was decreased by a factor of two.

7. Analysis of these RNA samples by Schlieren and U.V. optics in the analytical ultracentrifuge showed that nuclear RNA sedimented as 4 components in the ranges (i) 33-43S; (ii) 28-30S; (iii) 17-18S; and (iv) 4-7S. Cytoplasmic RNA was also found to separate into 4 components, in the ranges (i) 32-43S; (ii) 20-30S; (iii) 15-19S; and (iv) 4-5S. An approximate estimation was made of the proportions of each of these components in the respective samples. There was little difference in the patterns obtained for the nuclear RNA of the three dietary regimes, but the whole liver RNA of the protein-deprived group contained more heavy (33S+) RNA than the other two groups, and less 4S RNA.

8. The incorporation of ^{14}C-choline into the phospholipids of

nuclei was compared with that into corresponding components of smooth-surfaced vesicles and rough-surfaced vesicles, in a collaborative study. The results obtained were incompatible with the view that the nuclear membrane is the precursor of the cytomembranes. There is some evidence that the incorporation of choline into the nuclear membrane is influenced by amino-acid supply.

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