THE INFLUENCE OF CADMIUM ON HEPATIC PROTEIN SYNTHESIS IN THE RAT

Thesis presented for the degree of
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by

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ABBREVIATIONS

Met	Methionine
Lys	Lysine
Ala	Alanine
Arg	Arginine
Trp	Tryptophan
Val	Valine
Phe	Phenylalanine
A	Adenylic acid
U	Uridylic acid
T	Thymidylic acid
G	Guanylic acid
C	Cytidylic acid
GTP	Guanosine-5'-triphosphate
GDP	Guanosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
ADP	Adenosine-5'-diphosphate
GMPP(CH$_2$)P	5'Guanylylmethylenediphosphonate
Pi	Inorganic phosphate
PEP	Phosphoenolpyruvate
TCA	Trichloracetic acid
DOC	Sodium deoxycholate
EDTA	Ethylenediaminetetra-acetic acid
Tris	Tris-(hydroxymethyl)-amino methane
SDS	Sodium dodecyl sulphate
GSH	Gluthathione
RNAase	Ribonuclease
PMS	Post mitochondrial supernatant
ER	Endoplasmic reticulum
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
tRNA	Transfer RNA
mRNA	Messenger RNA
rRNA	Ribosomal RNA
pre-mRNA	Precursor mRNA
HnRNA	Heterogeneous RNA
dRNA	DNA-like RNA
aatRNA	Aminoacyl-tRNA
peptRNA	Peptidyl-tRNA
PhetRNA	PhenylalanyltRNA
AcPhetRNA	N-acetylphenylalanyltRNA
MettRNA	MethionyltRNA
MettRNA$_f$	Initiator MettRNA which can be formylated.
fMet	Formyl methionine
A site	Ribosomal Acceptor or Aminoacyl site
P site	Ribosomal Donor or Peptidyl site
Poly U	Polyuridylic acid
Poly A	Polyadenylic acid
hU	Dihydrouracil
Ψ	Pseudouridine
S	Svedberg units
MW	Molecular weight
g_{av}	Centrifugal force, gravity units, for average radius

cpm	Counts per minute
sp. act.	Specific activity
Ci	1 Curie = 2,22 x 10^{12} disintegrations per minute
rpm	Revolutions per minute
λ	Wavelength
$A_{254\ nm}$	Absorbance at 254 nm
ORD	Optical rotatory dispersion
CD	Circular dichroism
PMR	Proton magnetic resonance

PART I

INTRODUCTION
AND
LITERATURE REVIEW

CADMIUM

I 1. In the Environment

Cadmium is a metallic element which is placed between Zn and Hg in Group IIB of the Periodic Table. It is divalent, as are the Group IIA elements Ca and Mg. In order of electro-potential, Zn, Cd and Hg fall below Ca and Mg but above the transition elements, with which they do not have close relationships. They have low melting points so their volatility, as in the case of Pb, is an industrial hazard. Cd has recently received prominence as a cause of environmental pollution due to its accumulation in water supplies and crops from Zn mining operations and industrial effluents. In Japan, Itai-itai disease, which presents as bone deformation, proteinuria and usually glucosuria, has been ascribed to interference of Cd with Ca metabolism, particularly where Ca intake is restricted in the diet (Tsuchiya 1969). More recently, as a result of review of the urinary proteins of affected persons Nomiyama, Sugata, Murata and Nakagawa (1973), have concluded that Cd is unlikely to play an important role in the onset of Itai-itai disease. The high levels of Cd found among the patients studied has, however, focussed world attention on the dangers inherent in the uncontrolled discharge of industrial wastes into rivers and estuaries.

Cd certainly causes renal damage (Piscator 1962, Axelsson and Piscator 1966), and prolonged exposure results in proteinuria (Smith, Wells and Kench 1961, Kench, Wells and Smith 1962). It is deposited in the soft tissues (Smith, Kench and Smith 1957), and can cause necrosis through vascular damage. It accumulates in the liver and kidney (Bonnell, Ross and King 1960), where a large proportion of the Cd administered to animals has been found to be bound to protein (Piscator 1964, Pulido, Kägi and Vallee 1966). If exposure is discontinued it is slowly excreted but is still retained at a significant level for long periods. It is also a potent carcinogen (Gunn, Gould and Anderson 1967). Some of the effects of Cd in animals can be reversed by the simultaneous administration of Zn (Schroeder, Nason and Mitchener 1968), and thiol compounds (Gunn, Gould and Anderson 1968).

I 2. Chemistry and Biochemistry

Cd, in common with Zn, to which it is closely related chemically, forms tetrahedrally coordinated complexes. Bis-dithiocarbamates of Zn (2) and Cd (2), $[Zn(S_2CNEt_2)_2]$ and $[Cd(S_2CNEt_2)_2]$ for example, have been described and characterised as isomorphous and isostructural (Domenicano, Torelli, Vaciago and Zambonelli 1968, Shugam and Agre 1966). They are dimeric and the sulphur ligands form bridging groups between the two metal atoms.

Williams (1959), has made extensive studies of the relative stabilities of organic molecules when complexed by metal ions, and has placed the metals in a series showing their affinity for different types of ligands. When extended to biological systems the main classification holds but factors such as ion size and charge, geometry and electronic structure play a part in determining the final equilibrium between a cation and its complex. Further metalloenzyme studies have been reported by Vallee and Williams (1968), and Williams (1970), and the biochemistry of Hg, Cd and Pb has been recently reviewed by Vallee and Ulmer (1972).

Cd, Cu, Hg and Pb are strong Lewis acids and active in model systems where the intermediate is stabilised by chelation with the metal, thus accelerating the reaction. In enzymes, however, they rarely appear as cofactors whereas Zn and Co, with weaker binding capacity, are good catalysts for hydrolytic enzymes. Two main reasons for this could be, firstly, interaction with different basic centres as a consequence of superior electron affinity with consequent blockage of active sites, and secondly, binding at the active site but with alteration of its geometry in such a way as either to activate or inhibit the enzyme. An example is the contrasting effect of Hg, Cd and Pb and of Zn on the catalytic activity of carboxypeptidase. Substitution of any one of these ions for Zn at the active site converts the enzyme from a peptidase to an active esterase.

The position of Cd between the element Zn, essential to biological systems, and Hg, highly toxic, makes its study of considerable biochemical interest. Work with cases of industrial Cd poisoning in Manchester (Clarkson and Kench 1956, Smith and Kench 1957), initiated a long-term study of various aspects of Cd intoxication in these laboratories. In a review of the interaction of metals with proteins, Kench (1972), extended Williams (1970), biological classification of the metals to include Cd and Co along with the super-acid catalyst Zn. The members of this group are inactive as redox catalysts, form strong complexes, and are slow to exchange, in contrast to the highly mobile ions Na and K involved in transport, and the trigger ions Mg and Ca also concerned with structure stabilisation. Their preferred attachment to proteins is by complexing to nitrogen or sulphur ligands rather than anionic-binding to oxygen. Thus, interaction with a vicinal dithiol is thought to be the mechanism of the uncoupling of oxidative phosphorylation in mitochondria by Cd (Fluharty and Sanadi 1960). Recent work (Diamond and Kench 1974a) has shown this effect to be on a respiratory step prior to electron transfer through the cytochrome system.

I 3. Effects on Protein Metabolism

The protein metallothionein which binds both Zn and Cd, the synthesis of which is induced by Cd (Webb 1972), excess Zn, and Hg (Jakubowski, Piotrowski and Trojanowska 1970), contains a high proportion of sulphur (8 - 9%) mainly as cysteine residues (Pulido, Kägi and Vallee 1966). From all sources from which it has been purified it has been found to have a molecular weight of 6,000 to 10,000 (Kägi, Himmelhoch, Whanger, Bethune and Vallee 1974). It has been isolated from liver and kidney cortex in a fatal case of poisoning by Cd (Wiśniewska-Knypl, Jabłónska and Myślak 1971). The induction of metallothionein by Cd has been demonstrated in the perfused rat liver. (Diamond and Kench 1974b).

Cd causes either inhibition or stimulation of a number of enzymes (Vallee and Ulmer 1972), of which tryptophan oxygenase is stimulated both in rat liver (Kench, Gubb and Sutherland 1969), and chicks (Kench and Gubb 1970). It has been partially purified and features of its Cd binding described (Diamond, Jedeikin and Kench 1973). Low Cd concentration (1×10^{-7} to 1×10^{-5}M) had a stimulatory effect but higher concentrations progressively inhibited the enzyme indicating the presence of different types of binding sites.

I 4. Minialbumins

An interesting effect of Cd poisoning in mammals, including man, has been found to be the formation of a low molecular weight serum albumin deficient in lysine and cystine and containing no tryptophan (Kench, Gain and Sutherland 1965, Kench and Sutherland 1966, Kench and Sutherland 1967). These minialbumins, which are excreted in the urine, have a molecular weight of 10,000 to 20,000 and are immunologically indistinguishable from normal albumins. They are present in the circulation at an early stage of Cd intoxication before the appearance of proteinuria, which would dissociate their formation from renal tissue damage. The minialbumins have shown a more rapid turnover rate than normal albumin (Kench, Wells and Smith 1962), which would suggest an active process for their formation rather than interference with catabolism of albumin by inhibition of enzymes such as carboxypeptidase.

The main site of albumin synthesis is in the liver, it is also here that Cd accumulates on chronic intoxication, (Piscator 1964, Webb 1972), where a large proportion is bound by metallothionein. There is, therefore, a possibility that minialbumins could arise from a defect in protein synthesis resulting in release of shortened polypeptide chains into the circulation. Judah, Gamble and Steadman (1973) and Judah and Nicholls (1971), have observed different albumin species in rat liver, one of low-molecular weight and turning over rapidly and another albumin perhaps a precursor of serum albumin of large molecular weight.

Studies on rat liver preparations by Sutherland and Kench (1971) have shown, by atomic absorption analysis, attachment of Cd to isolated tRNA, and have also reported lowering of the extent of amino acylation of tRNA by Cd in the case of lysine and tryptophan, glycine and methionine being unaffected. It is also possible that the lack of tryptophan in minialbumins could be associated with stimulation of tryptophan oxygenase by cadmium.

I 5. Tryptophan

Tryptophan is one of the least abundant amino acids in proteins and also one of the most non-polar. A study by Swaney and Klotz (1970), of the amino acid sequence adjoining the single tryptophan residue in human serum albumin has shown it to be Lys-Ala-Trp-Ala-Val-Ala-Arg. The tryptophan is therefore in a cluster of non-polar residues bracketed by two cationic residues lysine and arginine, an environment which would enhance the binding of small anions and uncharged molecules such as steroids which is characteristic of albumin.

Tryptophan has been shown by Munro and co-workers to be the limiting amino acid in protein synthesis, possibly due to delay in translation caused by the low abundance of tryptophan-tRNA in the cell (Munro 1968). This has been demonstrated in bacterial systems by Anderson (1969).

I 6. Purpose of this Investigation

The main aim of this work was to search for some interpretation at the cellular level of the biochemical disturbances which have been observed in chronic Cd intoxication.

The aspects of particular interest were:-

1. The presence of albumins of low molecular

weight in the circulation.

2. The absence of tryptophan and the low content of lysine and cystine characteristic of these albumins.
3. The study of enzymatic effects of Cd, either stimulation or inhibition.
4. The binding of Cd to protein and its interaction with nucleic acids.

The particular area chosen for study was the translational step in protein synthesis as it was here that previous work had suggested that minialbumins might arise through synthesis of low molecular weight polypeptide chains. Albumin synthesis takes place mainly in the liver so the rat liver was chosen as a suitable animal system which would enable close comparison to be made with the untreated animal at all stages of the investigation. Protein synthesis in rat liver has been extensively studies in relation to various pathological conditions and metabolic disturbances, particularly through isolation of ribosomes and comparison of their polyribosome content and of their efficiency in the <u>in vitro</u> incorporation of amino acids into protein. Rats were injected with $CdCl_2$ at a level to induce a chronic level of intoxication over a period of 2 - 3 weeks. After this period of time minialbumins could be expected to appear in the circulation. Liver ribosomes and enzyme preparations for protein synthesis studies were then isolated from these treated rats and compared with preparations from control animals.

I 7. Conclusions from this Work

When efficiency in protein synthesis was compared using enzymes prepared from control animals, purified ribosome preparations from Cd-treated liver demonstrated a diminished rate of incorporation of amino acids into protein which levelled off at about 75% of the normal value. Enzymes prepared from liver of injected rats lowered incorporation levels when used with both normal and Cd-treated ribosome preparations. The lower incorporation level of the Cd ribosomes was found not to be due to polysome breakdown by fission of messenger. Gradient profiles showed a higher proportion of polysomes than in the control experiment after 1 hr incubation, but the ribosomes were labelled to a lesser extent. Further peptide bond formation would therefore seem to be inhibited by some defect on the ribosome. This could be in the messenger, or at peptide bond formation or translocation, both of which steps are dependent on ribosomal function.

Comparison between preparations of ribosomes from normal and Cd-intoxicated animals when isolation procedures were carefully controlled showed them to have similar polysome content, ratio of RNA to protein and requirement for Mg^{2+} in protein synthesis. The isolated RNA from the ribosomes in both cases showed the same pattern after polyacrylamide gel electrophoresis.

No difference could be demonstrated between the normal system, ribosomes and enzymes, and that from Cd-injected rats with respect to incorporation of the individual amino acids tryptophan, lysine and leucine.

Quantitation of RNA either by estimation as ribonucleic acid or totalling ribonucleoprotein particles from gradients showed a similar or slightly lower RNA content in homogenates and preparations from intoxicated animals. There is therefore possibly a slight decrease in the cellular content of ribosomes.

The general metabolic picture appears to be of inhibition of protein synthesis by a lowered rate of translation on the ribosome.

Significant amounts of Cd were found in purified ribosome and enzyme preparations so binding to nucleotides and proteins must have taken place. In the whole liver and in cell sap a high proportion is probably bound to metallothionein which would have increased as a consequence of the increase of Cd in the tissues.

Low levels of Cd were found to have a cytotoxic effect on HeLa cells in tissue culture and so further studies of the direct effect of Cd at the cellular level are planned.

PROTEIN SYNTHESIS

I 8. The Polyribosome

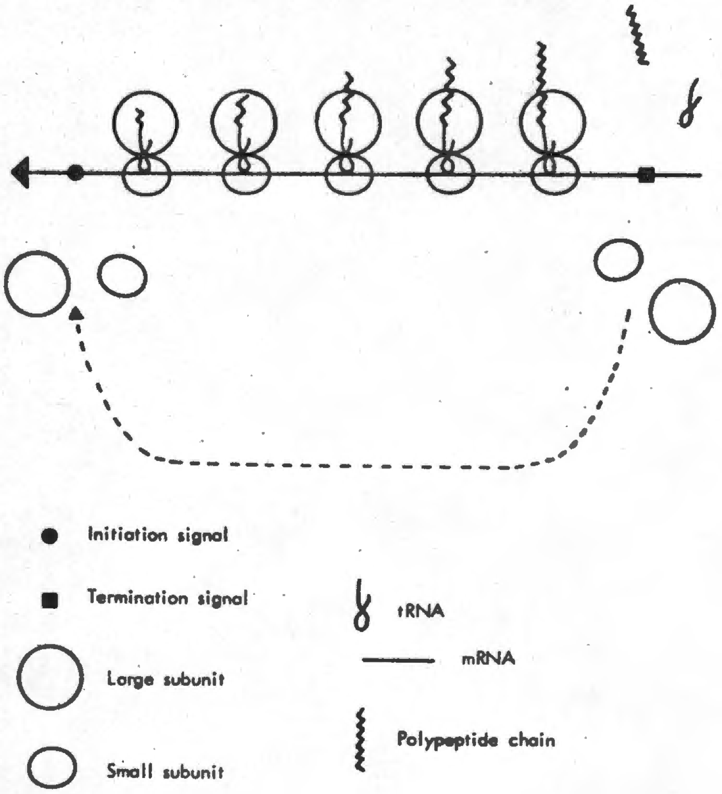
The site of protein synthesis in the cell is the ribosome, where each amino-acyl tRNA binds in turn to its codon on the attached mRNA, enabling peptide bonds to be formed between the amino acids, thereby resulting in translation of the message into a polypeptide chain. A diagram of a polyribosome is given in Fig. I showing the cycle of attachment and detachment of the ribosome. The mRNA is moving in the direction of the arrow relative to the ribosome. This diagram and that in Fig. 3 are adapted from Watson (1970).

When the polypeptide chain is completed, it is released from the ribosome, which becomes detached from the messenger before again initiating a fresh cycle.

mRNA varies in length with the molecular weight of the protein to be synthesized, and this in turn controls the size of the polyribosome, the group of

FIG. 1.

THE POLYRIBOSOME.



ribosomes attached to a single mRNA molecule. Polyribosomes containing 50 - 60 ribosomes have been identified by Heywood, Dowben and Rich (1967), as those synthesizing myosin (MW 170,000 - 200,000). The polysome for the synthesis of albumin would have 19 ribosomes (Campbell and Sargent 1964).

In a cell actively engaged in protein synthesis most of the ribosomes are attached to mRNA, and the extent of this attachment is a measure of the status of protein synthesis in the cell (Stanners and Becker 1971). Control of synthesis by decreased attachment of ribosomes to mRNA has been shown to be caused by slowing of initiation in the later stages of infection of HeLa cells by adenovirus. (Perlman, Hirsch and Penman 1972). It has been shown by Norman, Gamulin and Clark (1973), that single ribosomes not attached to mRNA accumulate as a result of starvation. Feeding a tryptophan-free amino acid mixture to previously-starved rats was found to cause polysome disaggregation which was reversible by refeeding with a complete mixture. It also gave rise to decreased amino acid incorporating activity and degradation of ribosomal RNA (Wunner, Bell and Munro 1966, Baliga, Pronczuk and Munro 1968). Several other amino acids were also found to be essential for restarting protein synthesis in an amino acid-depleted system. The availability of amino acids from the diet was found to have a daily cyclic effect on polysomal aggregation in rat liver. (Fishman, Wurtman and Munro 1969).

I 9. Ribosomes and Membranes

The liver cell has a well developed endoplasmic reticulum and ribosomes exist in two populations, one bound to form rough endoplasmic reticulum membranes, and the other free in the cytoplasm mainly as polysomal aggregates attached to mRNA. Another control mechanism for protein synthesis is the regulation of the degree of binding of ribosomes to the membranes. This binding can be enhanced by steroid hormones or inhibited by poisons or carcinogens such as aflatoxin (Williams and Rabin 1971).

Extensive studies comparing ribosomes free in the cytoplasm with those attached to the endoplasmic reticulum have not demonstrated any difference between them. The function of ribosomes attached to membranes has been shown to be that of synthesis of polypeptides for release directly into the cisternae for transport out of the cell. This has been confirmed in the case of immunoglobulin by Askonas and Williamson (1968) and Bernstein, Lamm and Vassalli (1970). Synthesis of the milk protein lactose has been shown to be controlled by α-lactalbumin through the endoplasmic reticulum membranes. α-lactalbumin is synthesized by membrane-bound polysomes and then interacts within the membranes with a transferase and by altering its specificity causes lactose to be synthesized and the onset of lactation. (Brew 1969).

In the rat liver, two proteins, ferritin, which is an iron storage protein retained in the cell, and albumin, secreted into the serum, have been the chosen ones for study to distinguish between the two classes or ribosomes. Sargent and Campbell (1965) found, after isolating the microsomal fraction, that newly synthesized albumin was retained within the endoplasmic reticulum membranes. Takagi, Tanaka and Ogata (1969), identified albumin by an immunological assay and showed its synthesis to be confined to the microsomal fraction. Redman (1969) and Hicks, Drysdale and Munro (1969) found ferritin synthesis associated with the free, and albumin synthesis with the bound ribosomal fractions.

In a recent series of papers, Shafritz (1974) and Shafritz, Drysdale and Isselbacher (1973), compared the translation of partially purified mRNA from bound and free polysomes from rabbit liver in both a homologous system, using rabbit liver polysomes, and a heterologous one using rabbit reticulocyte polysomes. Synthesis of both albumin and ferritin was demonstrated after rigid purification methods in the reticulocyte system. In the homologous liver system, membrane-derived messenger synthesized mainly albumin and messenger from free ribosomes mainly ferritin, but in the heterologous reticulocyte system no such distinction was found, ferritin and albumin being synthesized in similar ratios by both fractions. From these observations, they infer that the two classes of ribosomes differ not in their endogenous mRNA but in the control of its translation. In the heterologous system the normal control is relaxed and translation extended.

At the present time it is not known at which stage of its cycle the ribosome becomes attached to the endoplasmic reticulum membrane, but it has been demonstrated by Baglioni, Bleiberg and Zanderer (1971), that the large subunit binds first, suggesting that the small subunit with mRNA attached then joins the bound large subunit. Sabatini, Borgese, Adelman, Kreibich and Blobel (1972), discuss several models of attachment in the light of their own and other recent findings. Exchange of small subunits both into and out of microsomal preparations was shown to take place *in vitro*, but large subunits were not exchanged. This would mean continued attachment of the large subunit to the membrane through more than one cycle of protein synthesis. Other results suggest that the complete ribosome becomes attached with its messenger, or that mRNA is attached before initiation, but the final model has not been clearly elucidated.

I 10. Characteristics of the Ribosome

The ribosome is a roughly spherical cellular organelle of MW 3 - 4 x 10^6 consisting of two subunits of mass one-third and two-thirds of the total respectively. It is a porous structure consisting of RNA combined in a complex fashion with a number of different acidic and basic proteins. Regular tetramer crystals of ribosomes and of subunits have been obtained from cooled chick embryo cells and studied by electron microscopy. (Carey, Hobbs and Cook 1972).

Prokaryotes, for example bacteria, which have no differentiated nuclei, contain ribosomes of MW 2,7 x 10^6 and sedimentation constant 70S, those from *Escherichia coli* have an RNA to protein ratio of 2:1. The ribosomes from eukaryotic organisms, which have nucleated cells (higher organisms, mammals) are basically similar but larger (MW 4×10^6, 80S) and contain equal amounts of protein and RNA.

Ribosomes have a powerful capacity for binding proteins and in many cases it has been difficult to distinguish between constituent ribosomal proteins and those bound by contamination during isolation. An example is ribonuclease which is so tightly bound to ribosomes during isolation procedures that it has been thought to be a component of the ribosome. Although complete reconstitution from constituent RNA and proteins has been achieved in the case of both of the prokaryotic subunits (Nomura, Mizushima, Ozaki, Traub and Lowry 1969, Nomura and Erdmann 1970), the function of the individual proteins is still under study. Ribosomes are highly hydrated structures and hydrogen bonding both to associated water and between ribosomal constituents would therefore be important in maintaining their integrity. This has been shown by Petermann, Pavlovec and Hamilton (1972), in studies on rat liver ribosomes, which, like other mammalian ribosomes, dissociate on warming to 35°C and reassociate on cooling, without change in Mg^{2+} content. This reversible dissociation, which is attributed to hydrogen bonding effects, was found to be prevented by ethanol and ethylene glycol, both of which strengthen hydrogen bonds. On the other hand, urea which has a "structure-breaking" effect on water and is a commonly used protein denaturant, had the opposite effect, causing dissociation first to subunits and, on longer exposure, to loss of protein and unfolding of the subunits. Formamide, which is known to weaken RNA hydrogen bonds, had a similar effect. Treatment of subunits with formaldehyde, which reacts with the amino group of the RNA bases and so eliminates hydrogen bonding, prevented their reassociation.

The phosphate groups of the ribosomal RNA bind positively charged substances by electrostatic forces. Mg^{2+} is involved in maintaining ribosome structure, other metals are also bound, and polyamines have been found closely associated with ribosomes. Spermine and Spermidine can displace Mg^{2+} in binding to RNA, and have been used to prevent *E. coli* ribosomes from dissociating (Cohen and Lichtenstein 1960), and to inactivate ribonuclease (Thomas and Herbst 1963).

Dimers have frequently been reported as a high proportion of the ribosomes isolated from mammalian cells. Dimerisation is species-dependent and takes place with rat and hamster, but not with mouse or human ribosomes. The RNA of the large subunit provides the site for this phenomenon. (Reader and Stanners 1967).

Both types of ribosomes are dependent on bound Mg^{2+} for maintaining their integrity, and they can be dissociated by metal chelating agents such as ethylenediaminetetra-acetic acid (EDTA) into subunits sedimenting at 50S and 30S in prokaryotes and 60S and 40S in eukaryotes. Although subunits subsequently able to recombine to whole ribosomes active in protein synthesis can be obtained in this way from prokaryotes, ribosomes from eukaryotes require lower Mg^{2+} levels before dissociation takes place and then yield only inactive subunits. Martin, Wool, Rolleston and Low (1969), and Yang, Hamada and Schweet (1968), have prepared active eukaryotic subunits by dissociating ribosomes in high concentrations of KCl (0,5M) in the presence of Mg^{2+} ions.

The Prokaryotic Ribosome in Protein Synthesis

I II. The Ribosome Cycle

Extensive studies on prokaryotes, mainly *E. coli*, have enabled the part played by the ribosome in protein synthesis to be clarified in some detail. Dissociation into subunits was first demonstrated unequivocally to take place under physiological conditions by Kaempfer, Meselson and Raskas (1968), when E. coli cells were grown with heavy isotopes (^2H, ^{13}C, ^{15}N). Subsequent growth in "light" medium (of normal isotope content) and separation of ribosomes and "heavy" and "light" subunits showed the ribosomes to be intermediate in density. Thus dissociation and cycling through a subunit pool had taken place. Isolation of subunits from E. coli cultures and thus the establishment of the true distribution of polysomes and subunits was possible after the successful culture of "fragile" cells by Mangiarotti and Schlessinger (1966), such cells being easily lysed without destruction of the cellular organelles.

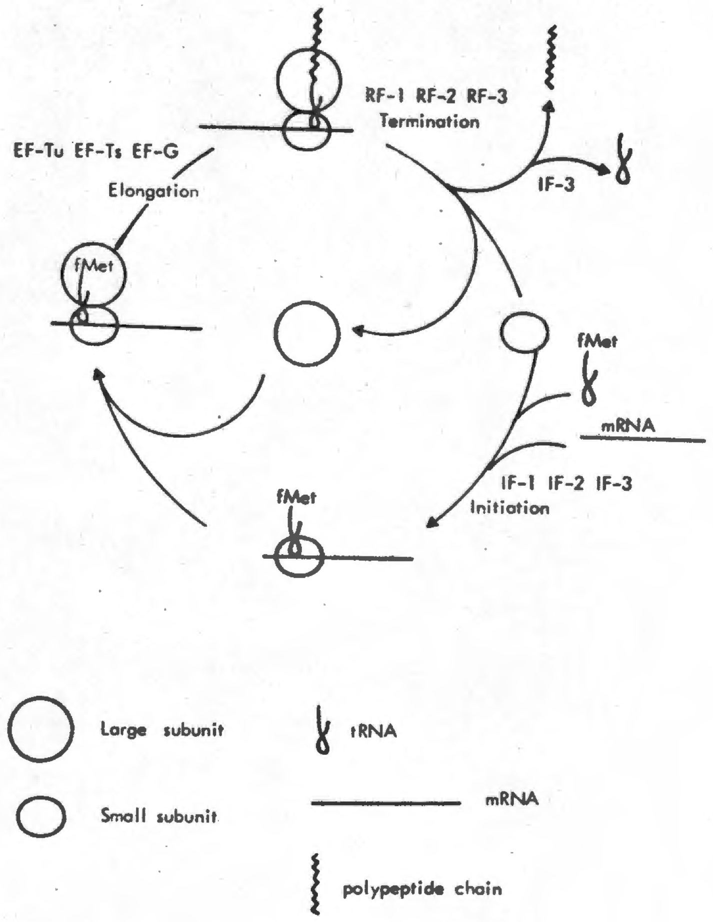
The part played by subunits and the interaction of specific factors during the cycle have been reviewed by Davis (1971).

A diagram of the ribosome cycle is shown in Fig. 2.

Protein synthesis is initiated by attachment of $fMettRNA_f$ and mRNA to the small (30S) subunit. Initiation factors IF-1 and IF-2 with GTP participate in this binding. GTP is hydrolysed to GDP and released with IF-2 in a bound form stabilised by IF-3. The 30S initiation complex is then joined by a large (50S) subunit to complete the 70S ribosome, which is then available for formation of the

FIG. 2.

THE RIBOSOME CYCLE
PROKARYOTES



first peptide bond between fMet and the incoming aatRNA, and further translation. This active ribosome may be part of a polysome since ribosomes attach as the mRNA is synthesized and initiation sites become available. The ribosome moves towards the 3' end of the messenger as the polypeptide chain it bears elongates. Elongation is catalysed by the three factors classified as EF-Tu, EF-Ts and EF-G. EF-Tu and EF-Ts are concerned with binding of GTP and aatRNA to the ribosome, with hydrolysis of GTP. After the formation of a peptide bond EF-G and GTP are required for translocation when hydrolysis of GTP again takes place. The factors RF-I and RF-2 interact with the release codons on the mRNA and, with RF-3, cause termination of the chain and release of the nascent polypeptide from tRNA. The factor IF-3 also interacts when ribosomal dissociation takes place before initiation of a new cycle.

I 12. Initiation

Purified initiation factors IF-I, IF-2 and IF-3 have been reported by Sabol, Sillero, Iwasaki and Ochoa (1970). Initiation was thought to proceed first through the binding of mRNA on the small subunit followed by attachment of the special initiation tRNA formylmethionyl tRNA ($fMettRNA_f$)

first isolated by Marcker and Sanger (1964). This step has recently been shown (Bernal, Blumberg and Nakamoto 1974), to precede the recognition of the initiator codon AUG on the mRNA in line with results found for eukaryotes. IF-3 is identical with the dissociation factor DF (Subramanian, Ron and Davis 1968). These protein factors associate reversibly with the ribosome and can be isolated in pure form from a IM NH_4Cl wash of ribosomal or 30S subunit preparations. The interaction of the factors involved in elongation and termination, as classified by Caskey, Leder, Moldave and Schlessinger (1972), is indicated at these two stages of translation, in the diagram in Fig. 2. Some of the factors interact with one another and the picture is not yet wholly clear.

Blockage of the ribosome cycle at various steps by antibiotics has been a useful technique, and the main effects of drugs on the cycle have been summarised by Schlessinger (1969). Examples are fusidic acid which "freezes" polysomes, tetracycline which causes accumulation of subunits and streptomycin which prevents dissociation of the monomer. Chloramphenicol blocks ribosome movement along mRNA and rifampycin prevents synthesis of new RNA. By these means, characteristic intermediates were prepared and studied, and the role of the two ribosomal sites involved in translocation, as proposed by Watson (1964), clarified.

I 13. Elongation

The Acceptor or Aminoacyl site (A site) binds the incoming aminoacyl-tRNA (aatRNA) for peptidyl transfer and then translocation of the peptidyl-tRNA (peptRNA) to the Donor or Peptidyl site (P site) takes place. At translocation the mRNA moves one codon along relative to the ribosome and the next codon is then available for binding of the incoming aatRNA on its entry into the A site. Puromycin reacts with the A site and mimics the incoming aatRNA, but, in so doing, terminates translation and causes release of nascent polypeptide chains from the ribosome as peptidyl-puromycin.

The elongation factors EF-Tu and EF-Ts control the binding of aatRNA to the A site and hydrolysis of GTP is involved at this step. After peptidyl transfer, which is catalysed by peptidyl transferase, an enzyme bound to the large subunit, the third protein factor EF-G and GTP are required for translocation, during which GTP is hydrolysed and the deacylated tRNA is released from the P site. (Lipmann 1969).

Studies on elongation have been facilitated by the availability of the artificial messenger polyuridylic acid (Poly U) (Nirenberg and Matthaei 1961), which operates by provision of the codon UUU for binding of phenylalaninetRNA (PhetRNA) and translation into polyphenylalanine. In the high Mg^{2+} buffers used for maintaining the integrity of the ribosomes in studies of protein synthesis, Poly U binds without initiation factors, thus simplifying the experiments.

I 14. Termination

Termination is specified on mRNA by three codons UAA (ochre), UAG (amber) and UGA, the nonsense codons (Garen 1968). These codons are recognised, not by tRNA, but by protein release factors which have been isolated from the ribosomal supernatant. RF-1 responds to the codons UAG and UAA and RF-2 to UAA and UGA. The third factor RF-3 has no activity alone but stimulates the action of the other two in the presence of the appropriate codons. Release activity is also exhibited by peptidyltransferase and termination may be associated with this site on the ribosome.

The Eukaryotic Ribosome in Protein Synthesis

I 15. Initiation and the Ribosome Cycle

As the process of protein synthesis in

eukaryotic systems becomes clarified it is seen to be fundamentally similar to that in prokaryotes. A well-substantiated ribosome cycle such as that drawn up for prokaryotes, where subunit exchange is rapid can not, however, yet be demonstrated, although initiation on the small subunit has been shown to be an obligatory step (Guthrie and Nomura 1968). The mammalian system requires a specific tRNA ($MettRNA_f$) for initiation, but it is not formylated. It can be distinguished from other MettRNA species by the fact that it can be formylated *in vitro* by *E. coli* transformylase (Caskey, Redfield and Weissbach 1967). Specific agents which block different stages of the cycle have again been useful in elucidating subunit exchange. Colombo, Vesco and Baglioni (1968), found that, on incubating reticulocytes with the inhibitor NaF, monomers accumulated, as their dissociation was inhibited, but the numbers of subunits declined, suggesting that they were used up in initiation. There was no decrease of subunits in the presence of NaF and cycloheximide as translation was then blocked at the polyribosome stage and initiation did not take place. Adamson, Howard and Herbert (1969), studied exchange between ^{32}P-labelled polyribosomes and the monoribosome and subunit pools, in a reticulocyte system with a high capacity for chain initiation.

In this system, steady-state protein synthesis functioned for 15 to 25 min at a rate comparable to that in the intact cell, and, during this period, 10 to 15 rounds of translation took place. Direct exchange with a portion of the subunit pool was found to be rapid, but the remainder of the subunits were inactive. Very little exchange with the monoribosomes took place, so there would seem to be a separate monoribosome pool exchanging only slowly, if at all, with the polyribosomes. Such monoribosomes are distinct from the inactive type of monomer artificially produced in the presence of drugs. The slow exchange of subunits was confirmed by Baglioni's group and, more recently, Baglioni (1972) has reported two types of small subunits, only one of which takes part in initiation. The results show that the active subunit only recognises the AUG codon through the initiator tRNA and mRNA must therefore bind after the MettRNA$_f$ is positioned on the ribosomal site.

This view has been confirmed by Schreier and Staehelin (1973), who separated four initiation proteins from reticulocytes, IF-E$_1$, IF-E$_2$, IF-E$_3$ and IF-E$_4$, one of which IF-E$_3$ is specifically required for MettRNA$_f$ binding in the absence of mRNA. Another, IF-E$_2$ can bind MettRNA$_f$ in the absence of IF-E$_3$ but in the presence of poly (A, U, G), but both, with GTP, are required for mRNA-independent binding of initiator tRNA. The initiator proteins are isolated from a high salt (0,5 M NH_4Cl or KCl) wash of ribosomes, and can be tested by their ability to bind Poly U at low Mg^{2+} concentration.

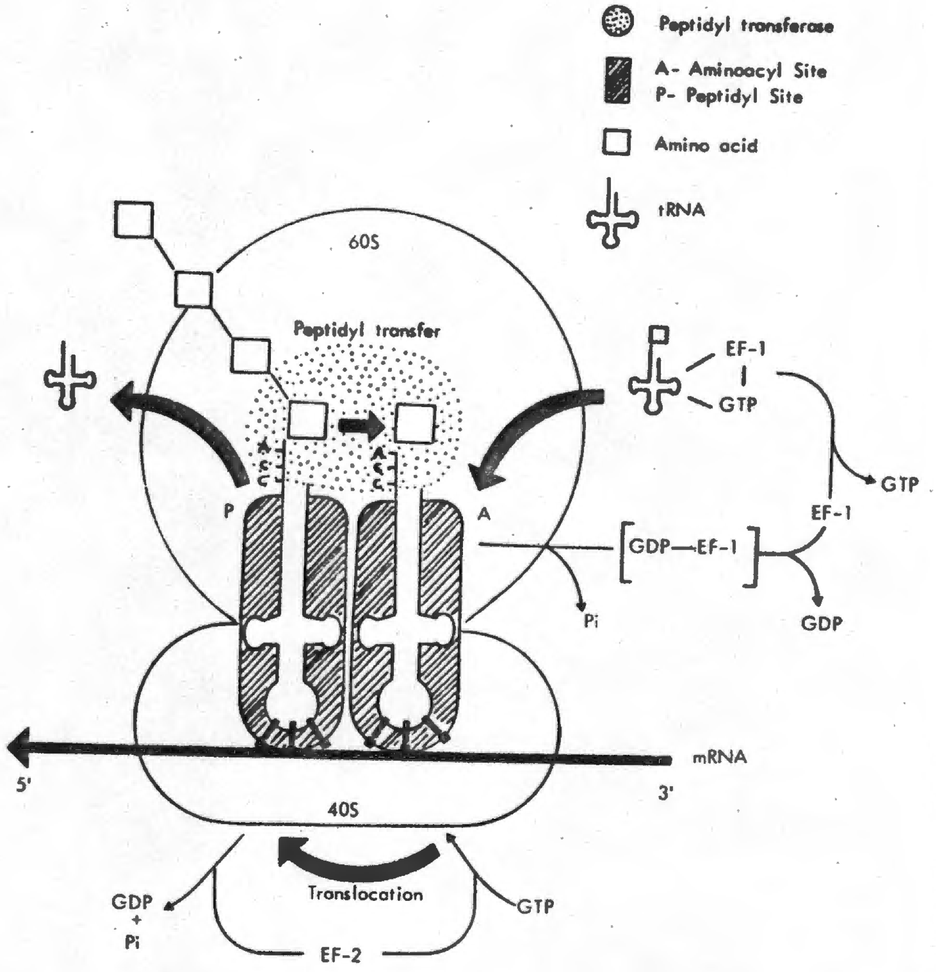
Eukaryotic factors have also been reported from the brine shrimp (Artemia salina) by Zasloff and Ochoa (1971), from embryonic chick muscle (Heywood 1970), and from rat liver (Grummt and Bielka 1971, Leader and Wool 1972). An initiation factor analogous to bacterial IF-3 has been isolated from rabbit reticulocytes by Kaempfer and Kaufman (1973), which is required for recycling of ribosomes and their binding to mRNA. This protein factor was found to complex with double stranded RNA, which implies that it normally recognises a double stranded region in mRNA.

I 16. Elongation

Elongation in eukaryotes follows essentially the same course as in prokaryotes. A diagram of the stages involved is given in Fig. 3. Factor EF-1 corresponds to the bacterial factors Tu and Ts and EF-2 to factor G. These proteins have been prepared from the soluble fraction of rat liver homogenates. EF-1 controls the binding of aatRNA to the ribosome (Arlinghaus, Shaeffer and Schweet

FIG. 3.

EUKARYOTIC RIBOSOME. ELONGATION



1964), and EF-2 the translocation of peptRNA and the release of uncharged tRNA from the ribosome (Skogerson and Moldave 1968).

Enzymatic attachment of aatRNA to the A site takes place in low Mg^{2+} concentration as a complex with EF-1 and GTP. This binding reaction does not depend on hydrolysis of GTP and can be studied separately by replacing GTP with its β-γ methylene analogue 5' guanylylmethylenediphosphonate (GMPP(CH_2)P) which cannot be hydrolysed and behaves as competitive inhibitor in reactions involving the hydrolysis of GTP. At peptide bond formation, EF-1 leaves the ribosome after GTP hydrolysis as a complex with GDP, which is subsequently released, freeing EF-1 for a fresh cycle (Moldave 1972).

Peptidyl transfer is an enzymatic reaction, independent of GTP hydrolysis, catalysed by peptidyl transferase which is located on the 60S subunit. No supernatant factors are required in forming the peptide bond (Neth, Monro, Heller, Battaner and Vasquez 1970). The carboxyl group of the partly synthesized polypeptide chain is activated through its attachment to the terminal adenosine of tRNA, and enzymatic cleavage of this ester linkage accompanies the formation of the peptide bond with the α amino group of the incoming aatRNA.

Translocation takes place with hydrolysis of

GTP following binding of EF-2. The activity of this factor is SH dependent and its binding, together with GTP, to the A site requires the presence of reduced thiol groups. The translocation reaction is inhibited by cycloheximide, which interferes at the binding site. These interactions have been clarified by study of the release of polypeptides by puromycin in a protein-synthesizing system from rat liver. (Baliga and Munro 1971, Baliga, Cohen and Munro 1970). When located at the A site, peptidyltRNA is not released by puromycin in the presence of cycloheximide, but if translocation to the P site is allowed to take place, freeing the A site for puromycin, the subsequent addition of cycloheximide has no effect on release.

Ribosomal intermediates at the various stages, from initiation to the post-translocational state have been prepared by Nombela and Ochoa (1973), employing N-acetyl-phenylalanyl tRNA (AcPhetRNA) as a model for chain initiation and PhetRNA with Poly U for elongation. Results of binding of EF-1 and EF-2 with these intermediates showed their interaction with the A and P sites to be mutually exclusive. The authors postulate that this selective control mechanism could best be explained by overlapping of the A and P sites and a conformational change in the ribosome at translocation. Regular pulsations of this nature during ribosome movement were originally proposed by Nishizuka and Lipmann (1966) by analogy with muscular contraction, energy being provided by hydrolysis of GTP instead of ATP.

The movement of mRNA relative to the ribosome has been demonstrated by Gupta, Waterson, Sopori, Weissman and Lengyel (1971), using E. coli ribosomes translating f2 bacteriophage RNA as messenger. Intermediates at different translation stages were prepared and the RNA not protected by the ribosome trimmed away with ribonuclease. Comparison of the protected sequences subsequently isolated from each stage with the known codons of the f2 phage confirmed that movement along the messenger by one triplet had taken place at translocation.

I 17. Termination

Termination in eukaryotes is specified by the same codons as in prokaryotes and the three codons have been identified in a reticulocyte system by Beaudet and Caskey (1971). They identified a protein-release factor (R) which is larger than the individual bacterial factors and may be a complex, since it recognises all three release codons.

Hydrolysis of peptRNA is ribosome-dependent and is thought to be linked with peptidyl transferase. This idea was strengthened by reports by Scholnick, Milman, Rosman and Caskey (1970) and by Fahnestock, Neuman, Shashona and Rich (1970), that ribosomes, presumably through the action of contained peptidyl transferase, can catalyse ester formation. This was demonstrated by substituting a derivative, hydroxypuromycin, in which the α amino group is replaced by a hydroxyl group, for puromycin. This compound was found to be active in the puromycin release reaction. It has been suggested by Vogel, Zamir and Elson (1969) that a release factor might alter the enzyme so that it catalyses the hydrolytic release of the polypeptide chain to water. An interesting analogy is the change in carboxypeptidase from a peptidase to an esterase by interaction with different metallic cofactors (Vallee and Williams 1968).

I 18. Proteins of the Ribosome

Work is actively in progress by a number of groups of investigators to study the proteins of ribosomes and to interrelate them, both spatially and functionally. The task is a formidable one as there is no repeating pattern or regular arrangement. The 70S ribosome has been mainly studied, since the 80S ribosome is even more complex, containing proteins exhibiting no similarities with those of the 70S ribosome by electrophoretic or immunological methods. Recent information on the ribosomal proteins and their function has been collected and reviewed by Wittmann (1972) and by Wittmann and Stöffler (1972).

True ribosomal proteins are regarded as those which are not removed by an NH_4Cl wash. Proteins from prepared subunits can be extracted by acetic acid at high Mg^{2+} concentration (Hardy, Kurland, Vognow and Mora 1969). Ca^{2+} and Mn^{2+} can substitute for Mg^{2+}. LiCl and urea, or digestion by ribonuclease, provide alternative means for extraction. Subsequent fractionation, chromatography and polyacrylamide gel electrophoresis are used to separate the mixture into its constituent proteins.

There is general agreement that there are 21 proteins associated with the small (30S) subunit (S1 - S21) and 34 with the large (50S) subunit (L1 - L34) in E. coli ribosomes.

The combined masses (400,000 daltons) of the 21 proteins found in 30S subunits considerably exceed the known mass (250,000 daltons) of protein of the particle, proving that the subunits are heterogeneous, confirming other studies that had already led to this conclusion. Seven proteins are found to occur only once per particle, these are designated "unit-proteins", but others are "fractional" or "marginal", present significantly less than once. In the 50S subunit the pattern of distribution of some of the proteins suggests the presence of repeated structures. Study of the function of some of the proteins has enabled progress to be made in understanding ribosomal function in protein synthesis. One of the 50S proteins, (L6) is possibly the peptidyl transferase, or is closely concerned at least with its activity. Another two (L7 and L12) have similar structures and play a role in translocation and the EF-G factor-dependent GTPase reaction. The proteins of the 30S subunit have been studied in more detail, many of these being involved in binding to the RNA and they act cooperatively in reconstitution of the subunit. One large protein (S1) is essential for the binding of Poly U and this protein is one of those "fractional" proteins observed to be absent from those subunits which form complexes on columns containing immobilised initiation codons (Bollen, Pêtre and Grosjean 1972). 30S subunits can be separated into active and inactive fractions in this way. Another protein (S21), when bound to the 30S subunit, prevents added 50S subunits from forming 30S - 50S couples able to bind fMettRNA$_f$. This interesting finding, together with the information collected from protein studies, has enabled Kurland, Donner, van Duin, Green, Lutter, Randall-Hazelbauer, Schaup and Zeichhardt (1972) to discuss models which have been proposed for translocation. The reversible binding of the S21 protein could be directly related to a conformational change in the ribosome, which would in consequence adopt two states alternately, the pre- and post-translocational states. These two alternating states would then accomodate in turn one of each of the two possible configurations of the anticodon loop of tRNA proposed by Fuller and Hodgson (1967). The resulting allosteric transition would enforce anticodon-codon interaction by each tRNA in turn and the mRNA would be moved through the ribosome by the ratchet mechanism proposed by Woese (1970).

I 19. Transfer RNA

Transfer RNA was first proven to be the molecule responsible for bringing free amino acids to the messenger template on the ribosome by Hoagland, Stephenson, Scott, Hecht and Zamecnik (1958). This RNA molecule (4S, MW 25,000) functions as an adaptor between the codon of the messenger RNA, the amino acid which it selects, and the site for peptide bond formation on the ribosome. To fulfil these separate functions it has a complex threedimensional structure which in plan is represented by the shape of a clover leaf. Each of the three lobes has a specific function: one (the TψC loop) probably binds to the ribosome; adjacent to it is the anticodon region, and this is followed by the dihydrouracil (hU) loop which is thought to bind to the amino acyl synthetase. The 3' end always has the sequence CCA. The 3' OH group of the ribose with which the amino acid is esterified by its synthetase is on this terminal adenylic acid. The 5' end, at which a triphosphate is attached to the 5' OH of the ribose, is usually guanylic acid (G). The lobes and the minor unpaired regions contain a number of N-methylated bases and also unusual bases such as pseudouridine (ψ), dihydrouridine (hU) and inosine, which disrupt base-pairing and so maintain an open structure. The remainder of the molecule is held in hairpin loops by Watson-Crick base pairs. The anticodon loop in the Fuller-Hodgson (1967) model is closed by two G-C base pairs and has seven unpaired bases, three of which comprise the anticodon. All tRNAs must be folded in a similar 3-dimensional shape since mixtures can form regular crystals. A structure of yeast PhetRNA as deduced from high-resolution x-ray crystallography has recently been described. The molecule has an L-shaped configuration with the anticodon opposite to the 3', 5' ends and the TψC and hU loops are folded across in a complex central region (Suddath, Quigley, McPherson, Sneden, Kim, Kim and Rich 1974).

There are two MettRNAs, one of which inserts methionine into the polypeptide chain while the other (fMettRNA$_f$) initiates in prokaryotes. Sequence analysis has revealed that these two tRNAs have the same anticodon (CAU). There are, however, differences in their bases and the initiator tRNA has cytidylic acid (C) at the 5' end instead of the usual guanylic acid (G).

In many cases, several tRNA species differ in their codon sequence but nevertheless select the same amino acid, which means that such an amino acid is specified by more than one codon. This constitutes evidence for the degeneracy of the genetic code. In addition, moreover, a single tRNA molecule can bind through its anticodon to more than one codon. These findings are explicable in terms of the "Wobble" concept of Crick (1966). He postulated that the antiparallel base pairing of the third base (5' end) of tRNA to the base at the 3' end of the codon is not as strict as in the case of the first two bases. Evidence derived from the anticodons in different tRNAs has confirmed this hypothesis. In spatial models of the anticodon loop, the third base has also been observed to be less confined than the other two.

I 20. Messenger RNA

The small contribution (<1%) of mammalian mRNA to total cellular RNA and its instability have made its isolation and study a difficult problem. The presence of such an intermediate in the control of protein synthesis was predicted by Jacob and Monod (1961) some years before refined techniques enabled detailed studies to be carried out with any degree of success. mRNA, as with other RNA species, is first transcribed in the cellular nucleus as very large molecules of variable size (20S - 100S), a component part of the so-called heterogeneous nuclear RNA (HnRNA) (Darnell, Jelinek and Molloy 1973). Label from RNA precursors is rapidly incorporated into this class of RNA and a portion of it is further modified to become finally the mRNA which becomes attached to ribosomesin the cytoplasm. This RNA has a G-C content of about 45% of the total nucleotides therein resembling DNA, which is to be expected since mRNA comprises a mixture of many different molecules transcribed from many different parts of the DNA genome. Consequently, it is sometimes known as DNA-like RNA (dRNA). A technique which has been much applied in prokaryotic systems is to hybridize dRNA with DNA and so to isolate mRNA for further study (Gillespie and Spiegelman 1965).

It has now been proved that nuclear HnRNA is the precursor of cytoplasmic mRNA by several groups of workers. Stevens and A. R. Williamson (1973) were able to purify a fraction of this pre-mRNA ($MW > 2 \times 10^6$) by making use of a binding site between the pre-mRNA and the H chain of immunoglobulin and isolation by antibody precipitation. The purified HnRNA fraction could then be employed to translate immunoglobulin in *Xenopus laevis* oocytes, a system which is often used for the translation of varied mRNA species. High MW HnRNA mouse globin sequences have also been translated in this way by R. Williamson, Drewienkiewicz and Paul (1973). The possibility that in such experiments a low MW nuclear RNA may have been associated with the high MW molecule through hydrogen-bonding or other non-covalent binding has been completely excluded by Ruiz-Carrillo, Beato, Schutz, Fiegelson and Allfrey (1973). They were able to demonstrate translation by a large nuclear RNA after centrifugal separation in a gradient containing 99% dimethyl sulphoxide. In this dipolar aprotic solvent no hydrogen-bonding is possible. (Parker 1962).

A characteristic of mRNA and its precursor HnRNA is the presence of regions of polyadenylic acid

(Poly A) about 200 nucleotides long at the 3' end of the molecule. Poly A is added in the nucleus, but following transcription. This sequence of events has been confirmed by the presence of Poly A in adenovirus - specific nuclear RNA. Poly A must have been added by a mechanism provided by the host since the virus is able to transcribe the mRNA sequences only derived from its own DNA (Philipson, Wall, Glickman and Darnell 1971).

The presence of the segment of Poly A on mRNA has been useful in its purification. Thus, Aviv and Leder (1972) used a column of oligo-thymidylic acid (Poly T)-cellulose for preferential binding of the Poly A-rich region of mRNA. In this way they separated globin mRNA from the bulk of rRNA in RNA extracts of rabbit reticulocyte polysomes. Non-absorbed material was eluted and the purified mRNA could then be washed from the column with buffers of low ionic strength.

Poly A has been found to be the binding site for two proteins (MW 78,000 and 52,000) by Blobel (1973). Proteins of similar molecular weight were isolated from mRNA released from polyribosomes of rat liver, mouse L cells and reticulocytes. No function for these proteins has been suggested, but complexes of proteins with mRNA have been reported previously. Particles containing dRNA and showing a polydisperse sedimentation pattern and not disrupted by removal of Mg^{2+} with EDTA have been isolated from various cell extracts and named "informosomes" by Spirin (1969). In the nucleus, globular protein particles called "informofers" by Lukanidin, Zalmanzon, Komaromi, Samarina and Georgiev (1972), have been proposed as sites for dRNA processing and transport to the nuclear membrane. Woodcock and Mansbridge (1971) isolated rapidly-labelled ribonucleoprotein particles, from rat liver cytoplasm, which became associated with polysomes. They contained 47% protein after purification, but the possibility of some adsorbed adventitious protein still remaining could not be excluded. They suggested that if the protein is functional in such particles it could protect the mRNA from degradation or prevent and so control its translation.

Messenger RNA is both synthesized and read in the 5' to 3' direction (Jorgenson, Buch and Nierlich 1969), and in prokaryotes ribosomes quickly attach to the nascent mRNA chain which is then degraded immediately after translation. Instead of having a lifetime of only minutes, eukaryotic mRNA survives for some hours, and estimates of its half-life are of great interest in the study of control mechanisms in protein synthesis. The drug actinomycin D, which binds to DNA and inhibits further mRNA transcription, has been used to measure the decay of mRNA in the cell. A lifetime of 5 hr has been calculated for rat liver mRNA by Tominagha, Aki and Natori (1971) using actinomycin D. There have, however, been reports of toxic effects such as inhibition of respiration and glycolysis (Soeiro and Amos 1966) caused by this drug, and of inhibition of protein synthesis due to failure of initiation rather than mRNA decay (Singer and Penman 1972).

New estimates have been made using the drug cordycepin, the 3'deoxy analogue of adenosine, which selectively inhibits the addition of Poly A to HnRNA in the nucleus and blocks transport of mRNA to the cytoplasm and its appearance in polyribosomes (Penman, Rosbash and Penman 1970). Grayson and Berry (1973) using specialised glandular secretory cells, a type of system which shows a long mRNA half-life in the presence of actinomycin D, found this estimate to be reduced from 12 hr to 2 hr in the presence of cordycepin. They concluded that there is a pool of mRNA in the nucleus which can be transported to the cytoplasm in the presence of actinomycin D but not when Poly A synthesis is inhibited by cordycepin.

The average mRNA half-life in non-specialised cells has been estimated at 2 - 3 hr using actinomycin

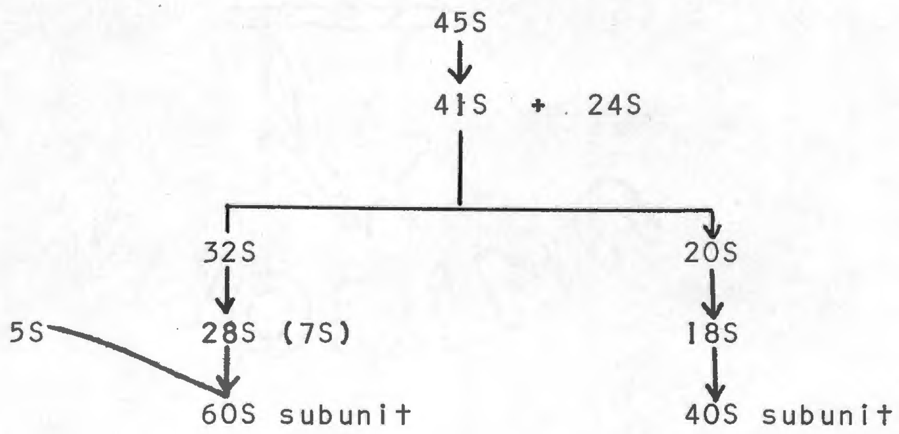
D. This has been extended to an estimated 12 - 14 hr by Cowan and Milstein (1974) without the use of drugs. They followed the labelling of mRNA for immunoglobulin light chain in cultured mouse myeloma cells by ^3H uridine in carefully timed pulse-chase procedures. The mRNA was shown to decay linearly throughout the cell cycle and so independently of cell division.

I 21. Ribosomal RNA

About 80% of the total ribonucleic acid of the cell is found in the cytoplasm in ribosomes. Of the remainder most occurs as tRNA and nuclear RNA represent only a small proportion of the total. Much of the information on the RNA of the animal cell and its characterization has come from cell culture, and Darnell (1968) is the author of a comprehensive review on the biochemistry and biosynthesis of RNA in cultured cells. Maden (1968) has summarised results on ribosome formation in various types of animal cells. Purified ribosomal RNA (rRNA) has no template role and the biological necessity for the high content of RNA in ribosomes has not been satisfactorily explained.

Together with the other classes, rRNA is transcribed from the DNA genes as very large precursor molecules (HnRNA). Most of this RNA never leaves the nucleus, but is degraded during processing of the various RNA species. At the time of transcription, rRNA is extensively methylated, by transfer of the terminal methyl group of methionine partly on the bases, but more especially at the 2'OH position of ribose. This methylation confers resistance to hydrolysis on the adjacent phosphodiester bond and therefore it plays an important part in rRNA processing. If methionine is withdrawn from the cell, precursor RNA is still synthesized, but it is degraded in the nucleus and not transferred to ribosomes (Vaughan, Soeiro, Warner and Darnell 1967). The base pseudouridine(ψ)is also present in significant amounts in precursor and rRNA.

The general scheme for rRNA processing proposed for HeLa cells is agreed to be as follows: (Mirault and Scherrer 1972, Wellauer and Dawid (1973).



The intermediate stages occur during cleavage of the unmethylated "spacer" sections of the precursor 45S molecule by endonucleases followed by trimming by exonucleases. During the transition of 32S to 28S RNA, a small portion, 7S, becomes hydrogen-bonded to the 28S molecule (Pene, Knight and Darnell 1968). This can be released from its association by gentle denaturation. A single 18S molecule (MW 0,7 x 10^6) becomes the constituent RNA of the small ribosomal subunit. The large subunit contains the 28S RNA molecule (MW 1,8 x 10^6) together with a small 5S RNA of 120 nucleotides (Brownlee, Sanger and Barrell 1968). This 5S RNA originates separately in the nucleus and not from processing of the precursor 45S RNA. It is also found in the large subunit of prokaryotic ribosomes; the other species is 23S (MW 1,1 x 10^6) and the small subunit contains a 16S (MW 0,55 x 10^6) molecule.

Proteins from the cytoplasm migrate to the nucleus and combine with the precursor RNA forming preribosomal particles in which processing of the RNA takes place. 80S preribosomes of HeLa cells have been found to be already associated with most of the large subunit proteins and some of those from the small subunit (Shepherd and Maden 1972). Other proteins associate during processing but do not leave the nucleus: they probably have specific functions in ribosome assembly up to the time of release of completed ribosomal subunits into the cytoplasm (Kumar and Warner 1972).

5S RNA has been shown to be part of the large subunit of all ribosomes, both prokaryotic (Marcot-Queiroz, Julien, Rosset and Monier 1965) and eukaryotic (Comb, Sarkar, De Vallet and Pinzino 1965). It can be dissociated from prokaryotic subunits by removal of Mg^{2+} or exposure to high salt concentrations. The subunit retains its structure but loses its activity in protein synthesis (Sarkar and Comb 1969). In eukaryotes, however, 5S RNA can be separated only under denaturing conditions with EDTA at elevated pH (Zehavi-Willner 1970, Petermann and Pavlovec 1969). It is released as a complex with a protein of molecular weight about 35,000. (Blobel 1971). Bacterial 5S RNA always has 120 nucleotides, but in mammals an extra nucleotide is often found (Forget and Weissman 1969, Williamson and Brownlee 1969), and there are many differences in the nucleotide sequence between bacterial and mammalian 5S RNA. This RNA has a number of base-paired regions and is folded in a rigid conformation which would play a part in its important structural role. It is the required binding site for at least four important proteins, and these were found not to bind during reassembly of the large subunit of <u>Bacillus stearothermophilus</u> if 5S RNA was omitted from the mixture (Erdmann, Fahnestock, Higo and Nomura 1971).

Forget and Weissman (1969) suggested 5S RNA as the binding site of the TψC loop of tRNA. It has been shown (Erdmann, Sprinzl and Pongs 1973) that in the 5S RNA sequence UGAAC the two adenines are exposed on the surface of the ribosome and these two residues are essential for biological activity and the binding of the nucleotide fragment TψCG. Extension of this work by the same group has shown recently (Grummt, Grummt, Gross, Sprinzl, Richter and Erdmann 1974) for a eukaryotic system that, whereas MettRNA binding to the ribosome is inhibited by this TψCG fragment, binding of MettRNA_f is not, so the TψC sequence is not needed by eukaryotes for tRNA binding to the ribosomes. They conclude that the initiator tRNA binds to a different site from the other tRNAs. Initiator binding must, therefore, take place, not at the aminoacyl (A) site, but directly into the peptidyl (P) site.

The long RNA molecules of the ribosomal subunits are known to have considerable secondary structure which is essentially maintained in the free state in solution. The presence of helical regions in the RNA in ribosomes has been shown by X-ray diffraction patterns (Zubay and Wilkins 1960, Klug, Holmes and Finch 1961). Digestion of ribosomes by ribonucleases of different strengths and specificities results in specific changes in RNA content in the remaining particles. Such studies have shown definite regions of RNA to be exposed on the surface of the ribosome (Hartman, Amaya and Schachter 1970), and these exposed sites are preferentially attacked in the isolated rRNA (Cahn, Schachter and Rich 1970).

Another technique, used particularly for studying the binding of proteins to DNA, has been adapted to rRNA by Bollen, Herzog, Favre, Thibault and Gros (1970). This involves use of ethidium bromide, which intercalates as a fluorescent complex with double-helical regions of nucleotides. They first complexed 16S RNA with the reagent, and changes in fluorescence were then followed as the 30S proteins were added and the subunits reconstituted. Displacement of the ethidium bromide from its binding sites showed that some of the proteins were attached to double helical regions of the rRNA. Previously it had been thought that binding sites for protein were only on single stranded regions in rRNA.

I 22. Spectrophotometric Studies

Extensive spectrophotometric studies have been made on ribosomes and their isolated RNA (Schlessinger 1960, Petermann 1964, Cox 1969).

Analysis of melting spectra and denaturation curves enables correction to be made for spectral changes caused by stacking of bases in single stranded regions. Reticulocyte ribosomes were found by Cox (1970) to have up to 70% of their RNA folded in double helical structure, probably as short helical hairpin loops. The rRNA molecule from the large subunit had a more stable structure than that from the small subunit, due to the higher proportion of guanine and cytosine in its bases, these forming the more stable G-C base pairs. Melting behaviour was correlated with helicity by changes in the absorbance, the hypochromic effect of the reversal from a disordered to an ordered form being followed by the lowering of the absorbance during cooling.

Such hypochromic effects are accompanied by an increased Cotton effect centred around 260_{nm} due to the stacking interactions of adjoining bases in the more rigid helical conformation.

The techniques of optical rotatory dispersion (ORD) and, more recently, circular dichroism (CD) provide a sensitive index of changes in geometry. If RNA is base-paired, a large Cotton effect is observed, of greater magnitude than in the case of double-helical DNA. This has been shown by McMullen, Jaskunas and Tinoco (1967), and Samejima, Hashizume, Imahori, Fuji and Miura (1968) for fully-paired viral RNA, which had a CD curve of about twice the intensity of that of DNA reported in their earlier studies. This technique has been used to predict the conformation of transfer RNA from studies on oligomers of ribonucleic acids (Cantor, Jaskunas and Tinoco 1966). Denaturing of tRNA has been traced to conformational changes in this way by Adams, Lindahl and Fresco (1967), and the influence of cation interactions on changes in the secondary structure of ribosomal RNA from mouse liver has been studied by Watson and Kidson (1969). Ionic concentration is thought to influence the tilt of the base with respect to the helical axis (Willick and Kay 1971).

Further confirmation of the partial double-helical structure of ribosomal RNA was obtained by Bush and Scheraga (1967), who used ORD studies on *E. coli* rRNA to show that in ribosomes it was largely in a base-paired double helical form with mainly G-C base pairs. McPhie and Gratzer (1966) found yeast, *E. coli* and reticulocyterRNA all to have similar ORD curves. In all cases the extracted RNA showed a positive ultraviolet Cotton effect in the same position as the intact ribosomes, centred around $266_{nm.}$ Removal of the protein caused small changes around 233_{nm} but the main Cotton effect was unchanged, showing that the RNA conformation remains the same in the free state.

I 23. Metals and Polynucleotides

The interaction of metal ions with polynucleotides has been studied by Eichhorn and Shin (1968). They place metals in a series showing their relative effect on DNA helicity based on melting curves and absorption spectra as follows:

Mg(2), Co(2), Ni(2), Mn(2),	Zn(2), Cd(2), Cu(2)
⟵	⟶
Increasing Tm	Decreasing Tm
Stabilisation	Destabilisation
Reaction with phosphate	Reaction with base
λ max unchanged.	λ max shows red shift 258 - 262 nm.

A sharp change in melting temperature (Tm) on passing from Zn(2) to Cd(2) was found, and heating with zinc did not alter the Tm significantly. Zn(2) binds to melted DNA chains cooperatively and holds the two chains closely enough to regenerate the double helix on cooling. In the presence of Cd(2), however, the Tm rises at low concentration and then falls by as much as 10°C at a binding ratio of 3 mole/DNA(P). Thus, Cd(2) binds more strongly than Zn(2) but destabilises the ordered structure. On cooling, in contrast to Zn(2), no decrease in absorbance, which would indicate regeneration of the double helix, is found. The strong bonds of Cd(2) to the DNA bases therefore remain intact. Mg(2) by binding to phosphate stabilises the structure, and increasing concentrations therefore raise the Tm, but cooling results in random stacking as Mg(2) shows no tendency to hold unwound chains in proximity. The effects shown by Cd(2) are more marked in the case of Cu(2) which causes a 30°C decrease in Tm at 2M concentration. Renaturation in the presence of these two metals only takes place after the addition of the electrolyte, sodium nitrate, to the cooled solution, when renaturation is immediate in the presence of Cd(2) but takes 5 hr in the case of Cu(2). Cu(2) is therefore more firmly bound than Cd(2) to the hydrogen bonding sites on the DNA bases.

Hg is unique in that it can complex with native DNA (Yamane and Davidson 1961), in contrast to Cu which requires prior heating to partially unwind the helix. Its interaction with DNA has been extensively studied and it is thought to bind to the N atoms of the bases. Carrabine and Sunderalingham (1971) in a study of complexes of mercuric chloride with uracil and dihydrouracil found, from crystallographic work, that the chloride is present in $Hg\text{-}Cl_2$ chains bonded to the O(4) atoms of the uracil rings which are consequently pulled significantly out of plane, disrupting the hydrogen bonds. It is interesting that they report other studies (Cavalca, Nardelli and Fava 1960), which show bis (biuret) Hg(2) chloride to be isostructural with the corresponding Cd(2) compound, the latter closely resembling their uracil complex.

It is clear that interference by metals with hydrogen bonding in DNA and RNA can cause marked conformational changes and interfere with its functioning. Such changes, demonstrated by CD and proton magnetic resonance (PMR) studies of the binding of the carcinogen N-2-Acetyl-amino-fluorene to ribonucleotides (Nelson, Grunberger, Canton and Weinstein 1971), are associated with impairment of the template activity of RNA polymerase and the binding and codon recognition of transfer RNA.

PART II

EXPERIMENTAL AND RESULTS

II 1. Animals and Intoxication

Locally bred rats of a Wistar-derived strain were housed in cages 30 x 25 x 25 cm, fed a commercial brand of rabbit pellets and supplied with water ad libitum. On this diet young rats gained an average of 20 g per week.

Young adult male rats (200 - 250 g) were injected intraperitoneally twice weekly under light ether anaesthesia with a sterile 0,1% solution of cadmium chloride ($CdCl_2.2\frac{1}{2}\ H_2O$) in 0,85% w/v NaCl at a dosage level of 2 mg Cd/kg/week, usually 5 injections of 0,4 - 0,5 ml each being given over 2 - 3 weeks. This resulted in a slower weight gain as compared with controls or, in most cases, a marked loss in weight although food consumption remained at the same level.

Cadmium content of the liver after this treatment averaged 0,1 mg/g (10^{-3}M). Figures for individual rats are given in Table I, and liver weight compared to body weight is shown for normal and Cd-treated rats. Livers were heavier on the average in the treated animals.

TABLE I

	Rat Weight	Liver, Wet Weight		Liver		Cadmium Analysis
	g	g		% of Rat Weight		mg/g/liver Wet Weight
Cd	196	5,81		2,91		0,18
	250	7,50		3,00		0,10
	254	8,00		3,15		0,10
	220	6,03		2,74		0,15
	226	6,91		3,06		0,13
	211	6,80		3,22		0,10
	146	5,15		3,53		0,19
	200	7,44		3,72		0,07
	240	8,95		3,73		0,12
	215	6,60		3,07		0,14
	316	10,43		3,30		0,11
	218	6,90		3,16		0,17
			Mean	3,24	Mean	0,13
			±s.d.	0,30	±s.d.	0,03
N	290	8,26		2,85		
	270	5,78		2,14		
	315	9,57		3,04		
	210	5,43		2,59		
	260	5,70		2,19		
	320	8,23		2,57		
	329	8,48		2,58		
	298	7,70		2,58		
	280	7,15		2,55		
	262	5,98		2,29		
	287	5,60		1,95		
	230	6,02		2,62		
			Mean	2,49		
			±s.d.	0,30		

On completion of the course of injections both injected and control animals were fasted overnight with free access to drinking water. Fasting was necessary in order to diminish the concentration of hepatic glycogen (Huston, Schrader, Honold, Beecher, Cooper and Sauberlich 1969), which forms a pellet below that of the ribosomes and so contaminates the preparation. Fasting is known to cause some disaggregation of polysomes (Gamulin, Norman and Clark 1973), but recovery is facilitated (Lowe, Reid and Hallinan 1970), so, as the normal rats used as controls were treated in the same way, valid comparisons could still be made.

Animals were killed by a blow on the head, the liver quickly removed, weighed in buffer and washed four times in ice-cold buffer before homogenization.

II 2. Homogenization

Treated livers, due to their firm texture could not be homogenized simply by cutting up, adding buffer and pressing through a Dounce homogenizer. This method, (Dounce 1955), initially described for preparation of intact nuclei, would be expected to minimise damage to organelles and has been frequently used for preparation of polyribosomes. (Hawtrey, Schirren and Dijkstra 1963, Munro, Jackson and Korner 1964).

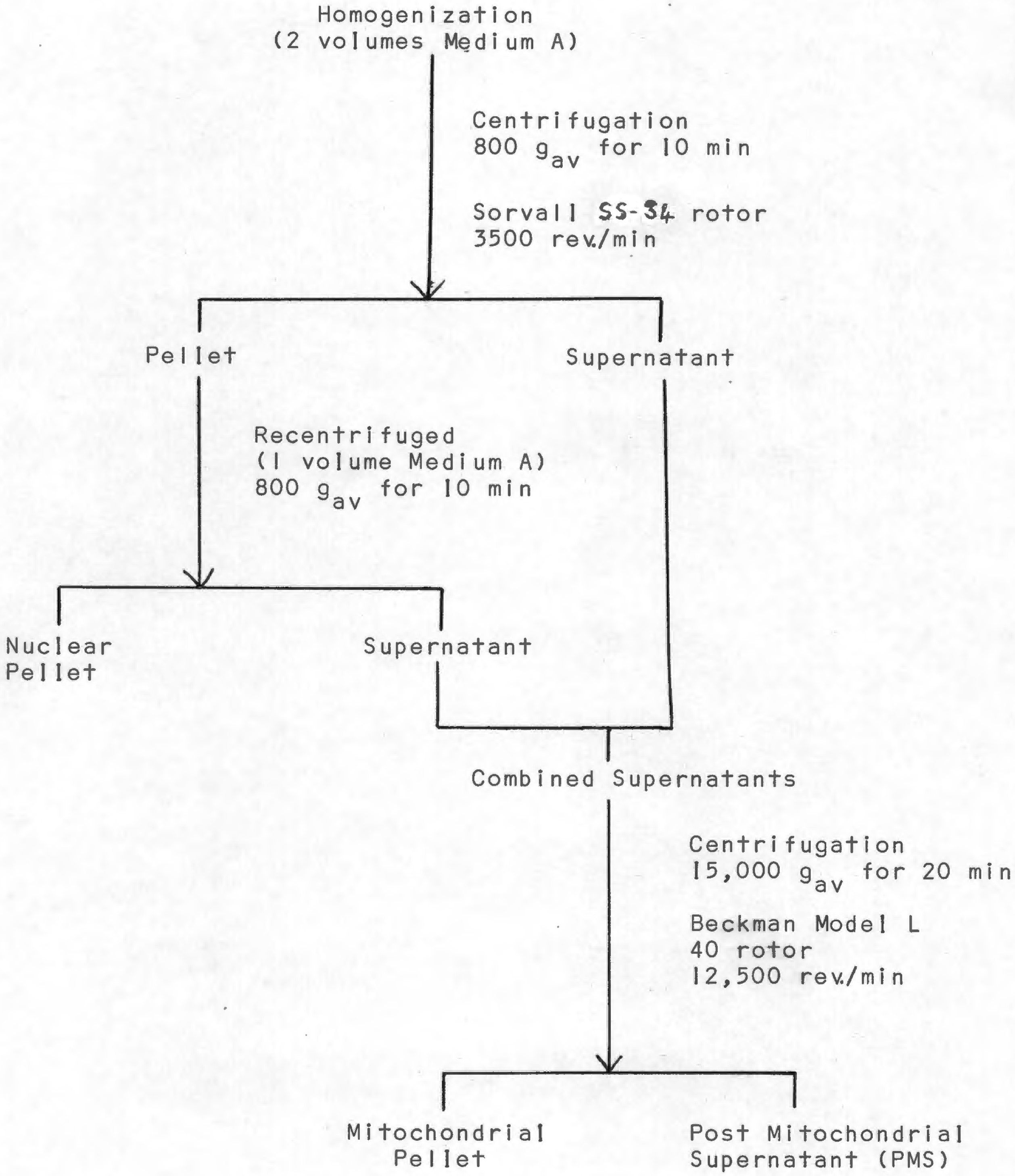
Blobel and Potter (1967), have made a critical study of the methodology of ribosome preparation from rat liver. They concluded that the use of the Potter-Elvehjem homogenizer with a motor-driven Teflon pestle did not cause detachment of ribosomes from the endoplasmic reticulum but resulted instead in more efficient fragmentation of the membranes to give smaller microsomes and therefore a higher recovery of ribosomes. This method of homogenization was therefore used, making 4 - 5 passes of the loose-fitting pestle (0,010 ins clearance). Filtration of the homogenate through cheesecloth to remove coarse particles caused variable losses in small scale work, so, instead, the homogenate was pressed through a small pad of glass wool placed inside a 20 ml disposable plastic syringe.

Medium A (0,05 M *tris* -HCl pH7,6(4°), (Hoagland, Stephenson, Scott, Hecht and Zamecnik 1958, Littlefield and Keller 1957), 0,025 M KCl, 0,005 M $MgCl_2$, 0,25 M sucrose) was used for homogenization. The high Mg^{2+} and K^{+} in this buffer are essential in order to maintain the integrity of the ribosomes, which dissociate in low Mg^{2+} buffer (Tissières, Watson, Schlessinger and Hollingworth 1959).

II 3. Preparation of Post Mitochondrial Supernatant

Blobel and Potter (1967) found that the minimal centrifugal force required to sediment nuclei caused a 35% loss of ribosomes, which by washing of the pellet could be brought down to 25%. As it was planned to make a quantitative comparison between normal preparations and those from Cd-treated rats the following procedure was initially used. (Hawtrey and Nourse 1966, Fitschen 1967). (See chart).

CHART


Homogenization
(2 volumes Medium A)
Centrifugation
800 g_{av} for 10 min
Sorvall SS-34 rotor
3500 rev./min
Pellet
Supernatant
Recentrifuged
(1 volume Medium A)
800 g_{av} for 10 min
Nuclear Pellet
Supernatant
Combined Supernatants
Centrifugation
15,000 g_{av} for 20 min
Beckman Model L
40 rotor
12,500 rev./min
Mitochondrial Pellet
Post Mitochondrial Supernatant (PMS)

II 4. Isolation of Ribosomes

It was decided that for general studies of protein synthesis the post mitochondrial supernatant (PMS) should be treated with detergent to produce a preparation containing the total ribosomes, that is to say both those free in the cytoplasm and those bound to the endoplasmic reticulum. Sodium deoxycholate (DOC) is most frequently used to release the bound ribosomes. This ionic detergent purifies ribosomes from contamination by lipoproteins with little loss of activity. (Munro, Jackson and Korner 1964, Korner 1961, Hallinan, Nagley, Murty, Bennett and Grant 1969, Olsnes 1971). In the standard method of Wettstein, Staehelin and Noll (1963), for the preparation of 'C ribosomes' the DOC-treated PMS is layered over a discontinuous gradient of 0,5 M over 1,0 - 2,0 M buffered sucrose. This results, particularly with the 2 M sucrose, in considerable purification of the ribosomes, most of the protein contaminants being retained on the interface of the lower layer. In this way, preparations containing a high proportion of polyribosomes are obtained which are active in protein synthesis.

The separation of ribosomes directly from the PMS, and not from isolated microsomes, protects the polyribosome from breakdown by fission of the messenger RNA (mRNA), as the cell sap contains a ribonuclease inhibitor (Bont, Rezelman and Bloemendal 1965, Lawford, Langford and Schachter 1966). The protective effect of the "high-speed" supernatant has been clearly shown by Blobel and Potter (1966), in their studies of the size distribution of ribosomes prepared with or without this cell sap supernatant.

II 5. Cadmium Analysis

Liver samples (0,2 - 0,4 g wet weight) were digested by an oxidative procedure essentially as described by Smith, Kench and Lane (1955) and adapted by Kench and Gubb (1970) and Gubb (1970). The method involved digestion in concentrated HNO_3 until brown fumes of nitrogen dioxide (N_2O_4) were no longer evolved. 0,1 - 0,2 ml of concentrated H_2SO_4 were then added and the digestion completed by adding H_2O_2 (30% w/v) in small portions to the cooled digest until on warming no further fumes were evolved and the digest remained colourless. This was then made to volume, (10 or 15 ml) with distilled water. Cadmium was then determined by atomic absorption spectrophotometry (Unican S.P. 90 or Techtron Type AA-5, Cd lamp, at 228,8 nm). Cd concentrations were read from a calibration curve drawn up from standard Cd solutions. The same method was used for Cd estimations on the supernatant from the ribosome pellet (0,09 mg/g liver) and on the mitochondrial pellet (0,02 mg/g liver). These analyses indicated a general distribution throughout the liver. Levels of Cd in normal livers were less than 10^{-7}M.

II 6. Histology

Livers of rats which had been injected 5 times with $CdCl_2$ were inspected in situ. The liver was found to be slightly enlarged and darker and firmer than normal. There were multiple adhesions between the liver and diaphragm, spleen, stomach and bowel. The lobes were bound together and showed pale fatty lesions on the inferior surface. The liver consistently presented a similar appearance. Histological examination at low magnification revealed no difference between the normal and Cd-treated liver. No excess fibrous tissue could be seen in the treated liver.

Following 5 injections with 0,85% w/v NaCl the liver of control animals was indistinguishable from untreated rats. There were no lesions in any of the lobes and no adhesions to any surrounding structures. Spleen and intestine appeared normal. The liver was soft and homogenized easily whereas liver tissue of Cd-treated rats was more resistant to homogenization.

II 7. Preparative Procedures

Careful treatment is necessary to prevent rupture of cellular components but at the same time to break up the maximum number of cells (Mathias 1966, Noll 1969, Blobel and Potter 1967). The ionic concentration is critical, particularly the ratio of K^+ to Mg^{2+} ions. High shearing forces must be avoided as these can cause release of degradative enzymes and mechanical disruption of mRNA. Rupture of lysosomes can release nucleases and proteases into the homogenate.

A large proportion of the ribosome population is attached to the membranes of the endoplasmic reticulum. (Campbell, Serck-Hansen and Lowe 1965, Bloemendal, Bont, de Vries and Benedetti 1967). These membranes must be broken into small fragments without damage to ribosomes or their attached proteins.

II 8. First Series of Experiments

One liver from a normal (N) and one from a cadmium-treated rat (Cd) were carried through the procedure concurrently under identical conditions. The temperature was maintained as close to 4°C as possible. A post mitochondrial supernatant was prepared as described (see chart) and a freshly prepared solution of DOC added to give a final concentration of 1,3%. Ribosomes were sedimented through 0,5 M (4 ml) and 1,5 M (3 ml) sucrose in Medium A buffer by centrifugation for 4 hr at 100,000 g_{av} (Beckman Model L ultracentrifuge 40 rotor, 39,000 rev./min). The supernatant, including the brown ferritin band at the top of the dense sucrose, was aspirated with a capillary pipette and the walls of the tubes wiped. The remaining sucrose was then poured off and the firm glassy pellets quickly rinsed and suspended in Medium A and kept overnight in ice. The preparation was then clarified by centrifugation at 800 g_{av} for 10 min and stored at 0°C at a concentration of 50 - 100 $A_{260\ nm}$ units/ml. In early experiments, ribosome pellets were yellow-brown in colour due to ferritin contamination, possibly caused by poor temperature control during centrifugation. Pellets from Cd-preparations were consistently browner than those of the controls indicating a higher ferritin content.

In the experiments reported in the first series (Table 2), centrifugation temperature was kept at 0 - 4^{o}C, and in only one case (Exp. 5) did pellets show some brown colour, again the Cd preparation being darker than the N. For the rest, pellets were almost colourless.

II 9. Analysis of Ribosomal Preparations

II 9. (a) Ultraviolet Absorption

Ribosomal preparations have a characteristic ultraviolet spectrum due to the high content of RNA which shows a maximum at about 260 $_{nm}$ and a minimum at about 230 $_{nm}$. The presence of protein, due to its high end absorption, shifts the minimum towards 235 $_{nm}$ and also raises the absorption in this region. The 260 $_{nm}$/235 $_{nm}$ ratio can therefore be used as a guide to the protein content of the preparation. Ribosomes from rat Jensen sarcoma with 50% protein have a ratio of 1,67 (Petermann 1964). Ultraviolet absorption measurements were made on all ribosome preparations and the 260 $_{nm}$/235 $_{nm}$ and 260 $_{nm}$/280 $_{nm}$ ratios are shown (Table 2)(Zeiss PMQII Spectrophotometer). Values found for the 260 $_{nm}$/235 $_{nm}$ ratio were low indicating a high protein content. Typical ultraviolet spectra are shown (Figs. 4 and 5) (Beckman DB recording spectrophotometer, 5in. recorder).

TABLE 2

Ribosomal Preparations First Series

		RNA mg/g Liver	RNA/Protein Ratio	Ratios of Absorption $260_{nm}/235_{nm}$	$260_{nm}/280_{nm}$
Exp. 1	N	0,81	1,04	1,48	1,65
	Cd	1,06	0,99	1,39	1,77
Exp. 2	N	0,97	0,75	1,46	1,76
	Cd	1,39	1,12	1,48	1,74
Exp. 3	N	1,07	1,01	1,46	1,79
	Cd	1,39	1,00	1,49	1,79
Exp. 4	N	1,00	0,99	1,47	1,74
	Cd	1,40	1,06	1,43	1,73
Exp. 5	N	0,73	0,52	1,58	1,77
	Cd	0,99	0,61	1,48	1,73
Exp. 6	N	1,16	0,75	1,50	1,80
	Cd	1,28	0,89	1,43	1,67
Mean	N	0,95	0,84	1,49	1,75
±s.d.		0,16	0,20	0,04	0,05
Mean	Cd	1,25	0,94	1,45	1,73
±s.d.		0,18	0,18	0,03	0,04

FIG. 4

ULTRAVIOLET SPECTRUM

N RIBOSOME PREPARATION

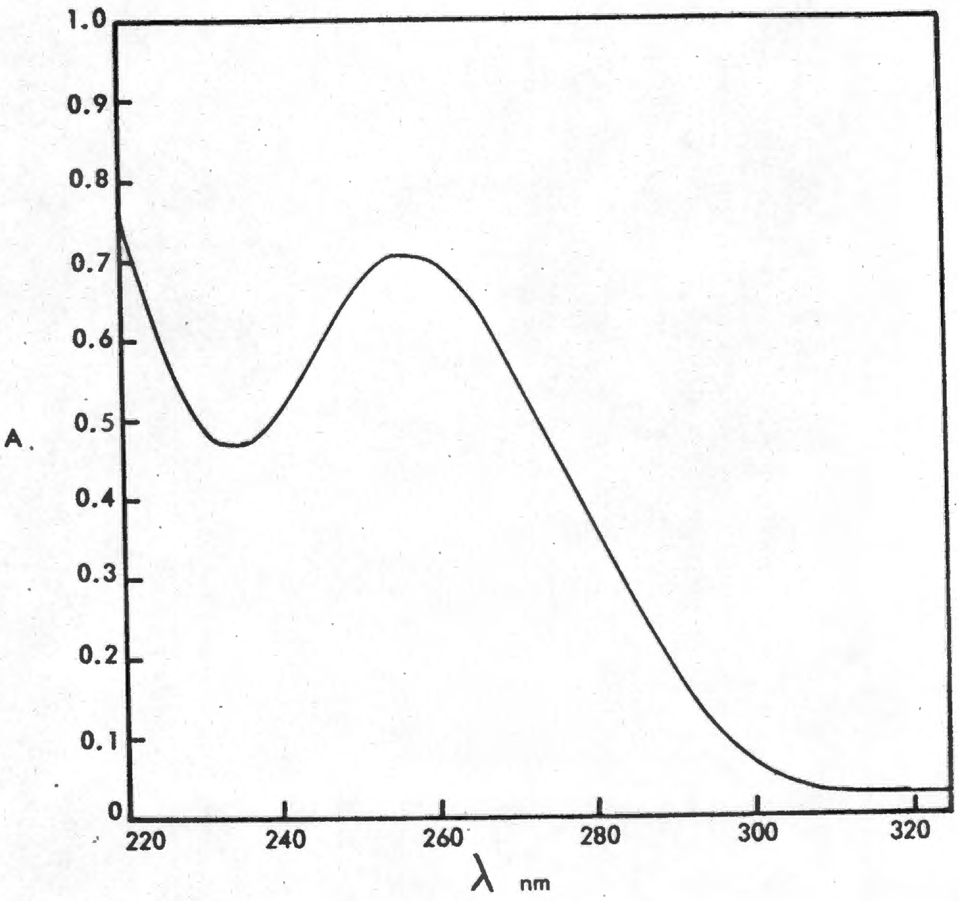
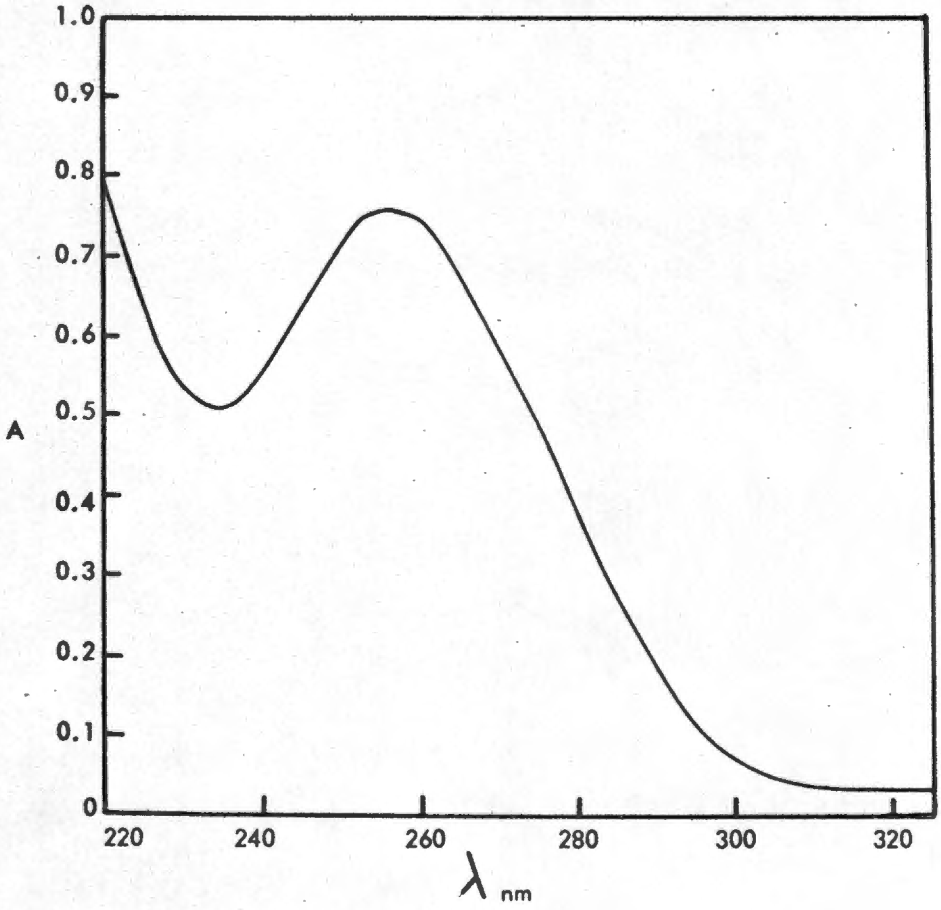


FIG. 5

ULTRAVIOLET SPECTRUM

Cd RIBOSOME PREPARATION



The 260 $_{nm}$/280 $_{nm}$ ratio can also be used to assess the purity of preparations and should approach 1,7 (Noll 1969). The absorption at 260 $_{nm}$ is quantitatively related to the RNA content of the ribosomal preparation. Holley (1961) found purified RNA from yeast to have an absorbancy of 1,9 for a 0,01% solution, 1 cm path, at 260 $_{nm}$. Absorbancy units (A.U.;20 A.U. = 1,0 mg RNA) can therefore be used for rapid estimation of the ribosomes in a preparation.

II 9. (b) RNA Analysis

RNA was determined by the method of Scott, Fraccastoro and Taft (1956) as modified by Munro and Fleck (1966). This method makes use of the different stabilities of RNA and DNA in dilute alkali. RNA is digested in 0,3 N KOH at 37° for 1 hr, and can then be estimated as nucleotides from the absorption at 260 $_{nm}$, while DNA is unaffected and can be separated with the protein by precipitation in perchloric acid. An absorption of 1,000 at 260 $_{nm}$ is given by a solution containing 32 µg of RNA per ml for rat liver (Munro and Fleck 1966). The procedure as described was suitable for RNA estimation in homogenates and supernatants. (See Section II 17.). For ribosomal preparations the procedure was scaled down to one tenth. Results are given as mg RNA per g liver wet weight. (Table 2). Sedimentation through 1,5 M instead of 2 M sucrose results in a less highly purified preparation but in a recovery of approximately 80% of the total ribosomes (Wettstein, Staehelin and Noll 1963). RNA analysis gave a consistently (27%) higher figure for RNA per g liver in the Cd preparations, which indicates a higher recovery of ribosomes. This was later investigated further. (See Section II 17.).

II 9. (c) Protein Determination

The Biuret method (Gornall, Bardawill and David 1949) was used for homogenates, supernatants and enzyme preparations. For ribosomal preparations the Folin method (Lowry, Rosebrough, Farr and Randall 1951) was used. Bovine serum albumin was used to draw up a standard curve for each batch of samples.

II 9. (d) RNA/Protein Ratio

The ratio of RNA to protein in the mammalian ribosome is approximately 1:1. The ratio found in preparations can vary considerably depending on the loss of protein from the ribosome by proteolytic enzymes during preparation, or on the other hand on adsorption of protein from the cytosol. (Petermann

1964). In this series of experiments ratios were in some cases higher for the Cd preparations than for the normal (Table 2). This could mean a loss of protein from the treated ribosome during preparation.

II 9. (e) Sucrose Density Gradient Centrifugation

The size range of polyribosomes in a preparation can be demonstrated by centrifugation through a sucrose gradient. (Martin and Ames 1961, Charlwood 1963). Linear sucrose gradients (15 - 30% w/w sucrose in Medium A buffer) were prepared by the procedure of Britten and Roberts (1960), using an apparatus described by Stead, Nourse and Hawtrey (1964), and adapted for the SW 39L rotor. Suitable aliquots were layered on the gradients and centrifugation was carried out at 125,000 g_{av} (Beckman Model L 39,000 rev./min) for 60 min at 0 - 2^{o}C. The bottom of the tube was pierced and approximately 30 fractions collected by drop counting, diluted with 1 ml of buffer, and their optical absorbance measured at 260 $_{nm}$ and 320 $_{nm}$. A raised absorbance at 320 $_{nm}$ indicated contamination by ferritin. This metalloprotein has a relatively high extinction at 260 $_{nm}$, which can distort the gradient profile in the monosome region. Use was therefore made of the ferritin correction proposed by Wilson and Hoagland (1965), and Jackson, Munro and Korner (1964). This involves correction of the absorbance at 260 $_{nm}$ according to the formula:

Corrected $A_{260\ nm}$ = total absorbance 260 $_{nm}$ - (absorbance 320 $_{nm}$ x 1,59).

Graphs are given in Figs. 6 and 7 of an early experiment (60 A.U. of preparation in each case) showing the ferritin correction. Graphs were plotted and the area of the polysome (Fractions 1 - 12) and oligosome (Fractions 12 - 24) regions measured and compared with the total area by the procedure of Wunner, Bell and Munro (1966), employing a compensating polar planimeter.

The sucrose gradient profiles were more variable in the Cd preparations and in two cases a high oligosome fraction approximately 60% of the sum of the two areas was found. Figures for oligosome and polysome content of the gradient profiles of preparations of ribosomes isolated in Expts. 1 - 6 (First Series) are given in Table 3. Graphs of the sucrose gradients of the preparations from Expt. 5 are shown in Fig. 8. Plots represent 20 A.U. of ribosomal preparations in each case (N and Cd).

Fractions from the gradients were bulked and their ultraviolet spectra recorded. Ratios of

FIG. 6.

SUCROSE GRADIENT. N PREPARATION.

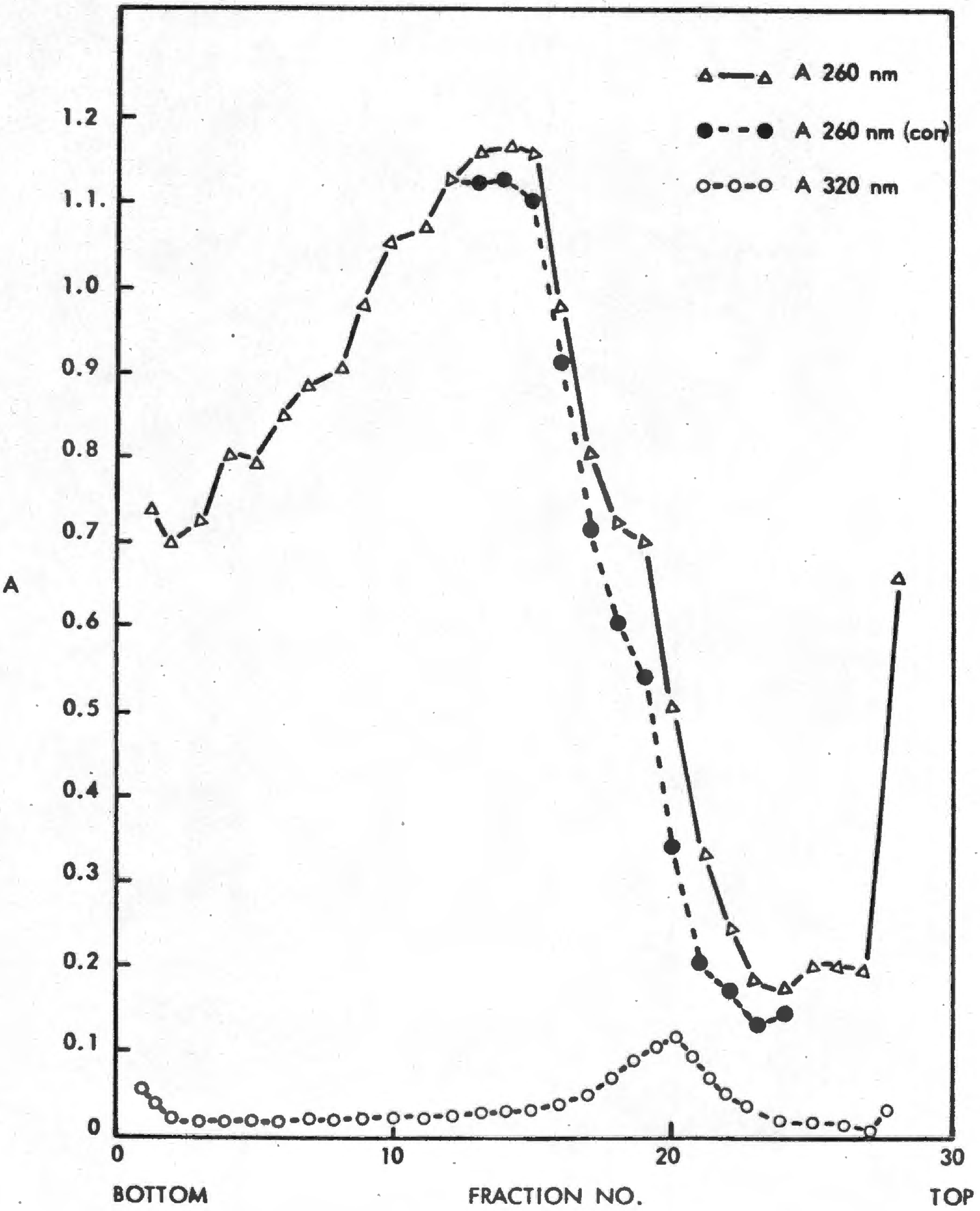


FIG. 7

SUCROSE GRADIENT. Cd PREPARATION.

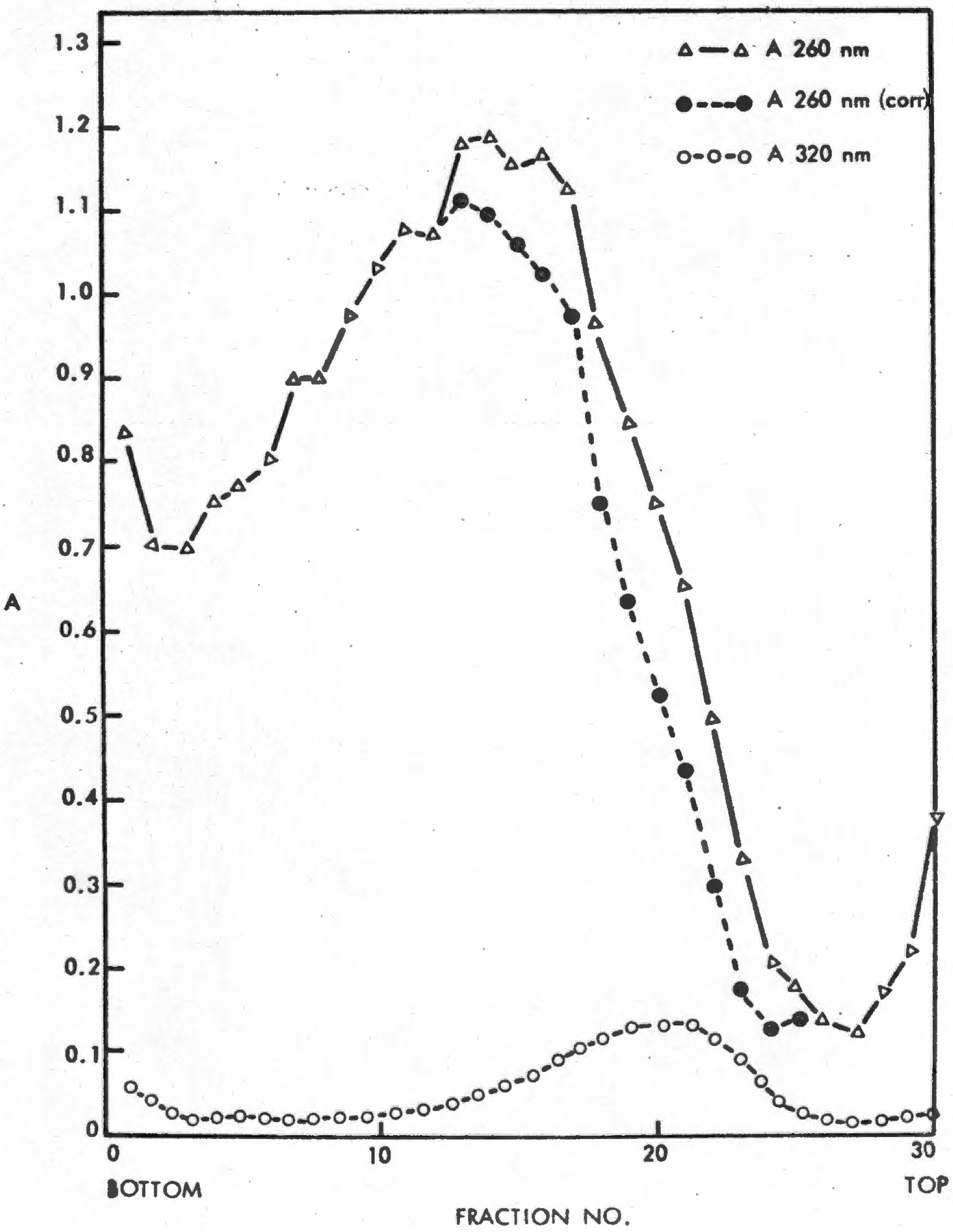


TABLE 3

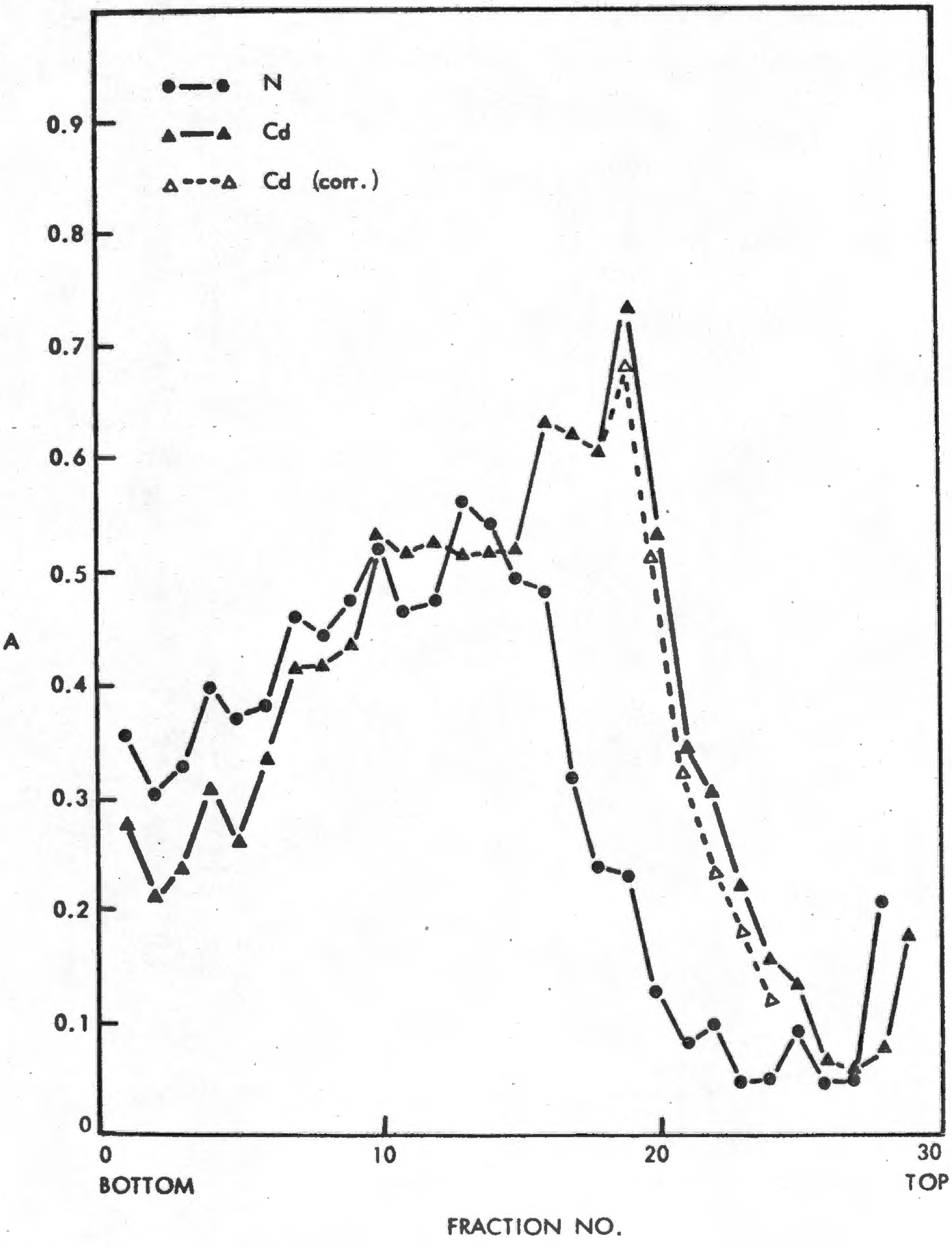
First Series

Sucrose Gradient Analysis of Ribosome Preparations

		Percentage of Total Absorbance	
		Oligosomes	Polysomes
Exp. 1	N	51,2	48,7
	Cd	55,5	44,5
Exp. 2	N	52,2	47,8
	Cd	44,8	55,2
Exp. 3	N	57,0	43,0
	Cd	59,4	40,6
Exp. 4	N	51,2	48,8
	Cd	44,6	55,4
Exp. 5	N	45,6	54,4
	Cd	59,5	40,5
Exp. 6	N	50,8	49,2
	Cd	46,5	53,5
Mean	N	51,33	48,65
±s.d.		3,63	3,63
Mean	Cd	51,71	48,25
±s.d.		7,20	7,16

FIG. 8.

SUCROSE GRADIENTS. N AND Cd PREPARATIONS.



absorbance at 268 $_{nm}$/280 $_{nm}$ and 260 $_{nm}$/235 $_{nm}$ are shown in Table 4 for some experiments. The values for fractions 16 - 18 in the oligosome region are in reasonable agreement with the figure 1,67 given by Petermann (1964), for rat Jensen sarcoma. Treatment of the ribosomal preparation with ribonuclease resulted in a shift to a high peak in the monosome region of the gradient. The method of Fitschen (1967) was used, which involved incubation of ribosomes at 18°C for 10 min with and without ribonuclease. Graphs are shown in Fig. 9 of sucrose gradients of samples (30 A.U.) of a Cd preparation kept at 0°C, at 18°C for 10 min and after incubation with ribonuclease (10 μg) at 18°C for 10 min.

FIG. 9

SUCROSE GRADIENTS. TREATMENT WITH RNAase

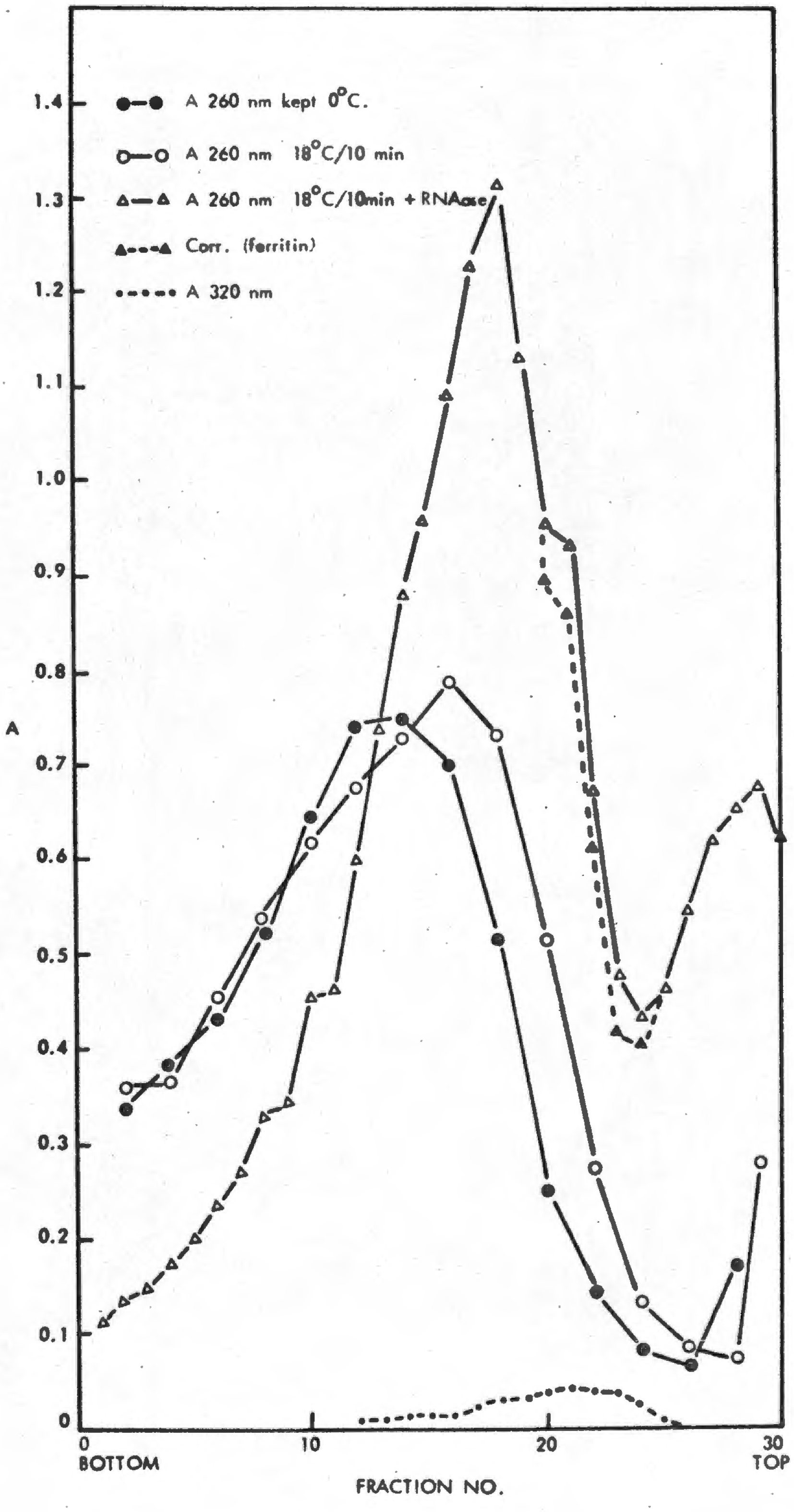


TABLE 4

First Series

Ratios from Ultraviolet Spectra of Gradient Fractions

Gradient Fractions		Ratios $260_{nm}/280_{nm}$ 6-8	9-12	13-15	16-18
Exp. 2	N	2,06	2,02	2,00	2,04
	Cd	2,00	1,94	1,96	1,91
Exp. 4	N	2,02	2,02	1,98	1,97
	Cd	2,06	2,14	2,08	1,68
Exp. 5	N	1,92	1,93	2,10	1,90
	Cd	2,06	1,97	1,96	1,95

Gradient Fractions		Ratios $260_{nm}/235_{nm}$ 6-8	9-12	13-15	16-18
Exp. 2	N	1,50	1,53	1,57	1,52
	Cd	1,59	1,57	1,56	1,54
Exp. 4	N	1,55	1,59	1,60	1,62
	Cd	1,53	1,59	1,59	1,22
Exp. 5	N	1,62	1,69	1,61	1,65
	Cd	1,61	1,67	1,69	1,65

II 10. Second Series of Experiments

In this series, 2 M sucrose was used in the lower layer of the discontinuous gradient to obtain purified ribosomes, "C ribosomes", for protein synthesis studies. This resulted in preparations with a higher proportion of protein, reflected also in slightly lower $A260_{nm}/235_{nm}$ ratios, and, in most cases, a lower $A260_{nm}/280_{nm}$ ratio. The RNA/Protein ratios in the treated animals were more closely similar to the normal than those in the first series. Recovery of ribosomes was lower, as assessed by RNA analysis, and subject to variable losses due to difficulties in separating the loose pellet quantitatively from the dense sucrose solution. Values of RNA content, ratios of RNA to protein and absorption ratios for Expts. 7 - 12 (Second Series) are shown in Table 5. Samples from the monosome -disome regions from the gradients (Expts. 9 - 11) had ratios of absorption averaging

	$260_{nm}/235_{nm}$	$260_{nm}/280_{nm}$
N	1,69	1,86
Cd	1,68	1,88

TABLE 5

Second Series

Ribosomal Preparations

		RNA mg/g Liver	RNA/Protein Ratio	Ratios of Absorption	
				$260_{nm}/235_{nm}$	$260_{nm}/280_{nm}$
Exp. 7	N	0,87	0,88	1,38	1,70
	Cd	0,73	0,99	1,48	1,77
Exp. 8	N	0,79	0,64	1,44	1,72
	Cd	0,93	0,83	1,47	1,69
Exp. 9	N	0,65	0,63	1,43	1,73
	Cd	0,72	0,62	1,45	1,68
Exp. 10	N	0,73	0,80	1,37	1,77
	Cd	0,97	0,72	1,48	1,82
Exp. 11	N	0,84	0,96	1,47	1,73
	Cd	0,77	0,93	1,44	1,73
Exp. 12	N	0,68	0,66	1,36	1,77
	Cd	0,62	0,68	1,38	1,68
Mean	N	0,76	0,76	1,40	1,73
±s.d.		0,08	0,13	0,04	0,02
Mean	Cd	0,79	0,79	1,45	1,72
±s.d.		0,13	0,14	0,03	0,05

Sucrose density gradient analysis was carried out as before, but the gradient was sampled by pumping downward through a 3 mm flow cell and the absorbance monitored at 254 $_{\text{nm}}$ (LKB Uvicord, Beckman recorder). Drop counting and an event marker enabled individual fractions to be recorded and collected. For accurate comparison, profiles were replotted and the area under the monosome and disome peaks measured and compared with the total area by use of the planimeter (Table 6). Gradients were resolved into well-defined peaks, with a consistently low proportion of monomers in all preparations, but a higher and more variable content of dimers and fewer polysomes than the normal were observed on the average in those from Cd-treated rats. Individual Cd preparations were observed, however, to have a polysome content comparable to or higher than the N in the same experiment. Gradients from a typical experiment are shown in Fig. 10, (N preparation 6 A.U. Cd preparation 7 A.U.). The dotted vertical lines show the demarkation of areas for estimation of monosome, disome and polysome content. The peak given by ferritin in this gradient system is also shown in this figure. Ferritin has a sedimentation constant of 65S in Svedberg units (S). (Petermann and Hamilton 1957, Takamami 1957). It provides a useful marker on

TABLE 6

Second Series

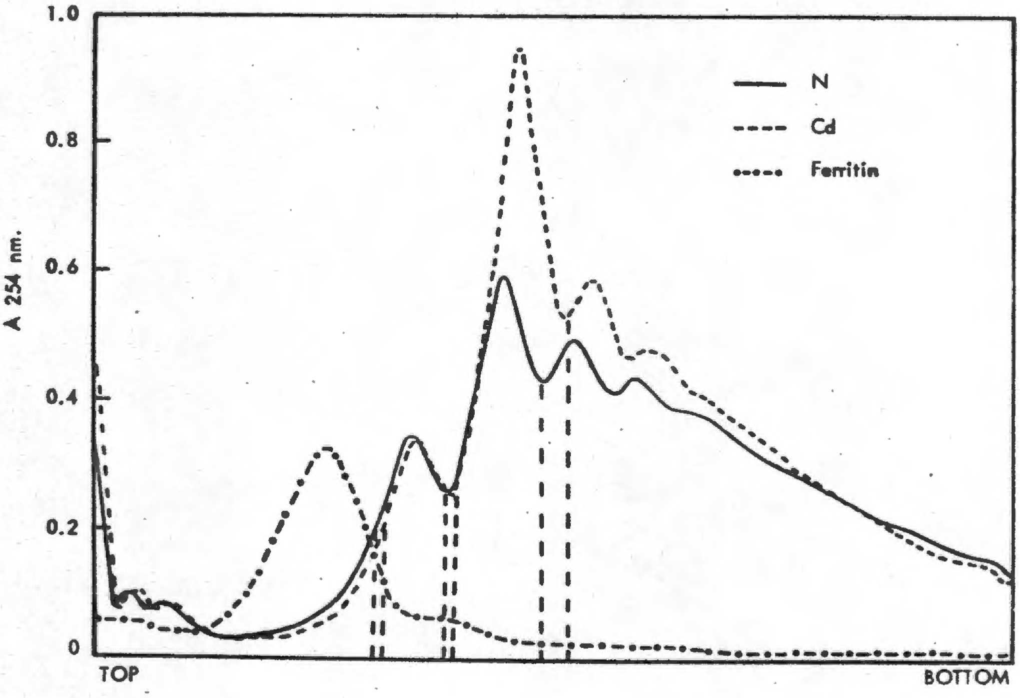
Sucrose Gradient Analysis of Ribosome Preparations

		Percentage of Total Absorbance		
		Monosomes	Disomes	Polysomes
Exp. 7	N	8,7	17,1	74,2
	Cd	8,0	17,3	74,7
Exp. 8	N	9,6	21,1	69,3
	Cd	8,2	31,3	60,5
Exp. 9	N	8,3	23,8	67,9
	Cd	8,9	37,2	53,9
Exp. 10	N	10,4	23,9	65,7
	Cd	10,7	17,4	71,9
Exp. 11	N	8,2	19,9	71,9
	Cd	7,2	31,5	61,3
Exp. 12	N	7,2	15,1	77,7
	Cd	8,4	18,6	73,0
Mean	N	8,7	20,2	71,1
±s.d.		1,12	3,55	4,39
Mean	Cd	8,6	25,6	65,9
±s.d.		1,18	8,79	8,46

FIG. 10.

SUCROSE GRADIENTS.

N AND Cd PREPARATIONS COMPARED WITH FERRITIN.



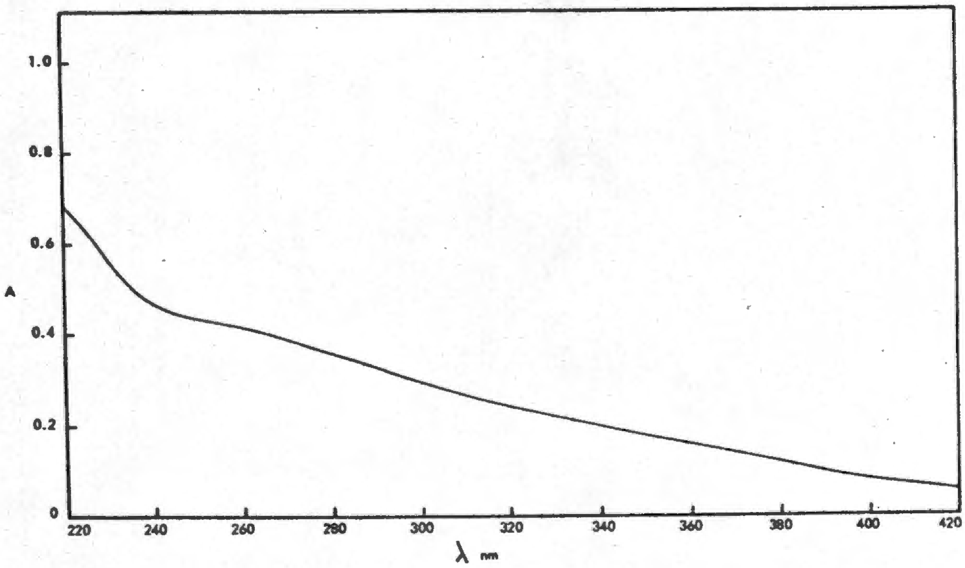
sucrose gradients for the monoribosome fraction (80S) as it appears in the centrifuge tube as a coloured band just above it.

Ferritin was prepared from yellow material collected from the interface between the discontinuous sucrose layers of a ribosomal preparation from a normal rat. This material was sedimented at 100,000 g_{av} for 2,5 hr from Medium A. It gave a single peak in the sucrose gradient in the position shown in the figure. The ultraviolet absorption spectrum of a sample from the gradient peak is shown in Fig. 11. It had a 260 $_{nm}$/320 $_{nm}$ ratio of 1,75 (Beckman DB recording spectrophotometer, 5in. recorder). The low shoulder below the monosome peak in the gradient and the high ratio (Wilson and Hoagland 1965: 260 $_{nm}$/320 $_{nm}$ 1,59) are presumably caused by contamination with ribosomes.

To investigate further the effects of sedimenting ribosomes through 1,5 M or 2 M sucrose in the lower layer of the discontinuous gradient, a comparative experiment was carried out at this time on a normal rat. The deoxycholate-treated PMS was divided in half and two ribosome preparations made using 1,5 M and 2 M sucrose. The 2M preparation gave a pale-coloured, loose pellet, and the 1,5 M a firm pellet browner in colour. Results of analysis

FIG. 11.

ABSORPTION SPECTRUM OF FERRITIN.



are shown below:

Preparation	RNA mg/g Liver	RNA/Protein Ratio	Ratios of Absorption $260_{nm}/235_{nm}$	$260_{nm}/280_{nm}$
1,5 M	1,01	1,14	1,52	1,74
2 M	0,82	1,06	1,57	1,79

Sucrose gradient profiles of the two preparations on the first day were similar, the 1,5 M showing a 3% lower percentage of polysomes (Fig. 12) (1,5 M 9A.U., 2 M 8A.U.). After 24 hrs at 0°C, the 2 M preparation showed some breakdown to give the type of pattern shown above in Fig. 10 which had been commonly encountered in other experiments (9,4% monosomes, 22,2% disomes, 68,4% polysomes). On warming to 37°C for 2 min, the two preparations gave similar patterns both with a high monomer peak (Fig. 13) (1,5 M 5A.U., 2 M 4A.U.). After long storage (42 days at 0°C), the 2 M preparation had a high proportion of dimers and still a low monomer peak, whereas the 1,5 M preparation showed a higher proportion of monomers than dimers (Fig. 14) (1,5 M 5A.U., 2 M 4A.U.). Percentages of monomers, dimers and polysomes for these gradients are given in Table 7. Addition of Mg^{2+} to a portion of this preparation resulted in only a slight shift from monomers to dimers. In these figures 65S marks the position of ferritin in

FIG. 12.

SUCROSE GRADIENTS OF 1.5 M and 2 M PREPARATIONS.

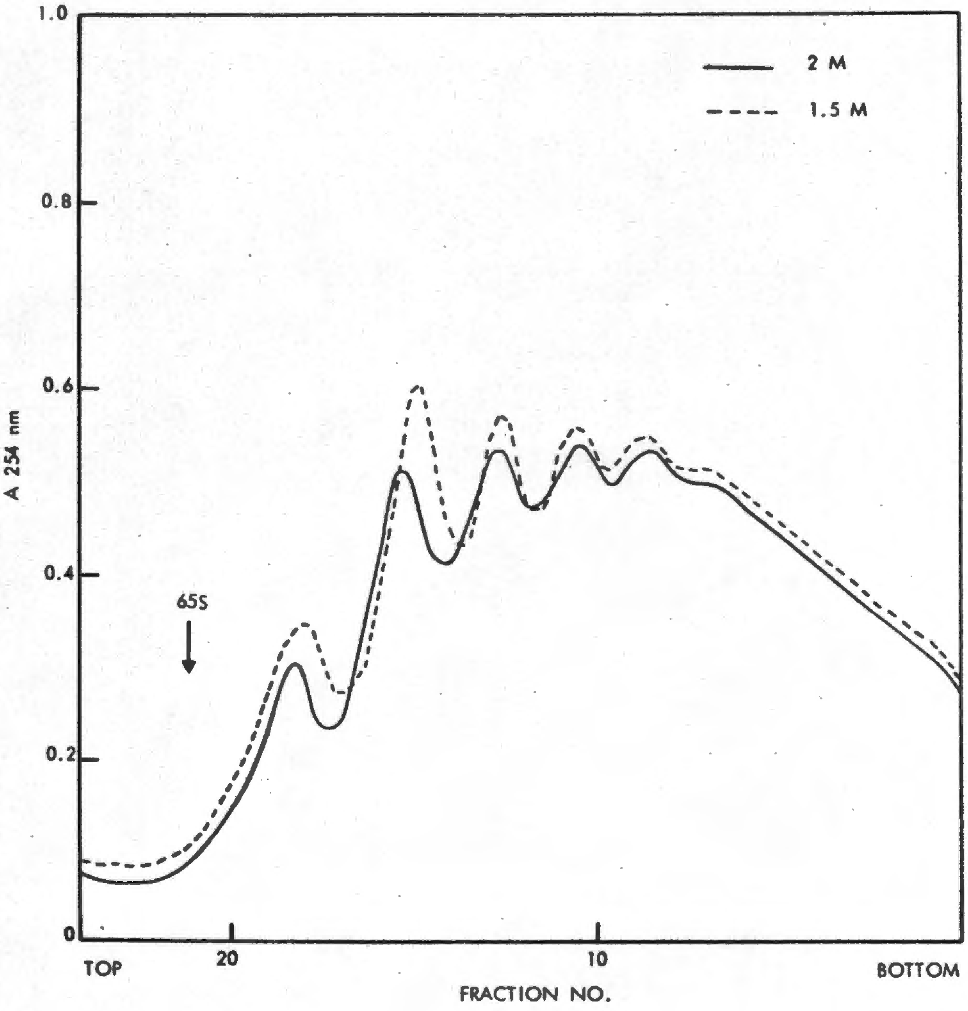


FIG. 13.

SUCROSE GRADIENTS. 1.5 M and 2 M PREPARATIONS. 2 min/37°.

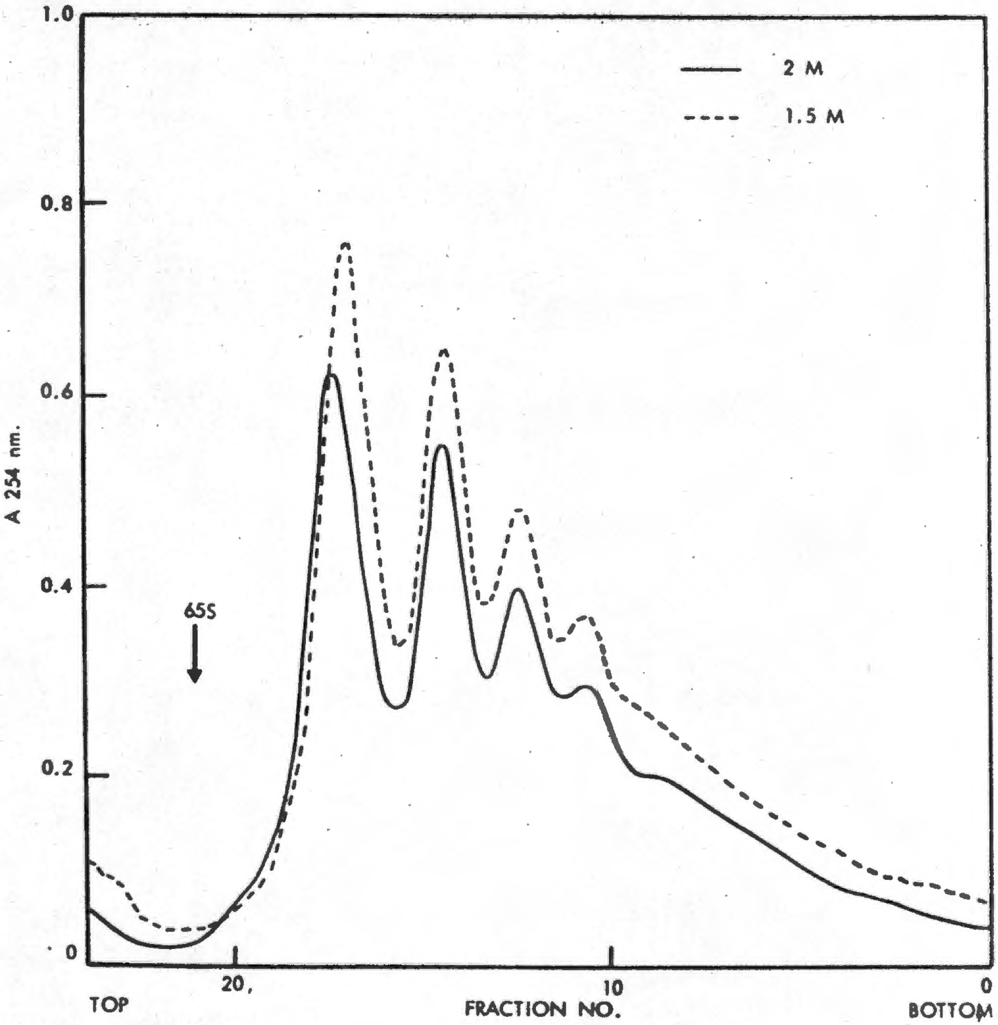
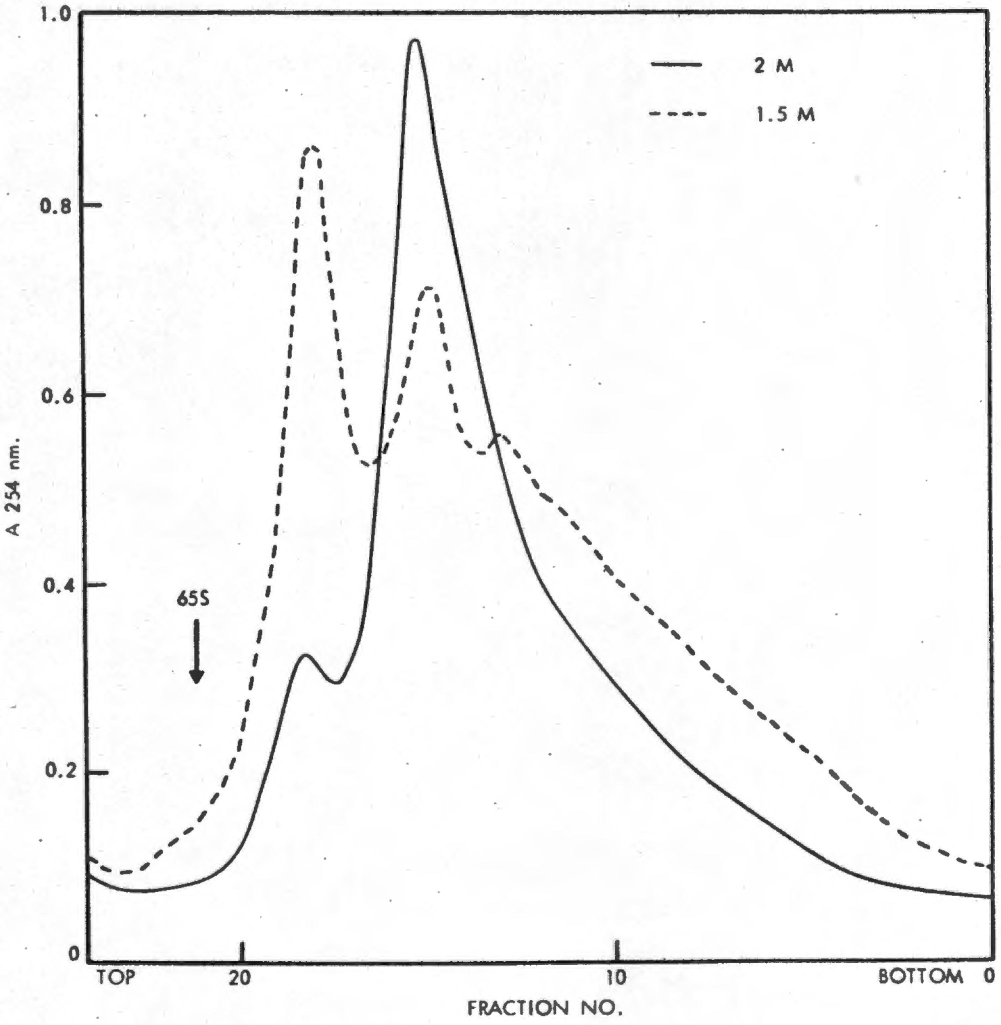


FIG. 14.

SUCROSE GRADIENTS. 1.5 M and 2 M PREPARATIONS. 42 DAYS/0°C.



this gradient system.

The dimer is known to be stabilised by high Mg^{2+} concentration, above 0,02 M, and to be stable only at low temperature (Reader and Stanners 1967). The two ribosomes are not attached through mRNA but at sites on the large subunits through interaction between the 28S RNA (Stanners, Eliceiri and Green 1971). Free unprogrammed ribosomes originally present in the cell can dimerise *in vitro*, and polysome breakdown during isolation procedures can produce dimers in a preparation. The extent of this breakdown has also been related to the activity of the ribonuclease present by Brewer, Foster and Sells (1969). The slow accumulation of dimers in the 2 M preparation and monomers and dimers in the 1,5 M might result from differing activities of ribonuclease in the two preparations.

Release of active ribonuclease during preparation could also cause the high disome content of the preparations in Table 6. On the average, the Cd preparations appear to contain more ribonuclease activity than the N.

TABLE 7

Sucrose Gradients of 1,5 M and 2 M Preparations

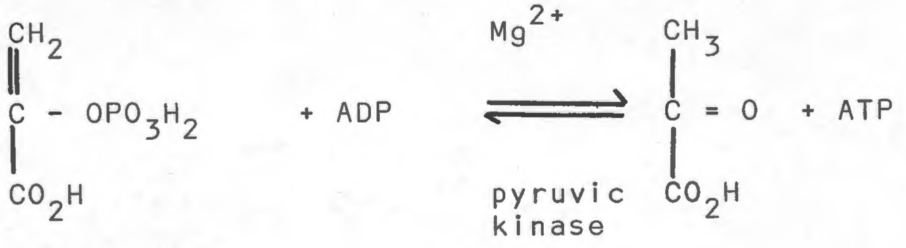
Preparation		Percentage of Total Absorbance		
		Monosomes	Disomes	Polysomes
1,5 M	First Day	7,8	17,1	75,1
2 M		6,6	14,8	78,6
1,5 M	24hr/0°C	-	-	-
2M		9,4	22,2	68,4
1,5 M	2min/37°C	27,5	22,0	50,5
2 M		26,9	22,2	50,9
1,5 M	42days/0°C	27,3	21,2	51,5
2 M		7,7	48,0	44,3

II II. Protein Synthesis Studies

A measure of the efficiency of an isolated ribosomal preparation is given by a study of the extent of the incorporation of radioactive amino acids into protein in a cell-free system. A number of the essential factors required in these systems have been identified, and their function described (Lucas-Lenard and Lipmann 1971, Lengyel and Söll 1969).

The standard incorporating mixture is based on the Medium A buffer system (Hoagland et al 1958), as used for the ribosomal preparation but with a raised Mg^{2+} content. The complete system used was that of Fitschen (1968). It contained (0,4 ml or 0,5 ml per assay tube), 1,25 mM ATP, 0,5 mM GTP, 5 mM reduced glutathione, (GSH), 5 mM phosphoenolpyruvate (K salt), 20 μg phosphoenolpyruvate kinase, 53 mM KCl, 7,5 mM $MgCl_2$, 300 mM sucrose, pH5 enzyme (0,6 - 1,2 mg protein), 0,4 or 0,5 μCi ^{14}C-labelled protein hydrolysate, and 65 mM Tris-HCl, pH 7,6 at 20°C.

Phosphoenolpyruvate (PEP) and PEP kinase provide an ATP generating system:-



$$CH_2{=}C(OPO_3H_2)CO_2H + ADP \underset{\text{pyruvic kinase}}{\overset{Mg^{2+}}{\rightleftharpoons}} CH_3C({=}O)CO_2H + ATP$$

ATP is required for adenylation of amino acids with subsequent transfer of each amino acid to its specific acceptor transfer RNA (tRNA):-

$$\begin{matrix}\text{ATP +} \\ \text{amino acid}\end{matrix} \xrightleftharpoons[\text{synthetase}]{Mg^{2+}} \left(\begin{matrix}\text{amino} \\ \text{acid}\end{matrix} - \underset{\text{complex}}{\text{AMP}} - \text{enzyme}\right) + \begin{matrix}\text{PPi} \\ \text{pyro-} \\ \text{phosphate}\end{matrix}$$

$$(\text{amino acid - AMP}) + \text{tRNA} \xrightleftharpoons[\text{synthetase}]{Mg^{2+}} (\text{aminoacyl-tRNA}) + \text{AMP}$$

GTP hydrolysis to GDP provides energy for translocation of aminoacyl-tRNA from the aminoacyl site (A site) to the peptidyl site (P site), and is involved with its binding to the ribosome.

Glutathione (GSH) is a source of thiol groups and a buffering system.

The high-Mg^{2+} buffer facilitates *in vitro* binding of aatRNA and translation factors on the ribosome.

An excess of amino acid activating enzymes, transfer enzymes and tRNA is provided by the pH5 enzyme, an enzyme fraction separated from inhibitors in the cell sap by precipitation at pH5. This can be replaced by cell sap treated to remove inhibitors and amino acids (See Section II 13.).

II II. (a) Preparation of pH5 Enzyme

(Moldave 1963, Holley and Goldstein 1959). Procedure was the same for normal and Cd-treated rats. In the latter case, livers were taken the morning after the final injection. Animals were not starved overnight.

The livers of 2 or 3 rats were weighed and homogenized as above in two volumes of Medium A. The homogenate was centrifuged at 20,000 g_{av} (Sorvall, 13,000 rev./min) for 15 min. This PMS was then centrifuged at 95,000 g_{av} (Beckman Model L, 40 rotor 37,000 rev./min) for 2 hr. The surface lipids were removed and the supernatant aspirated leaving a small residue above the pellet. This cell sap supernatant was brought to pH5,2 by addition of IN acetic acid, while stirring, at 4°C. The precipitated enzymes were sedimented by centrifuging at 20,000 g_{av} for 15 min, resuspended and washed with ice-cold water, spun down, and reprecipitated as before. This final precipitate was suspended in Medium A (1 ml/4 g liver), homogenized gently in a small glass homogenizer, the pH adjusted to 7,5 with IN KOH, and clarified by spinning at 20,000 g_{av} for 10 min after standing for 30 min. The protein concentration of the clear enzyme solution was determined (Biuret method) and it was stored at -15°C for up to two days, or at -80°C for longer periods.

II II. (b) Incorporation of Amino Acids into Protein

Reaction was started in tubes containing the above mixture by addition of ribosomes (50 - 200 µg RNA) and warming to 37°C. Values for zero time incorporation were obtained by precipitation of proteins immediately after addition of ribosomes with an equal volume of 10% w/v trichloracetic acid (TCA) containing 20 unlabelled amino acids (1 mg each/ml.) Incorporation was for 30 min, after which proteins were precipitated in the same way. Tubes were kept at 4°C overnight, the precipitate was isolated by centrifugation, washed twice and then heated in 5% TCA for 15 min at 90°C to hydrolyse amino-acyl tRNA. The precipitate was transferred to a Millipore filter (0,45 µm pore size), washed with 100 ml 5% TCA, dried on planchettes at 110° for 10 min to remove TCA, and counted in a Beckman LS 233 liquid scintillator with external standardisation. Results were corrected for zero time incorporation. Toluene containing a 40:1 mixture of 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazole-2-yl) benzene (POPOP) was used for counting medium. PPO is an efficient proton emitter and POPOP is a secondary fluor used to shift the primary emissions to a longer wavelength closer to the maximum response of the photomultiplier of the scintillation counter. Counting efficiency was obtained from quench curves making use of the external standardisation facility of the instrument whereby the ratio of the counts in two channels is compared with a gamma source and an external standard ratio calculated. Efficiency for ^{14}C was 90% and for ^{3}H 50%. Absorption of counts within the TCA precipitate probably reduced efficiency particularly for ^{3}H to a much lower level.

Early experiments showed Cd preparations in most cases to have lower specific activity, expressed as counts per minute (cpm) incorporated per μg ribosomal RNA, compared with the N, but in some experiments the reverse was the case. These results could be correlated with the polysome profiles. As incorporation efficiency is dependent on the polysome content, a valid comparison would, therefore, need to be made between preparations with the same profiles.

Three of the experiments in the Second Series are illustrated in Figs. 15, 16 and 17.

Polysome profiles (a) are shown for comparison with incorporation (cpm) of amino acids (b) of the preparations containing increasing amounts of ribosomes (μg RNA) in the assay tube. Counts incorporated into protein increased, sometimes sharply, at higher ribosome levels, with a slight fall off in specific activity (Table 8).

In Expt. 10 (Fig. 15), a 6% higher polysome content in the Cd, as compared to the N, resulted in an average of 7% higher incorporation, whereas in Expt. 11 (Fig. 16) incorporating ability and polysome content were about 40% and 10% lower, respectively, in the Cd preparations.

Substitution in the system of pH5 prepared from Cd-treated rats caused a considerably lower level of incorporation for both N and Cd preparation in Expt. 10. In Expt. 11 (Fig. 16) however, the activity of the Cd pH5 preparation differed only slightly, being somewhat lower than the N when used with the N preparation but higher when combined with the Cd preparation.

Some refinements in procedure enabled improved and less variable preparations of ribosomes and enzymes to be compared in Expt. 12 (Fig. 17). The profiles of the preparations were similar, the Cd containing 4% fewer polysomes, and the Cd ribosomes showed a 7 - 12% lower incorporation over the range studied. The Cd pH5 caused further decreases in each case. (Norton and Kench 1973a).

The changes in procedure were as follows:

FIG. 15a.

SUCROSE GRADIENTS.

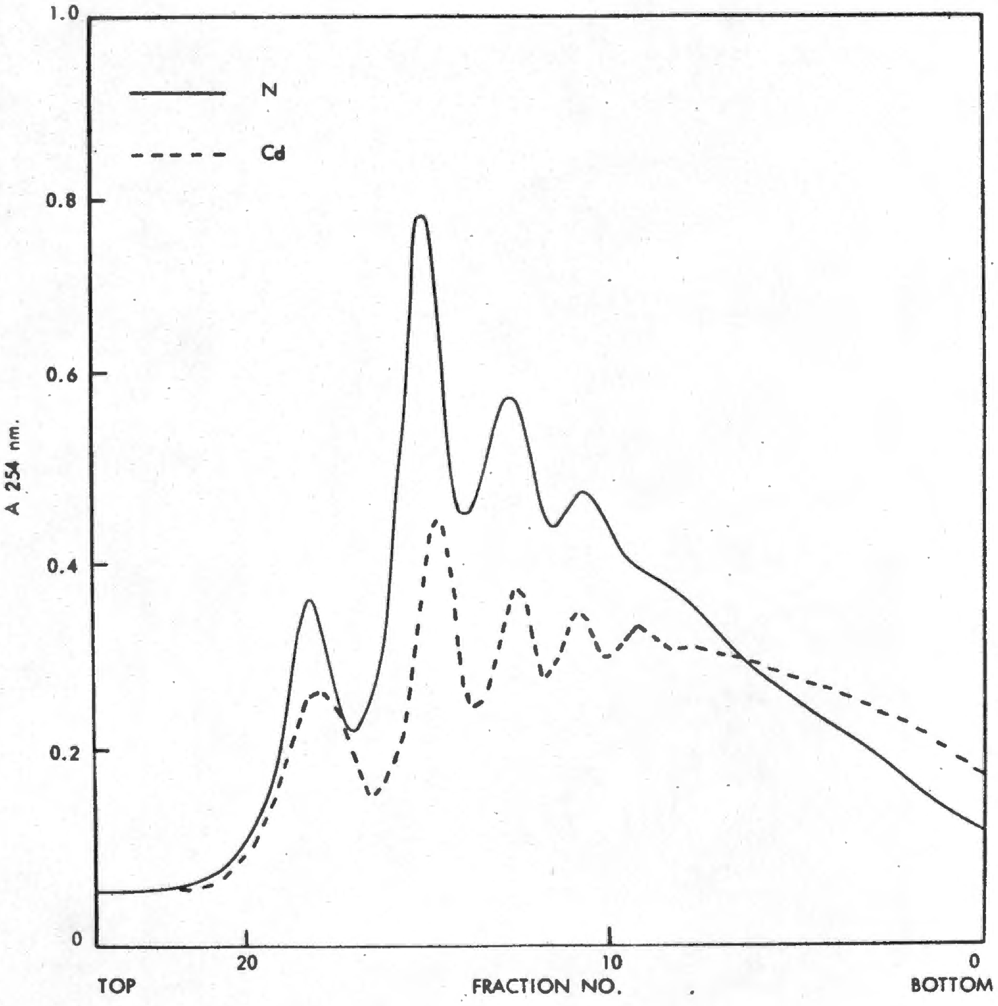


FIG. 15b.

INCORPORATION. EXP. 10.

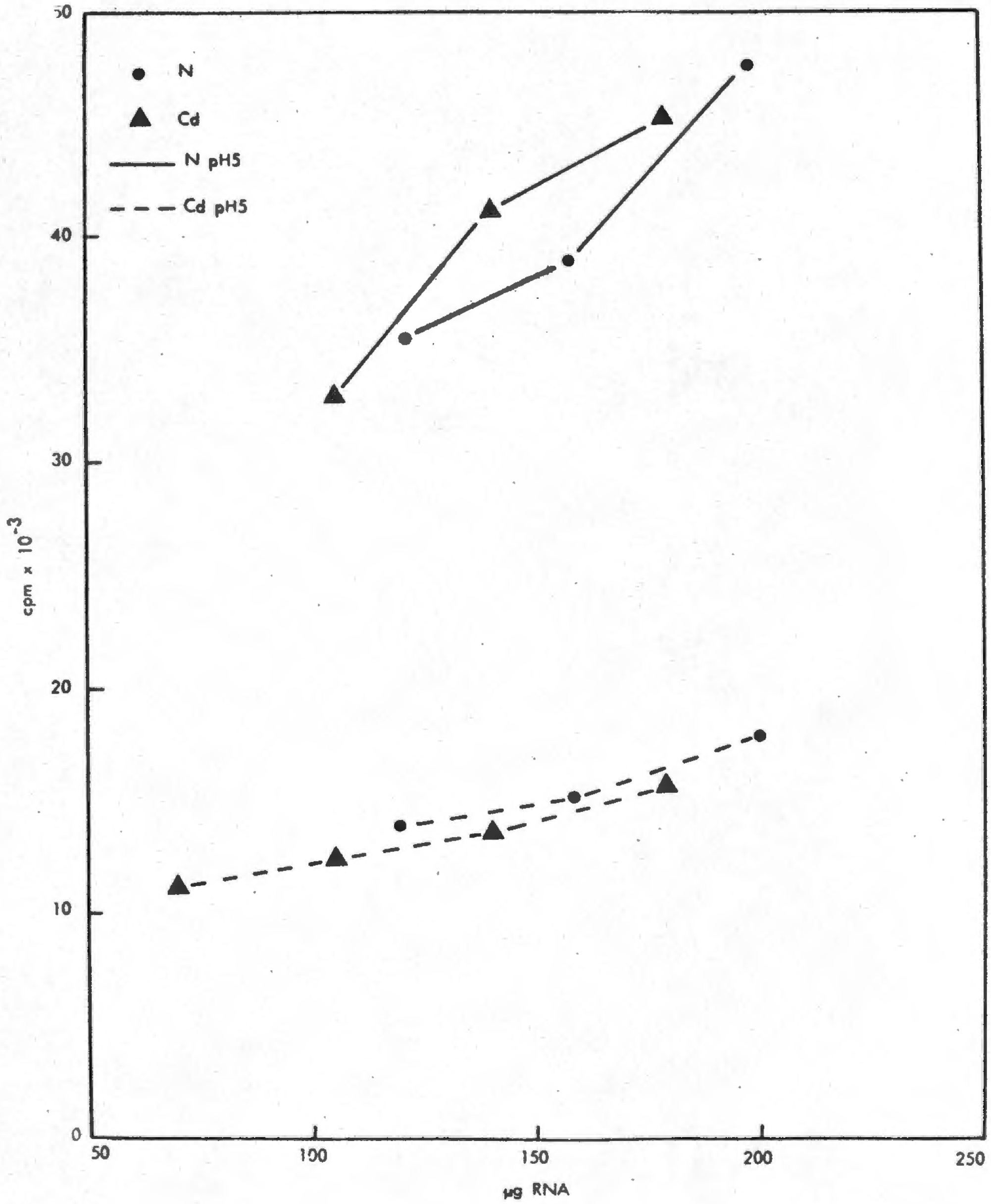


FIG. 16a.

SUCROSE GRADIENTS.

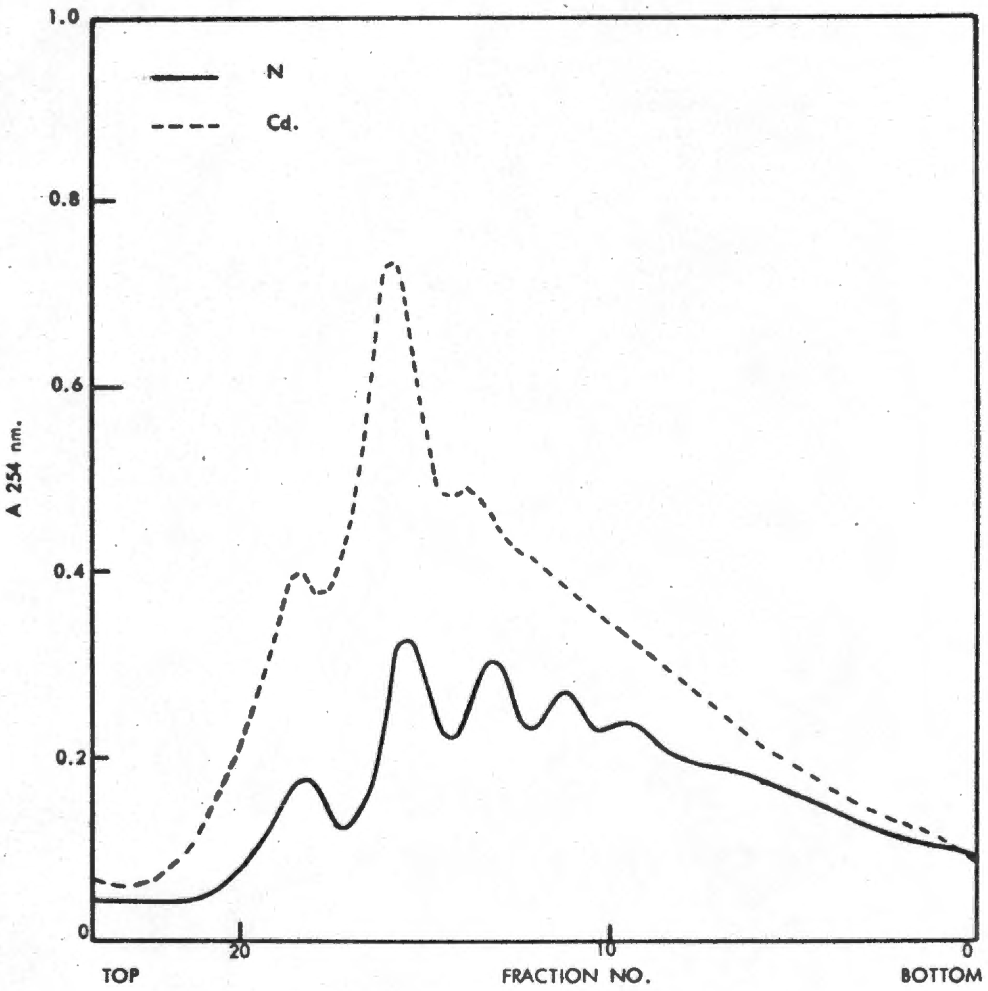


FIG. 16b.

INCORPORATION. EXP. 11.

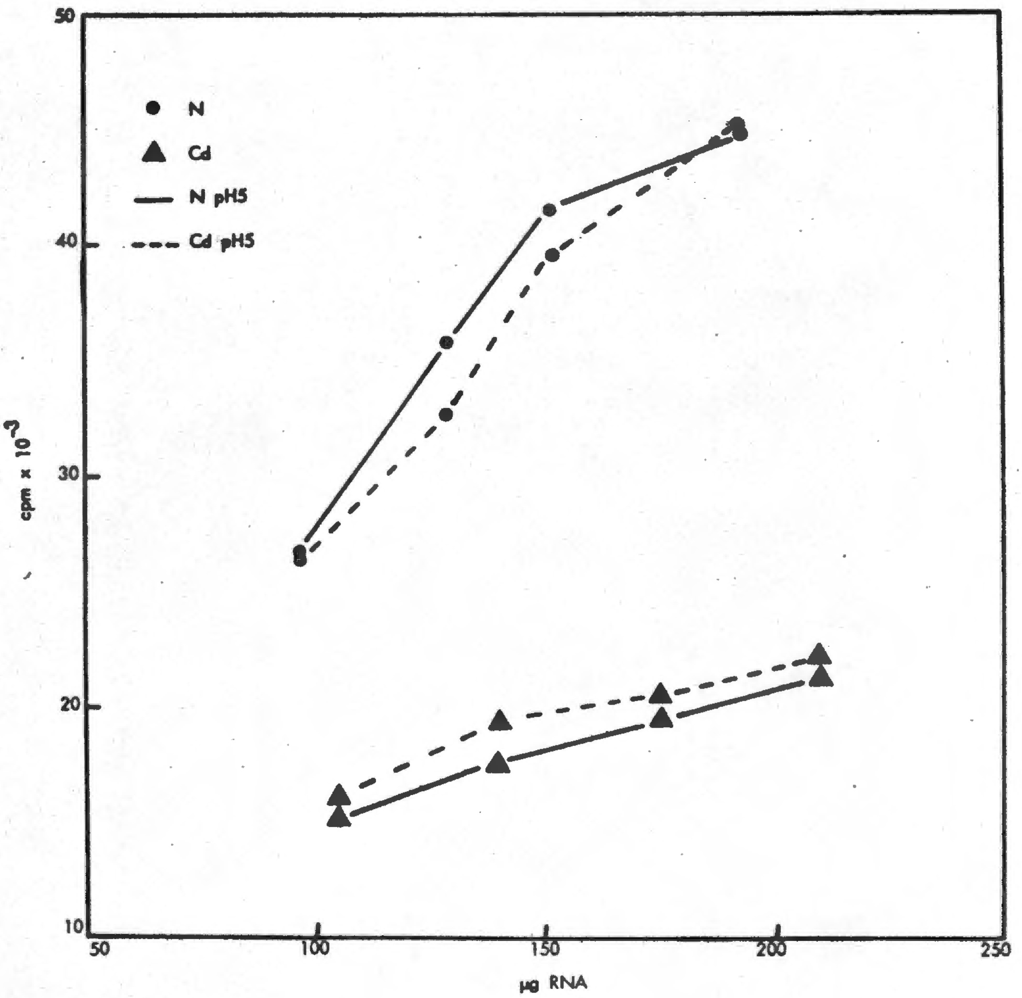


FIG. 17a.

SUCROSE GRADIENTS.

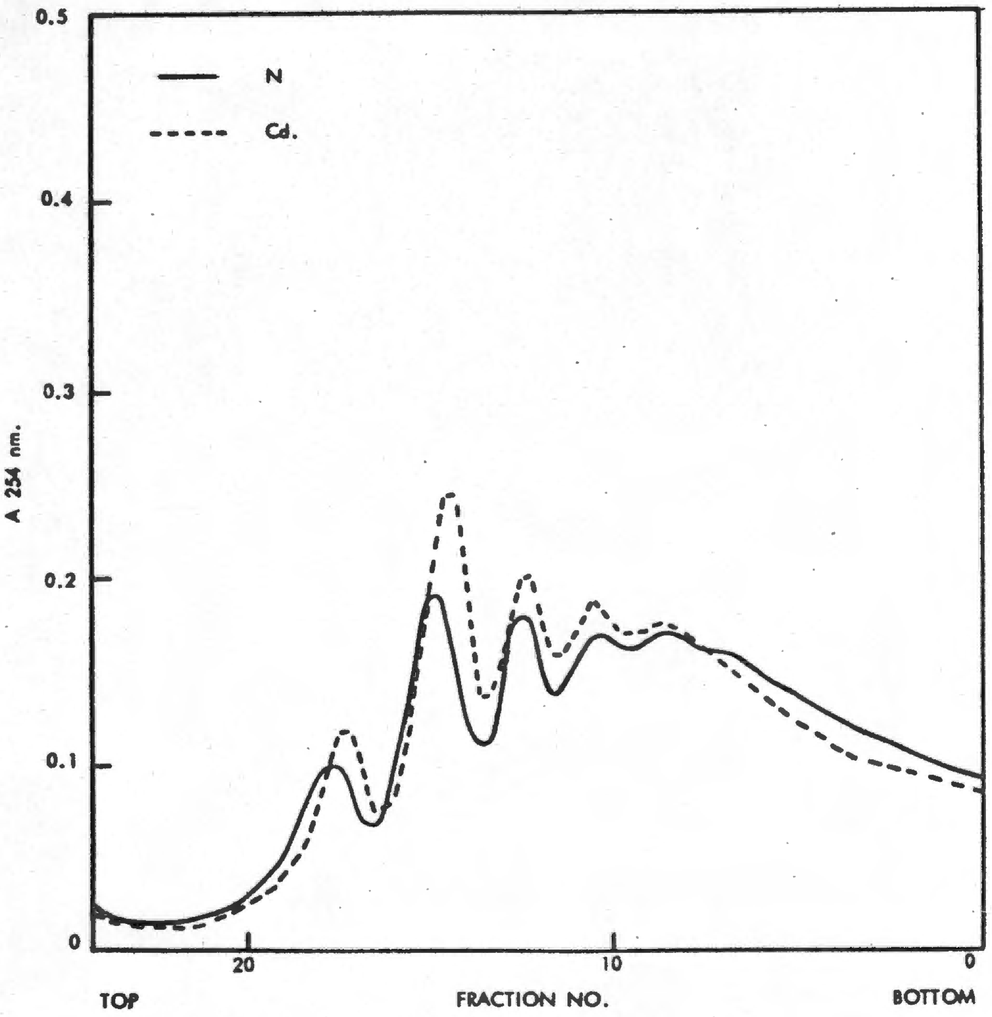


FIG. 17b

INCORPORATION EXP. 12

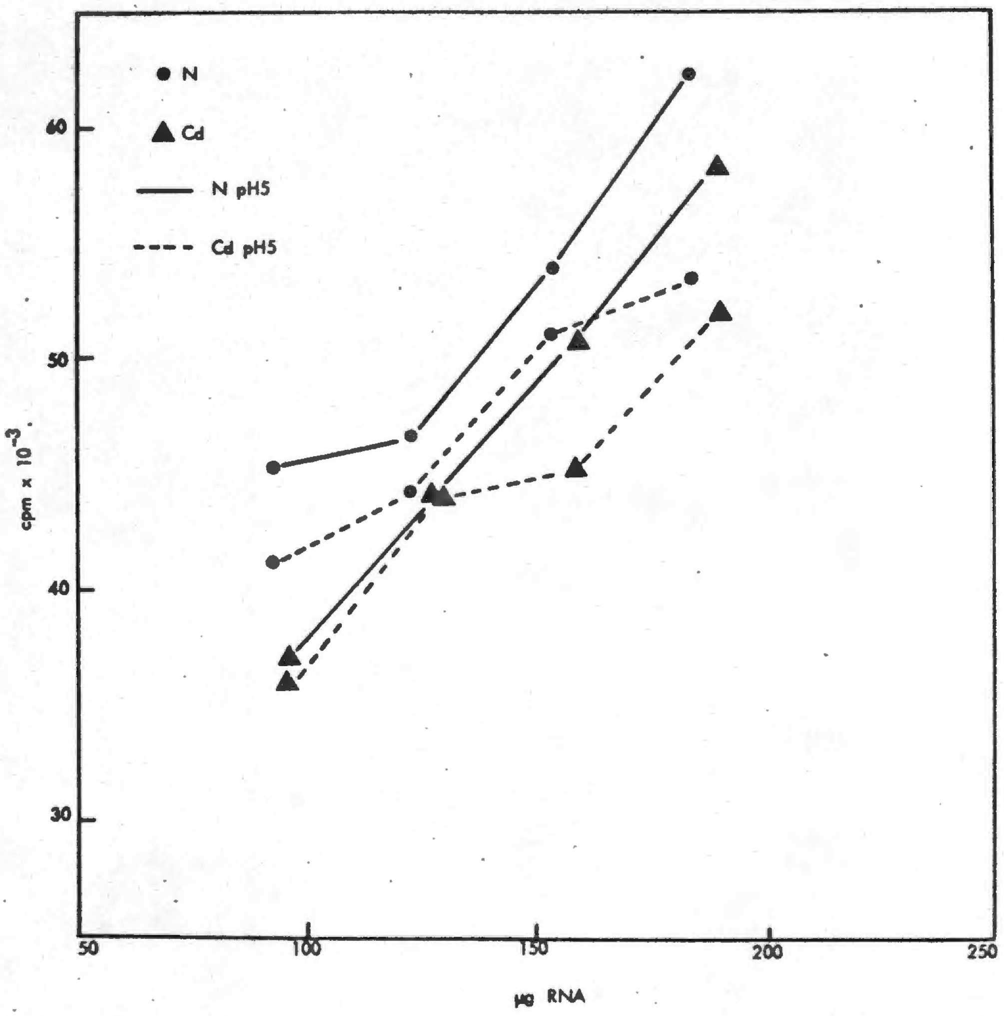


TABLE 8

Specific Activities

		NpH5		CdpH5	
		Ribosomes µg RNA	Sp. Act. cpm/µg RNA	Ribosomes µg RNA	Sp. Act. cpm/µg RNA
Exp. 10	N	121	293	121	115
		158	247	158	96
		198	241	198	90
	Cd			70	161
		105	314	105	119
		140	293	140	98
		179	253	179	87
Exp. 11	N	97	369	97	378
		129	355	129	330
		151	342	151	328
		193	284	193	285
	Cd	105	241	105	250
		140	215	140	210
		175	194	175	174
		210	162	210	153
Exp. 12	N	92	495	92	450
		122	384	122	365
		153	354	153	335
		184	340	184	293
	Cd	95	391	95	387
		126	352	126	351
		158	333	158	289
		189	310	189	277

1. The liver was cut up and passed through a Harvard tissue press (1,5 mm screen) before homogenization.
2. Homogenization was carried out by 4 - 5 passes in the Potter-Elvehjem homogenizer in 3 volumes of Medium A at 2,000 rev/min (set by means of a stroboscope), using a stronger (1/20th horse-power) motor to drive the pestle, to minimise shearing effects, and the homogenate was not filtered.
3. The nuclear pellet was not washed. This step had already been omitted at the start of the Second Series and 3 volumes of Medium A taken for initial homogenization.
4. Special grade ribonuclease-free sucrose was used throughout the procedures.

II 12. Third Series of Experiments

Further changes in procedure were introduced at this stage. Huston, Schrader, Honold, Beecher, Cooper and Sauberlich (1970), carried out some critical studies on the amino acid incorporation system and the isolation of active polysomes from rat liver. These authors found the addition of GSH (3mM) to all extraction media to enhance the activity of polysomes. It was decided to follow this procedure both for preparation of ribosomes and of enzymes. Thiol reagents had been omitted previously since it was possible that they might reverse some of the effects of Cd, which complexes with thiol groups (Fluharty and Sanadi 1960). Ribosomal preparations from Cd-treated animals had, however, already shown lowered activity in protein synthesis in the presence of GSH in the incorporation assay system. The presence of an additional buffering system and source of thiol groups might therefore protect apparently more fragile cellular constituents in the treated livers.

Tris buffer was still used, but the pH was adjusted, after addition of GSH, at 4°C for preparative solutions, but at room temperature for the incorporation mixture, to minimise the pH change on warming to 37°C.

<u>II</u> 12. (a) Preparation of Ribosomes

Two normal or two Cd-treated livers were combined and processed the same way but not concurrently.

Samples were taken during the preparation and supernatants kept for estimation of protein, total ribonucleoprotein particles and RNA (See Section <u>II</u> 17.).

A PMS was prepared directly from the homogenate by centrifuging at 20,000 g_{av} (Beckman Model J-21, 15,000 rev./min JA-20 rotor) for 10 min at 2°C. About two-thirds of the middle portion of the supernatant was aspirated, using a 20 ml plastic, disposable syringe with a thin teflon tube attached, and employed for ribosome preparation. The remaining supernatant was used for a separate ribosome preparation. DOC was added as before and ribosomes sedimented either at 100,000 g_{av} (40 rotor) or at 255,800 g_{av} (Beckman Model L2-65B 60,000 rev./min 60 Ti rotor) for 1,75 hr at 2°C. Pellets in 40 rotor tubes were quickly washed off with buffer if the pellet was firm enough and stored individually in glass tubes, closed with greased stoppers, in solid CO_2 (-80°C). When sedimented in the Ti 60 rotor the ribosomes were transferred to glass tubes in small portions, snap frozen in ethanol/solid CO_2 and stored in the same way. Trials had previously been made on lyophilization of ribosomes (Christman and Goldstein 1971), and storage frozen as pellets (Earl and Morgan 1968), and with the latter method they retained virtually full activity at -80°C for long periods. Preparations had a high polysome content and were evidently low in ribonuclease as they were relatively stable to incubation at 37°C. A typical gradient is shown in Fig. 18 (9A.U. of preparation). N and Cd preparations did not differ significantly, both showed slight variations in monomer and dimer content. Figs. 19 and 20 show the gradient profiles after warming samples of a preparation (10A.U.) to 37°C for 2 min and 15 min respectively.

11 12. (b) Protein Synthesis Studies

A series of experiments to study the time course of incorporation of amino acids was carried out on these improved ribosomal preparations. Conditions for incorporation of a mixture of ^{14}C-labelled amino acids (0,5 μCi/tube), were as before, but reaction was stopped by rapid cooling and precipitation of proteins with TCA after time intervals varying from 1 to 30 min. In order to confine the study to differences in the ribosomal preparations only, pH5 enzyme from N rats was used.

FIG. 18.

SUCROSE GRADIENT. THIRD SERIES EXPTS.

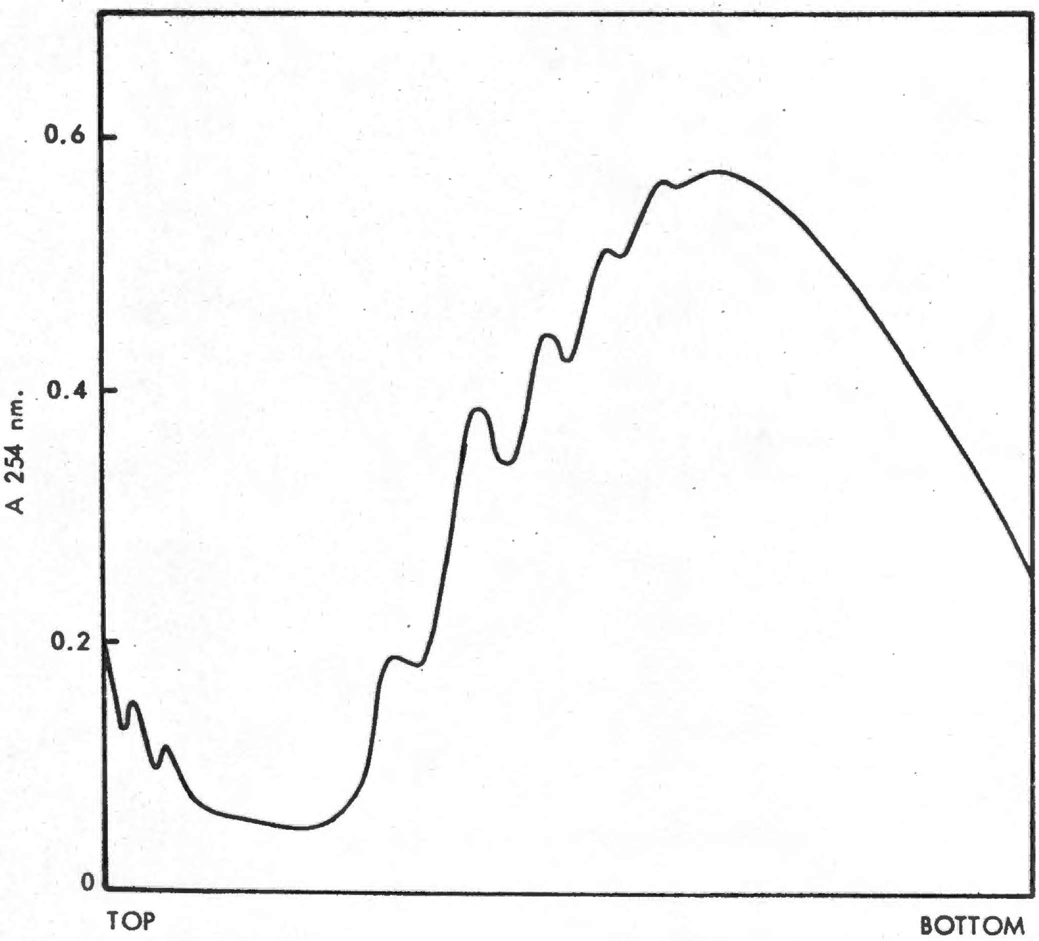


FIG. 19.

SUCROSE GRADIENT. THIRD SERIES. 2 min/37°.

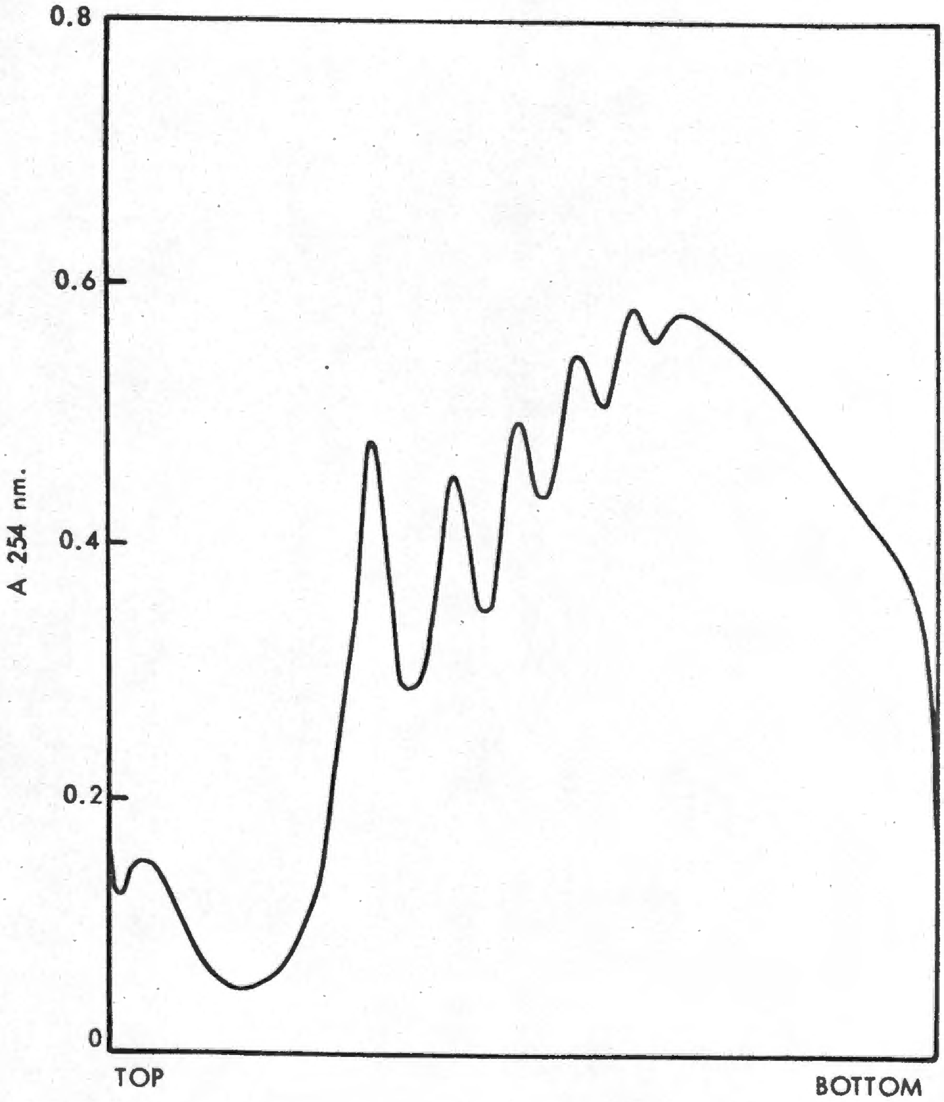
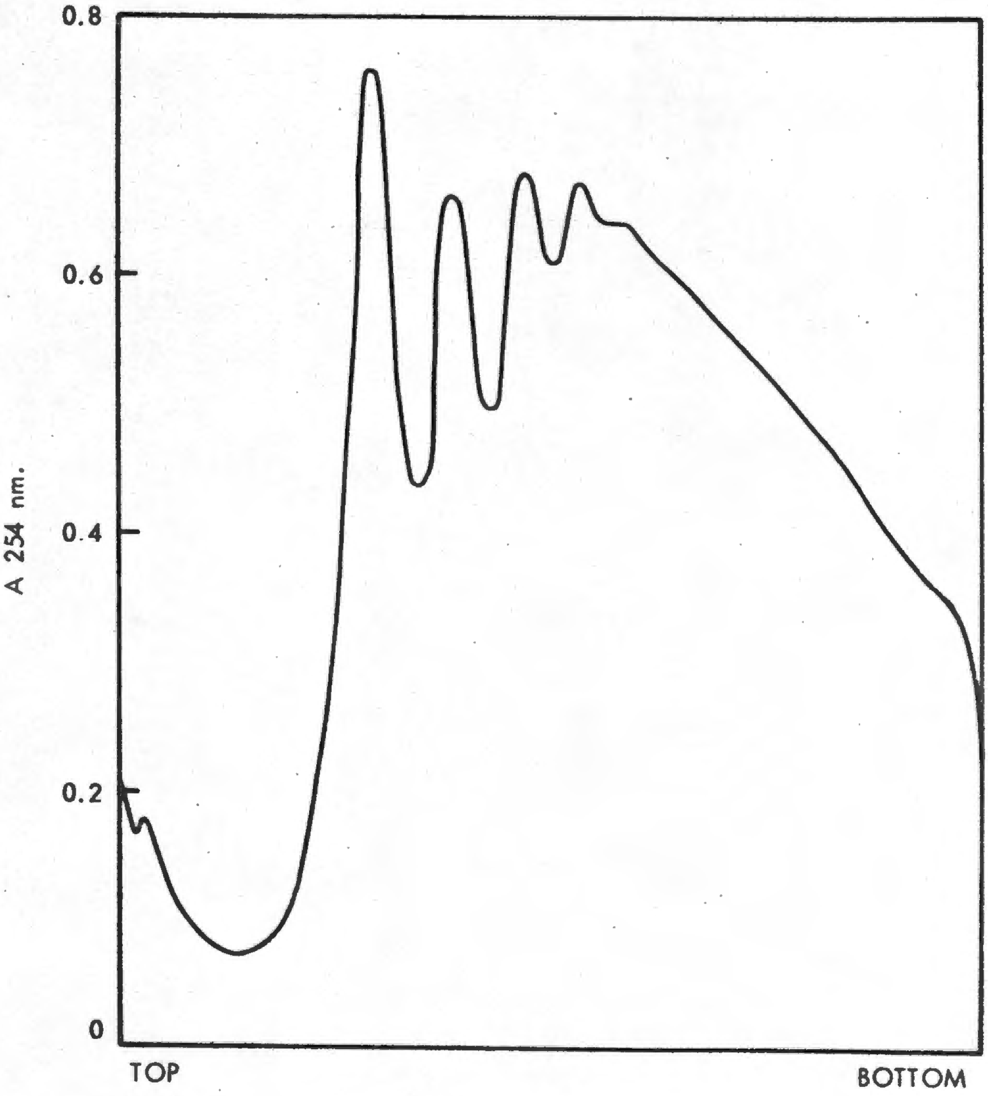


FIG. 20.

SUCROSE GRADIENT. THIRD SERIES. 15 min/37°.



Equivalent amounts (5A.U.), of ribosomes were added to each assay tube. Results are shown graphically in Figs. 21 (N preparations) and 22 (Cd preparations). Different symbols denote individual experiments, the curves indicate average values. Comparing the average cpm incorporated at each time interval, the Cd preparations show about 75% of the efficiency of the N, except at 2 min where it is 10% higher.

Time	Cd % of N
2 min	111
5 min	74
10 min	75
15 min	81
20 min	77
30 min	76

Incorporation is virtually linear in the N for 10 min and still rising at 30 min, but in the case of the Cd series incorporation has already slowed down after 5 min and continues thereafter at a lower level. (Norton and Kench 1973b).

FIG. 21.

INCORPORATION. TIME COURSE. N PREPARATIONS/N pH5

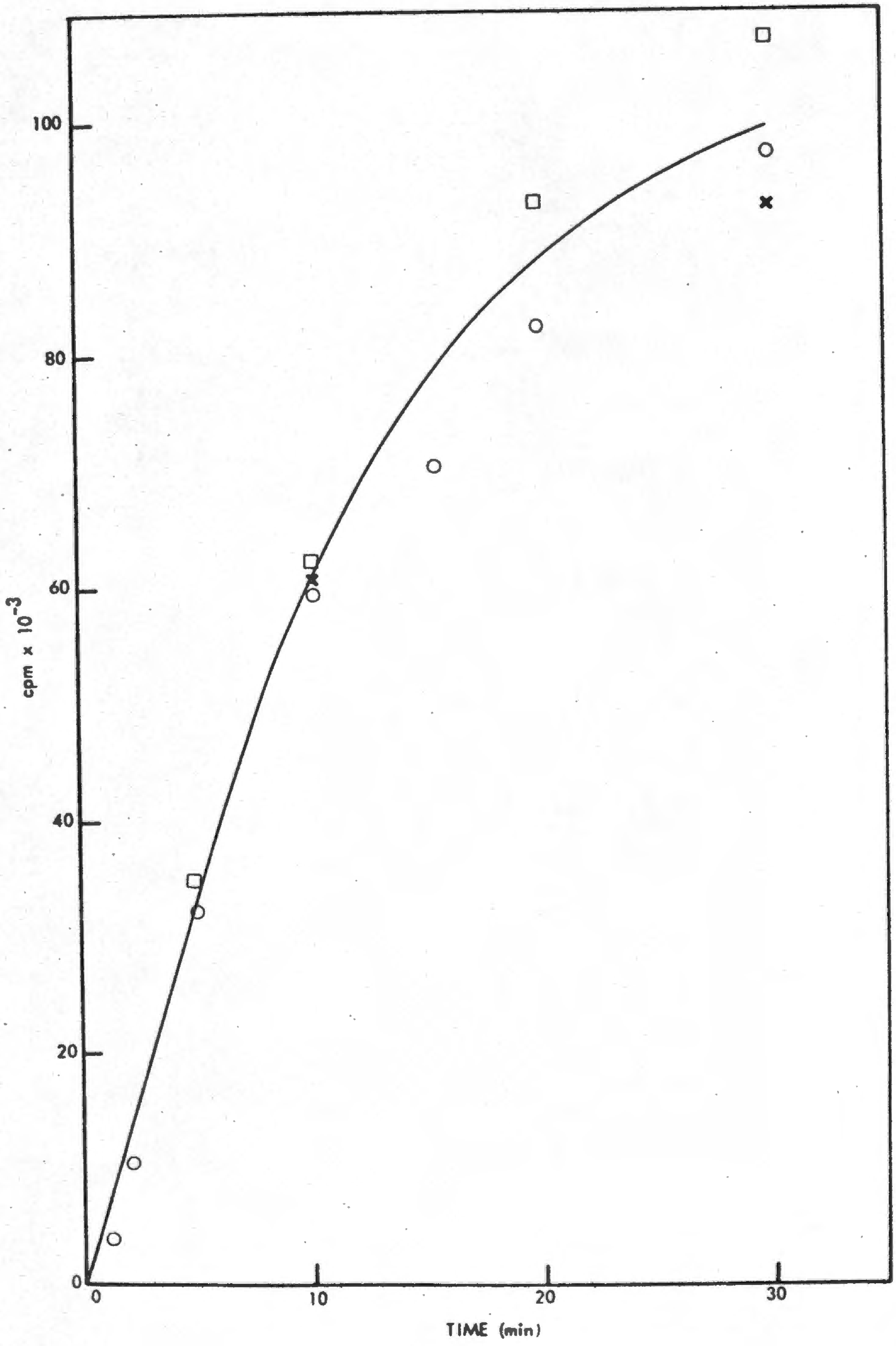
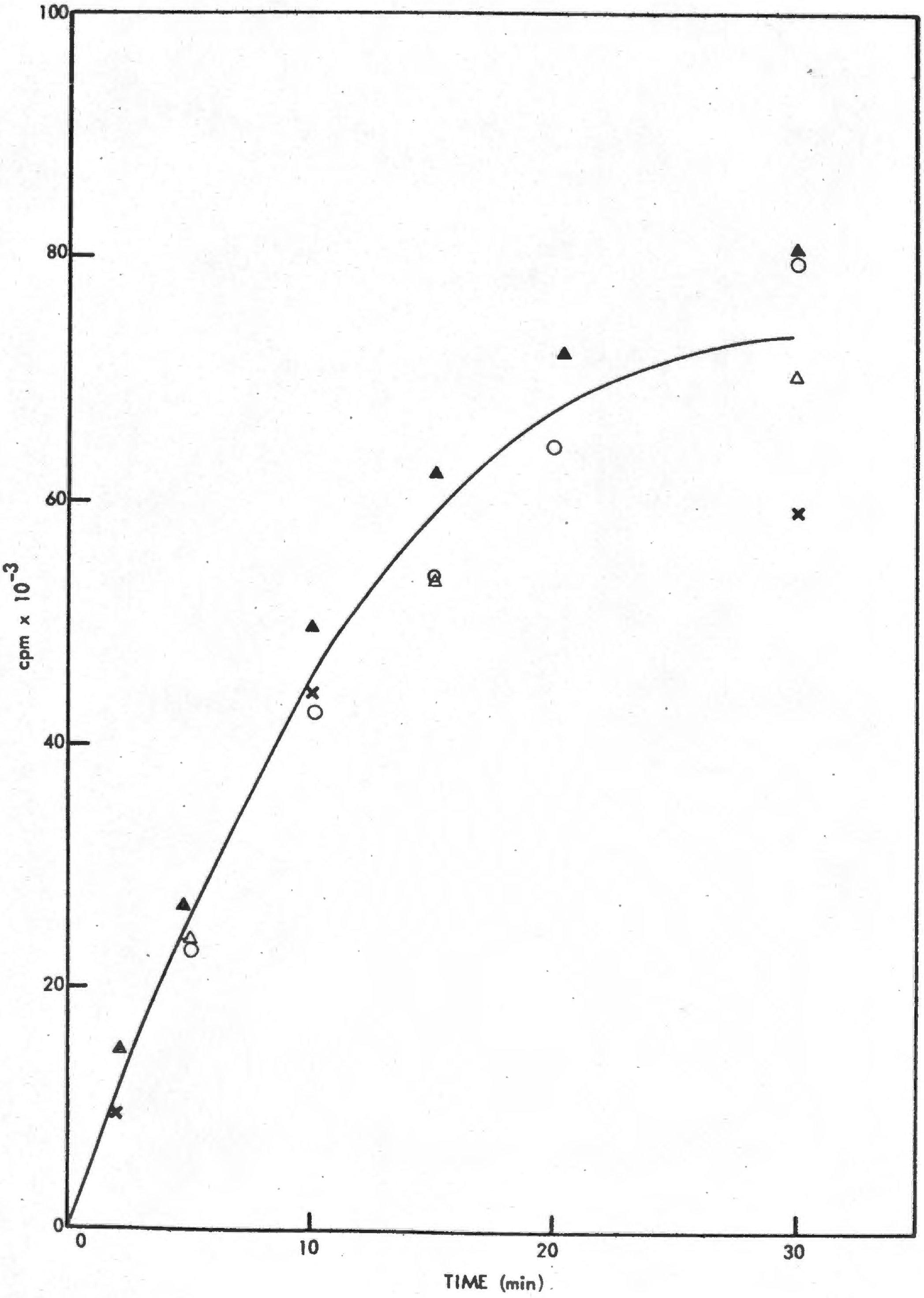


FIG. 22

INCORPORATION. TIME COURSE. Cd PREPARATIONS/N pH5



II 13. Experiments Using Cell Sap Instead of pH5 Enzymes in the Assay System

It was thought that cell sap treated to remove amino acids simply by gel filtration might provide a more active and complete enzyme source than pH5 enzymes in the incorporation system. Gel exclusion chromatography on Sephadex G-25 has been used by a number of workers for rapid removal of amino acids from the cell sap. (Mansbridge and Korner 1963, Baliga, Pronczuk and Munro 1968). Huston et al (1970), found after testing several gels that Sephadex G-10 filtration provided a more active preparation than G-25. This method was therefore used to prepare cell sap from both normal and Cd-treated rats.

II 13. (a) Preparation of the Sephadex Column

25 g of Sephadex G-10 was allowed to swell in Medium A buffer for 3 hr, the slurry was then degassed and poured into a 1,5 x 26 cm column. The column was kept at 4°C by a cooled water jacket. A small head of solvent was maintained by a pump of the flow inducer type (Watson-Marlow), and the eluant passed through a Uvicord and absorbance recorded at 254_{nm}. Void volume was checked with dextran blue and found to be 18,5 ml. A test run with a mixture of 10 mg bovine serum albumin and

2 mg tryptophan gave good separation and a sharp elution peak for the protein at the void volume. Immediately before use the column was equilibrated with Medium A buffer containing GSH (3 mM), pH 7,3.

II 13. (b) Preparation of the Cell Sap

Two livers were used for preparation of a cell sap supernatant in the same way as for the pH5 enzymes. 6 ml of this supernatant from the centre of the centrifuge tube were taken, sucrose was added to increase the density, and the mixture was pumped onto the top of the column. Elution speed was maintained at 5 ml per min. The first sample (0,5 ml), was collected after 19,5 ml had eluted as the absorption rose sharply. This and a second (0,6 ml) were colourless and were discarded. The third fraction (6,8 ml), of coloured eluant with high absorption was collected, sampled for protein analysis (100 μl = 3,8 mg protein), and stored at -80^{o}C in several portions. Later samples had lower protein content and were subsequently discarded. The column was flushed exhaustively with buffer and used for the preparation of cell sap from Cd-treated rats (Cd cell sap). This preparation was carried out as far as possible in an identical fashion. Again the two first samples (0,5 and 0,5 ml), were discarded and the coloured fraction

(7,0 ml), was sampled (100 µl = 4,0 mg protein) and kept in portions at -80°C.

II 13. (c) Protein Synthesis Studies

Incorporation of a mixture of ^{14}C-labelled amino acids into trichloracetic acid-precipitable protein was as before, using 0,5 µCi per tube and 5A.U. ribosomes N or Cd. Experiments (30 min incorporation), to find the optimum level of N cell sap for this system showed a slight rise in incorporation level from 2,0 mg cell sap protein to a maximum at about 3,5 - 4,0 mg protein per assay tube. Above this figure, there was a sharp drop indicating an inhibitory effect on both N and Cd preparations (Fig. 23). The curve was similar for normal and for Cd-treated ribosomes, the latter showing a drop of 10% in incorporating efficiency at the low level of cell sap protein to about 20% when 4 mg cell sap were taken. Maximum counts incorporated were only 60% of the figures using pH5 enzymes. For time-course experiments, 100 µl cell sap (3,8 mg protein) was added to each tube and reaction terminated as before after 2 - 30 min (Fig. 24). Both N and Cd ribosome preparations showed a rapid decline in rate after 2 min and efficiency of the Cd was an average of 65% of the N. Cd cell sap showed similar low activity with both N and Cd ribosomes when added to the assay system in the same amounts as the N (2,0 - 4,5 mg protein), (Fig. 25). Time-course experiments substituting Cd cell sap for the N demonstrated a rapid decline in the initial rate of incorporation. Curves for both N and Cd ribosomes only reached the same low level after 30 min as the Cd ribosomes with N cell sap (Fig. 26).

It is interesting to compare the incorporating efficiency of N cell sap with that of the pH5 enzyme preparations, using N and Cd ribosomes (Table 9). The initial rate (2 min) of incorporation for Cd ribosomes is only 6% higher using cell sap, but is doubled in the case of the N. Subsequent fall off is similar for both N and Cd.

It is clear that the cell sap is, over a short period, more active than the pH5 enzymes but is much more unstable, but Cd-treated ribosomal preparations are unable to take advantage of this initial high activity. Experiments (to follow) using these same cell sap preparations, varying the Mg^{2+} in the assay system, showed a further lowering of cell sap efficiency after storage for 1 - 2 months. The N preparation dropped to a level similar to and even slightly lower than the Cd. Ribosomal and pH5 enzyme preparations retained their activity over a similar period. Apparently some highly labile factors

FIG. 23.

INCORPORATION. N CELL SAP.

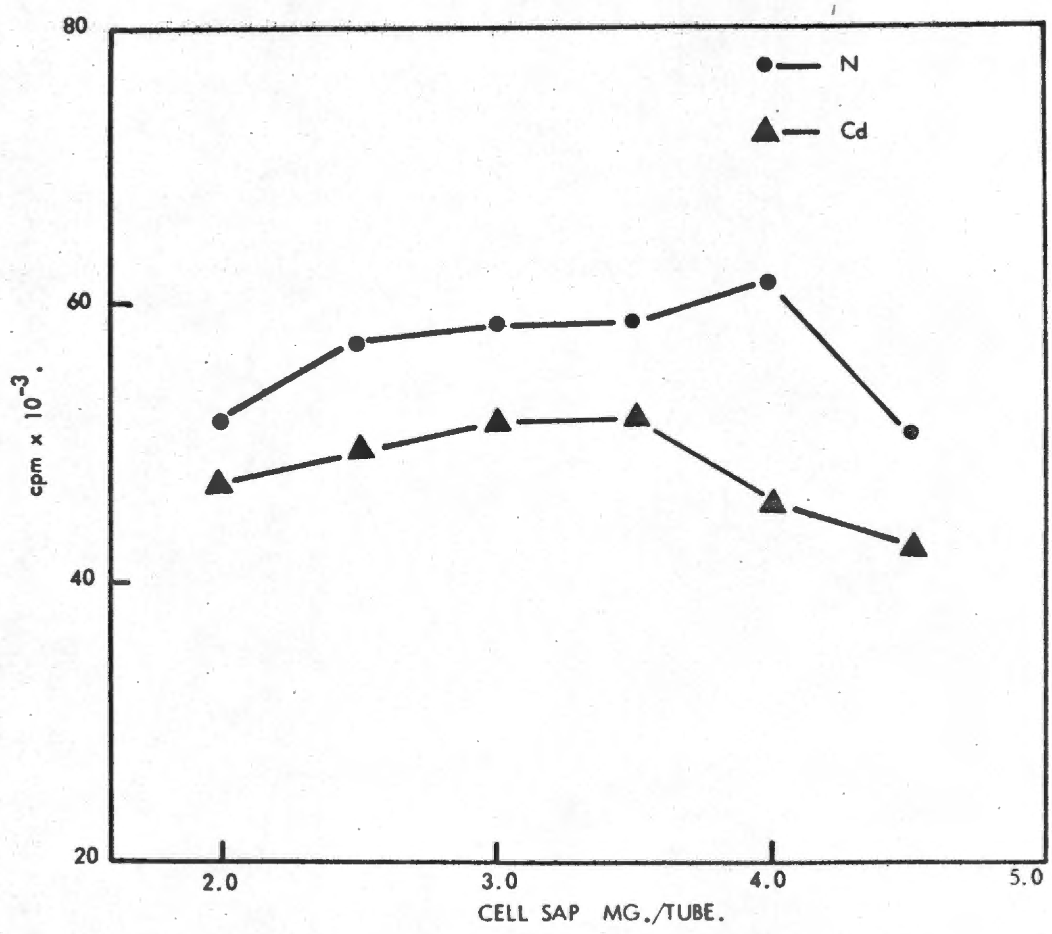


FIG. 24.

INCORPORATION. TIME COURSE. N CELL SAP.

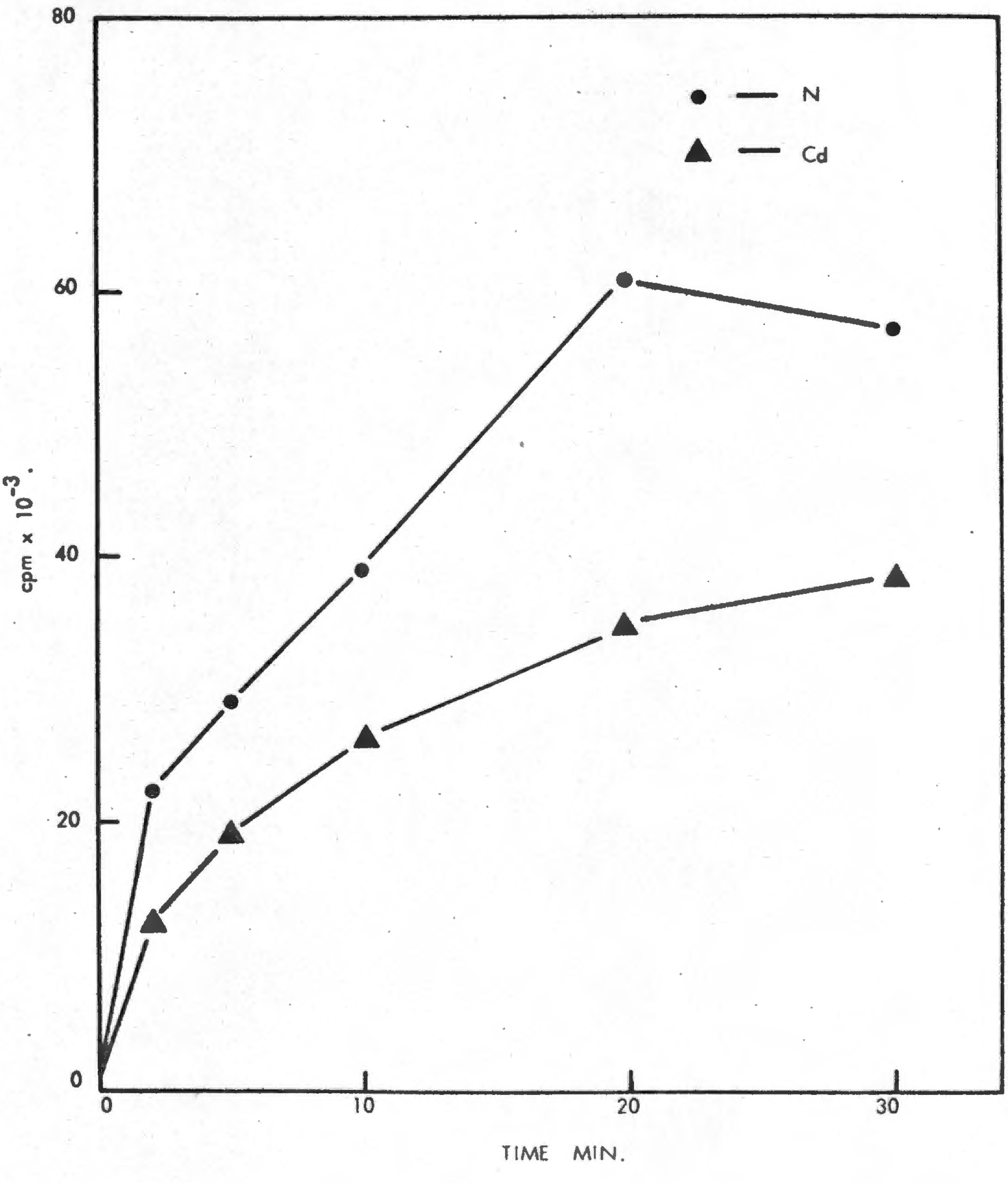
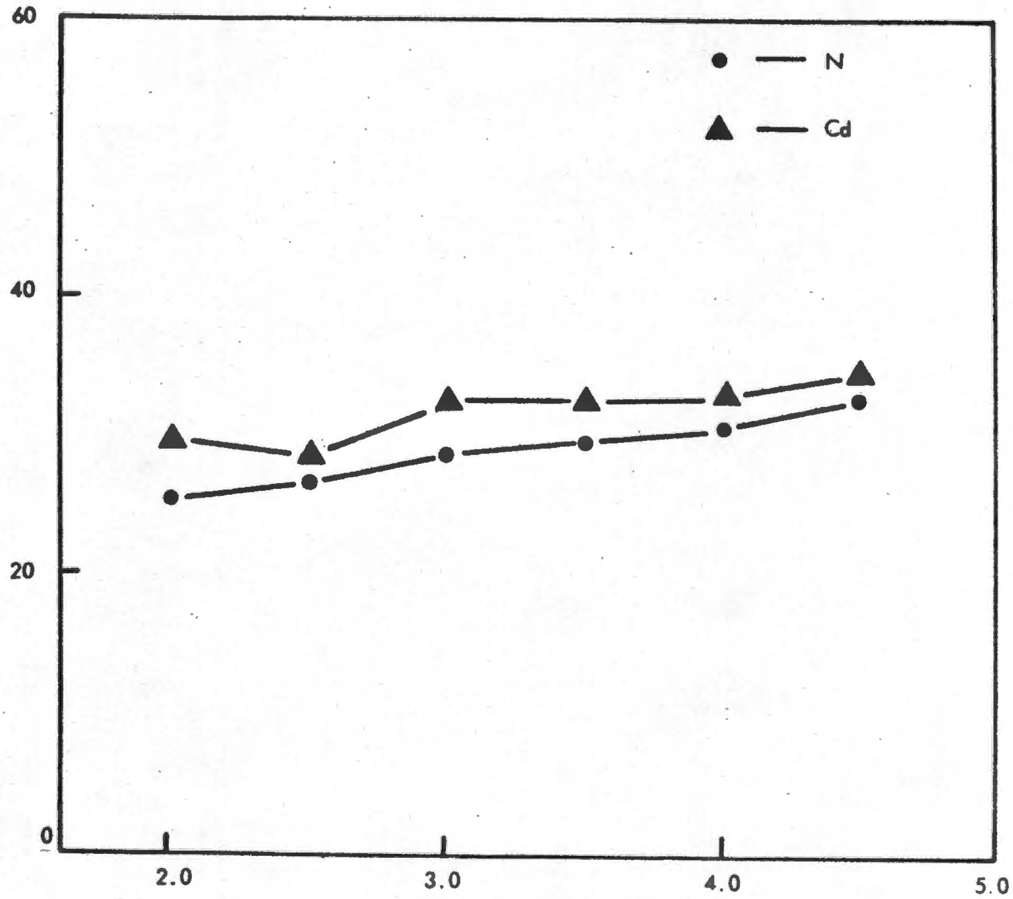


FIG. 25

INCORPORATION. Cd CELL SAP.



CELL SAP MG./TUBE.

FIG. 26

INCORPORATION. TIME COURSE. Cd CELL SAP.

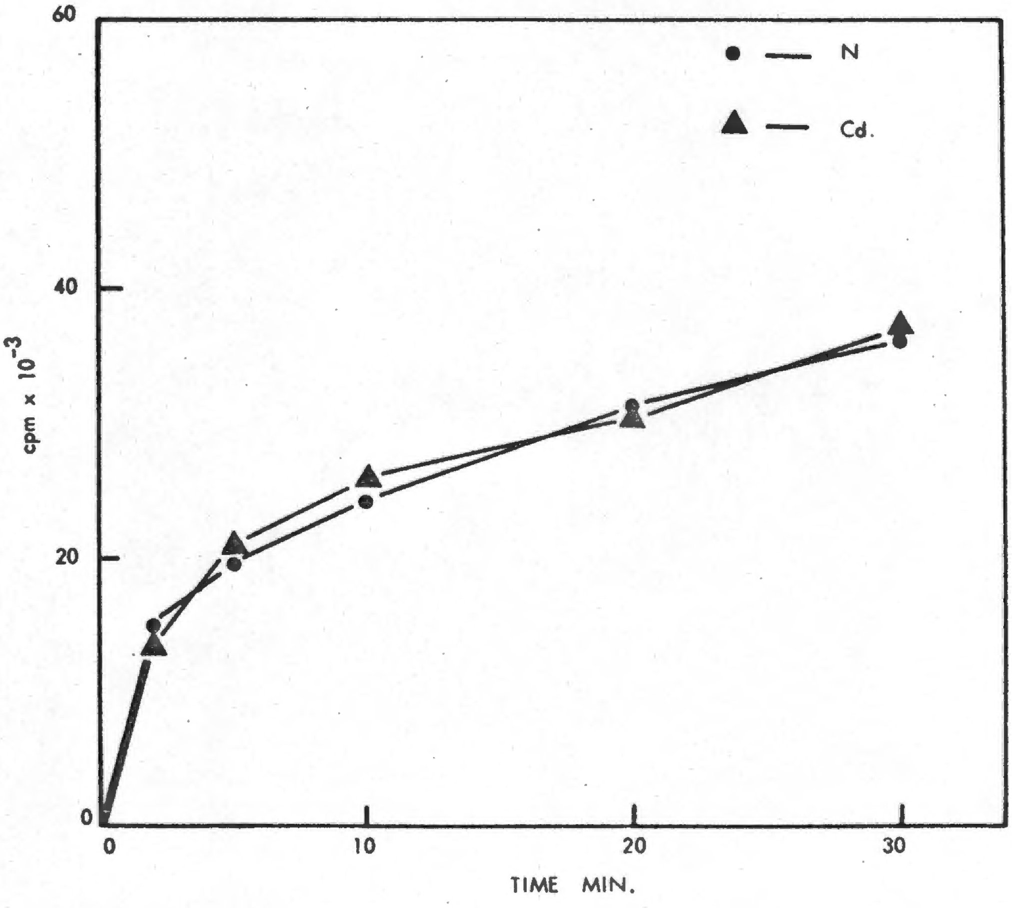


TABLE 9

	cpm Cell Sap	pH5 (Average)	Cell Sap % of pH5
Cd ribosomes			
Time (min)			
2	12490	11760	106
5	19334	25002	77
10	26431	45647	58
20	34507	67740	51
30	38222	78288	49
N ribosomes			
Time (min)			
2	22427	10673	210
5	29133	33790	86
10	38512	60486	64
20	60223	87855	69
30	56762	102298	56

deteriorate rapidly, even on storage at -80°C, and better comparison would be made using freshly-prepared cell sap in the assay system.

II 13. (d) Effect of Mg^{2+} Concentration in the Assay System

The N and Cd ribosome preparations were tested for incorporating efficiency using the cell sap preparations, at varying Mg^{2+} levels. Mg^{2+} is essential for stability of the ribosome and, at high levels, for binding of amino acyl-tRNA in *in vitro* studies before peptide bond formation can proceed. The presence of Cd or alterations in the ribosomes or proteins due to the *in vivo* influence of excess Cd might alter the requirement for Mg^{2+} in the assay system. Incorporation buffer was made up without Mg^{2+} and $MgCl_2$ was added to each tube to a final concentration of 1 - 10 mM, taking into account the Mg^{2+} already present from ribosomes (5A.U.) and cell sap preparations (3,8 mg protein), the final volume being adjusted to 0,5 ml (0,5 µCi per tube). Incorporation levels were low due to ageing of the cell sap but the curves (cpm) were similar (Cd preparation Fig. 27; N preparation Fig. 28). It therefore seems that a concentration of 5 - 8 mM Mg^{2+} is optimum for both N and Cd ribosomal preparations.

FIG. 27

INCORPORATION. Cd RIBOSOMES. VARYING Mg^{2+}.

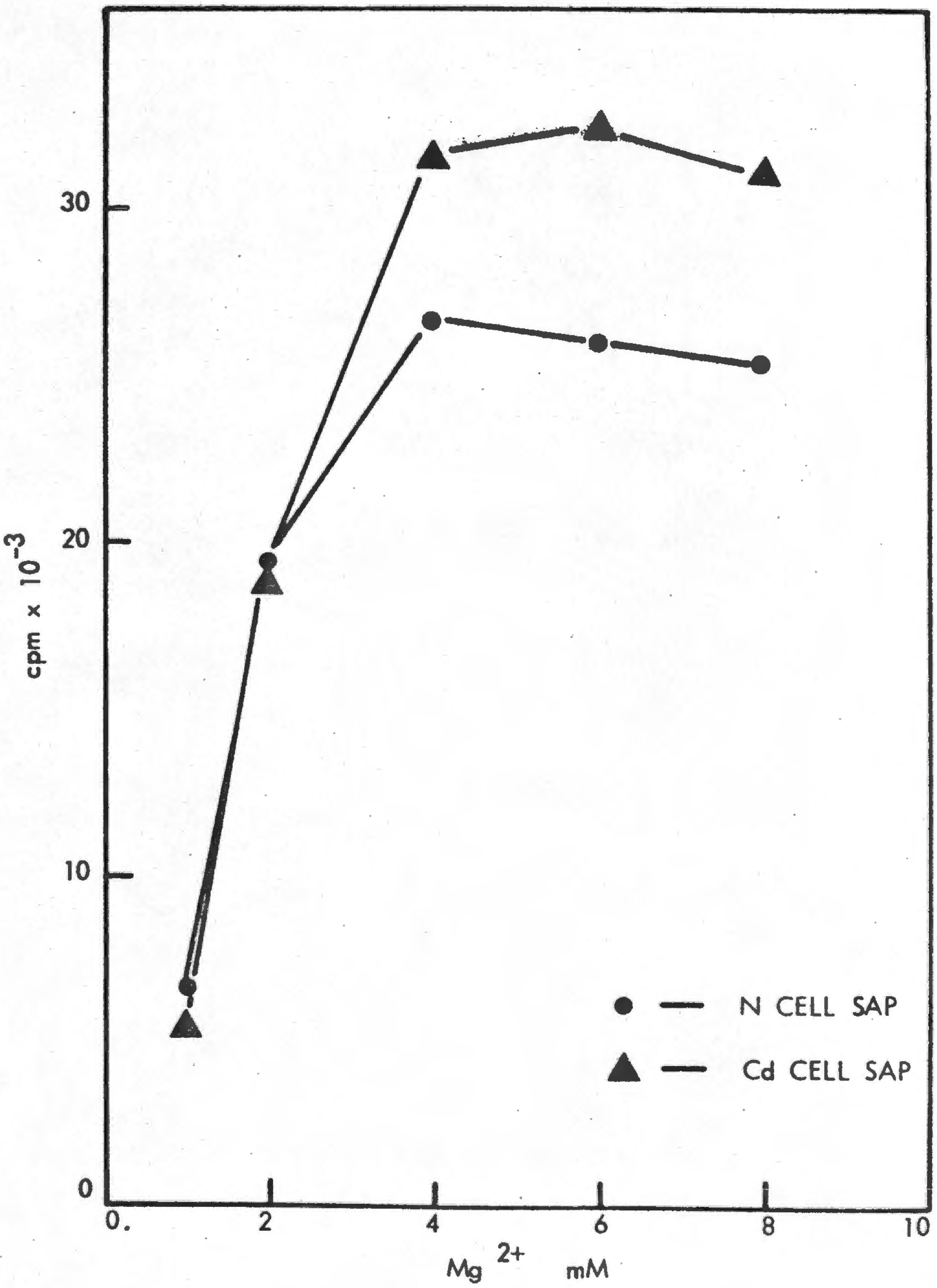
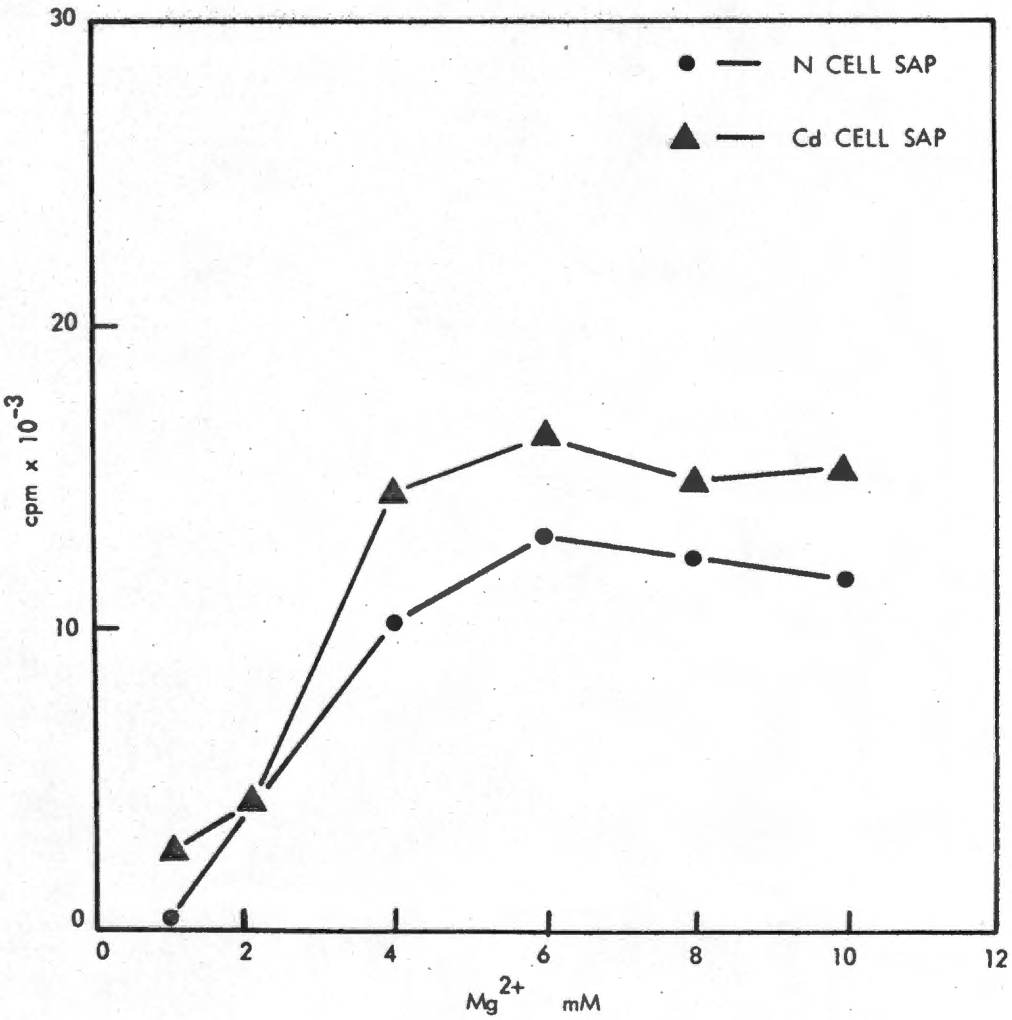


FIG. 28

INCORPORATION. N RIBOSOMES. VARYING Mg^{2+}.



II 14. Incorporation Gradients

The preferential labelling of polysomes over monosomes after amino acid incorporation demonstrates their higher activity in protein synthesis (Wettstein et al 1963). Defects might therefore alter the distribution of counts between these regions. It is important to have ribosomal preparations where the content of disomes formed as artefacts during preparative procedures is low, as these disomes would dissociate to monomers during incubation at 37°C and contaminate the monomer fraction. During the course of these protein synthesis experiments, a number of gradients had been run after incorporation of amino acids for various time intervals to study the distribution of counts over the monosome and polysome regions, and preferential accumulation of labelled amino acids into the polysome region had been demonstrated (Norton and Kench 1973b).

An interesting result had also been observed in several early experiments (First Series) showing fractions at the bottom of the gradient tube from Cd preparations to have a higher specific activity than the N after incorporation of amino acids for 10 min. This result seemed at variance with the instability and high oligosome content of the ribosomal preparations at the time, and indicated the presence of some heavy Cd polysomes more stable than the N.

II 14. (a) Methods

Ribosomes were incubated at 37°C in the incorporation mixture, as already described, and after the specified time the mixture was cooled rapidly to 0°C and carefully layered upon a cooled 15 - 30% gradient of buffered sucrose. For 5 ml tubes, 300 µl Medium A was layered on the gradient first and then 0,25 ml of cooled mixture (0,25 µCi). These tubes were centrifuged at 125,000 g_{av} (Beckman Model L, 39,000 rev./min, SW 39L rotor) for 60 min at 2°C. Gradients were monitored at 254 $_{nm}$ as before and about 30 fractions collected by drop counting. These fractions were counted directly with 90% efficiency in a toluene-based cocktail containing 7% BBS-3, a detergent solvent, 2% water and 0,8% TLA fluor, a 16:1 mixture (wt/wt) of, 2-(4'-t-butylphenyl)-5-(4"-biphenyl)-1, 3, 4-oxadiazole (butyl-PBD) and 2-(4'-biphenyl)-6-phenyl-benzoxazole (PBBO) (Beckman instruments).

Later gradients were run in 16 ml tubes by layering above 500 µl Medium A on 15 - 45% linear sucrose gradients made in an apparatus adapted from that described by Stead et al (1964). 0,5 ml (0,5 µCi) of the cooled mixture after incubation at

37°C for 2 min up to 1 hr were centrifuged at 52,400 g_{av} (Beckman L2-65B, 20,000 rev./min, SW27,1 rotor) for 17 hr at 2°C. Tubes were then placed in a holder cooled by an ice bag and pierced from the side near the bottom with a 23 g needle greased to prevent leakage and connected through a Uvicord to a peristaltic pump (LKB Perpex), the tubing and cell being previously filled with 45% sucrose. By this means a steady elution rate of 1 ml/min was maintained. Approximately 50 fractions were collected, at 15 sec time intervals, and counted in toluene cocktail as before. Fractions from the upper portion of the gradient, showing high 254_{nm} absorption, were bulked and the proteins precipitated with an equal volume of TCA for counting on a Millipore filter. Comparison of these counts with those of unfractionated duplicate tubes from which total polypeptides were precipitated after incorporation for the same time interval gave an estimate of the polypeptides released from the ribosomes. The pellet remaining at the bottom of the centrifuge tube was in some cases also recovered for estimation of radioactivity. Fluid remaining in the centrifuge tube was carefully aspirated and the pellet mixed up and transferred in Medium A to a tube for precipitation with TCA and counting.

II 14. (b) Results

The pH5 preparation, and not cell sap, was used as an enzyme source for incorporation gradients in order to provide a stable system to compare with the previous time-course incorporation experiments.

Three graphs are shown of gradients centrifuged in 5 ml tubes of Cd-ribosomal preparations incubated with N pH5 enzymes for different times: 1,5 min (Fig. 29), 5 min (Fig. 30), 10 min (Fig. 31). Comparison of the polysome profile with the cpm of each fraction shows the increasing labelling of the ribosomes with time and the higher specific activity of the polysome region. The lower specific activity of the monomer region is partially obscured by the high absorption and radioactivity of the supernatant in the top 10 fractions. The counts released into this fraction as TCA-precipitable polypeptides are given in Table 10A for the 10 min gradient and N and Cd 20 min gradients - being an average of about 10% of the total activity incorporated.

The next two gradients (Fig. 32 6 min, Fig. 33 15 min) show the much improved resolution in the 16 ml tube, and the counts associated with individual ribosome peaks. The lower number of counts associated with monomers as compared with the polysomes is clearly shown, and follows the normal

FIG. 29

INCORPORATION GRADIENT. 1.5 MIN.

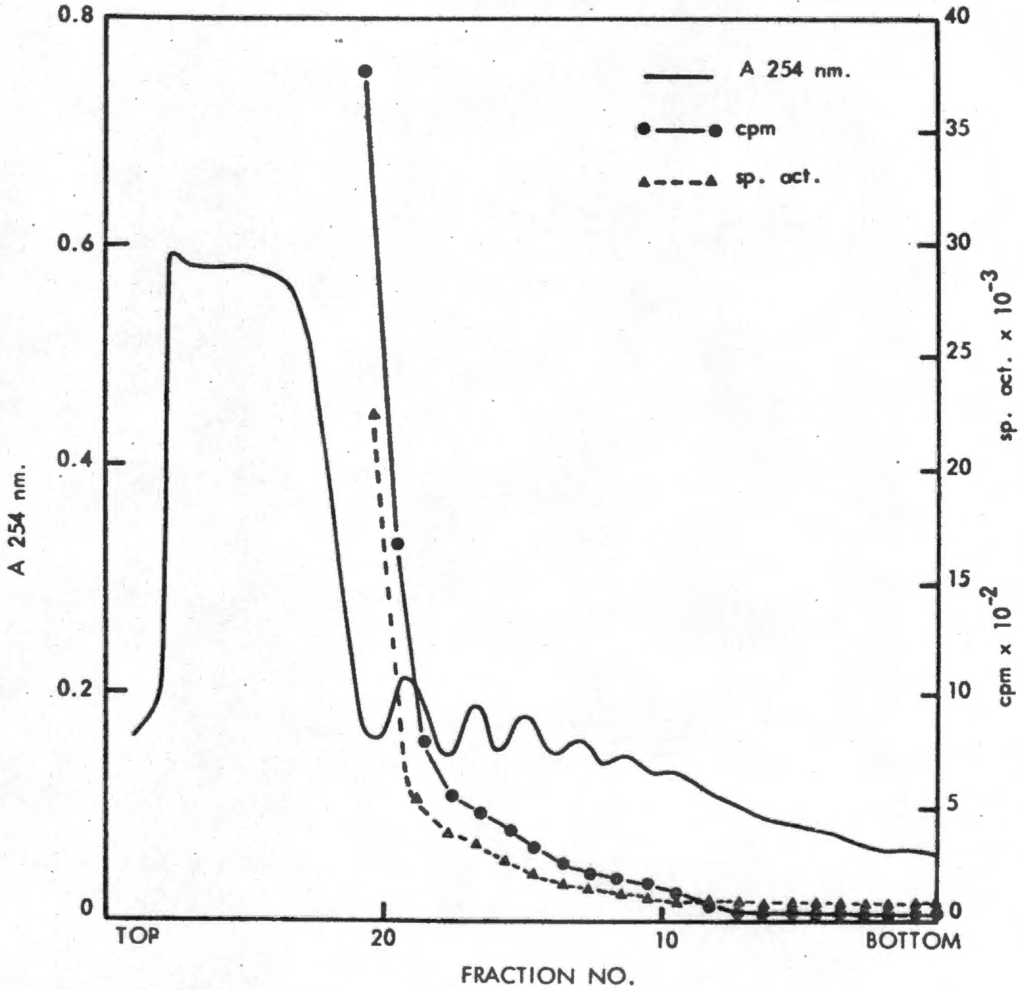


FIG. 30

INCORPORATION GRADIENT 5 min.

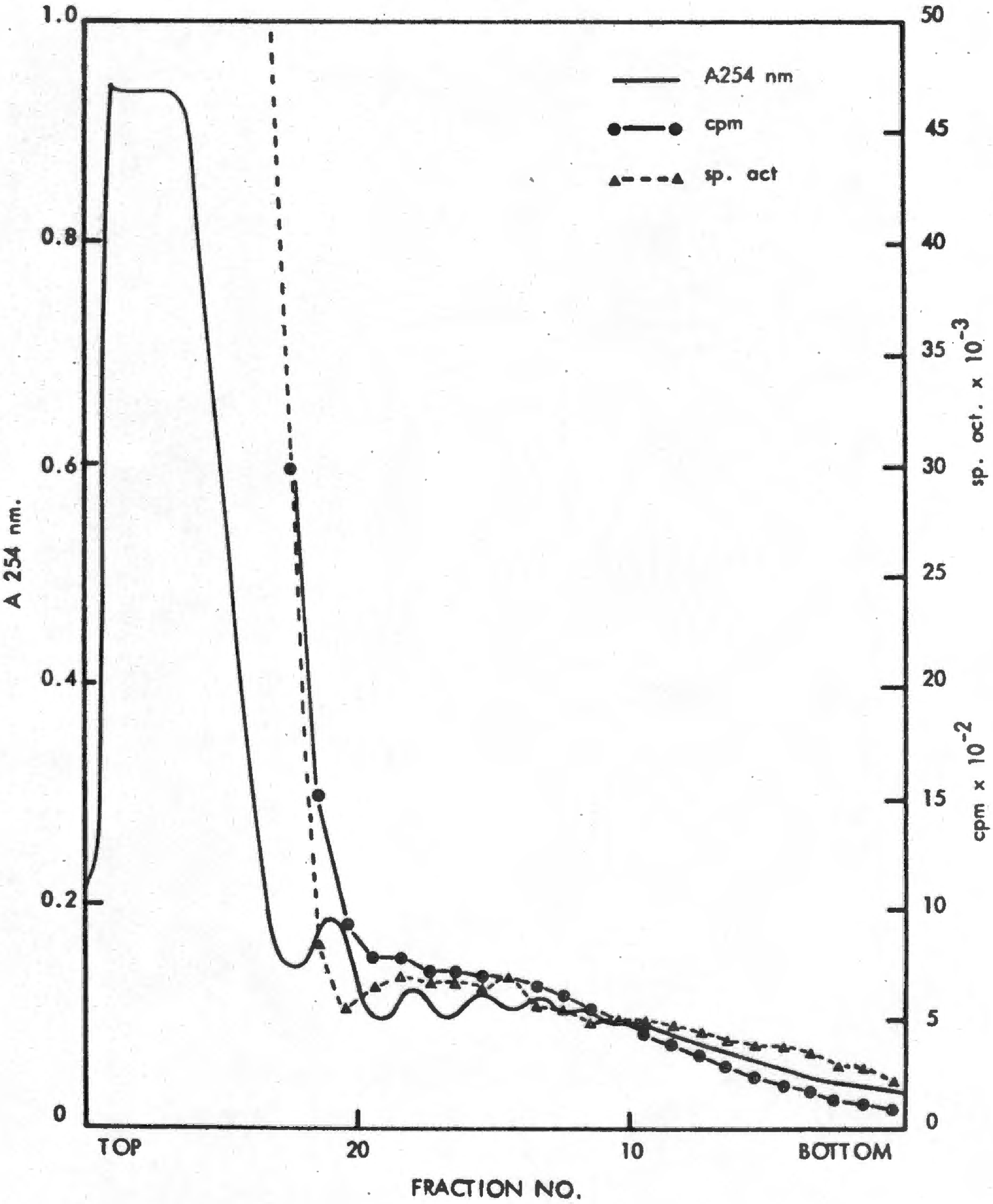


FIG. 31.

INCORPORATION GRADIENT. 10 MIN.

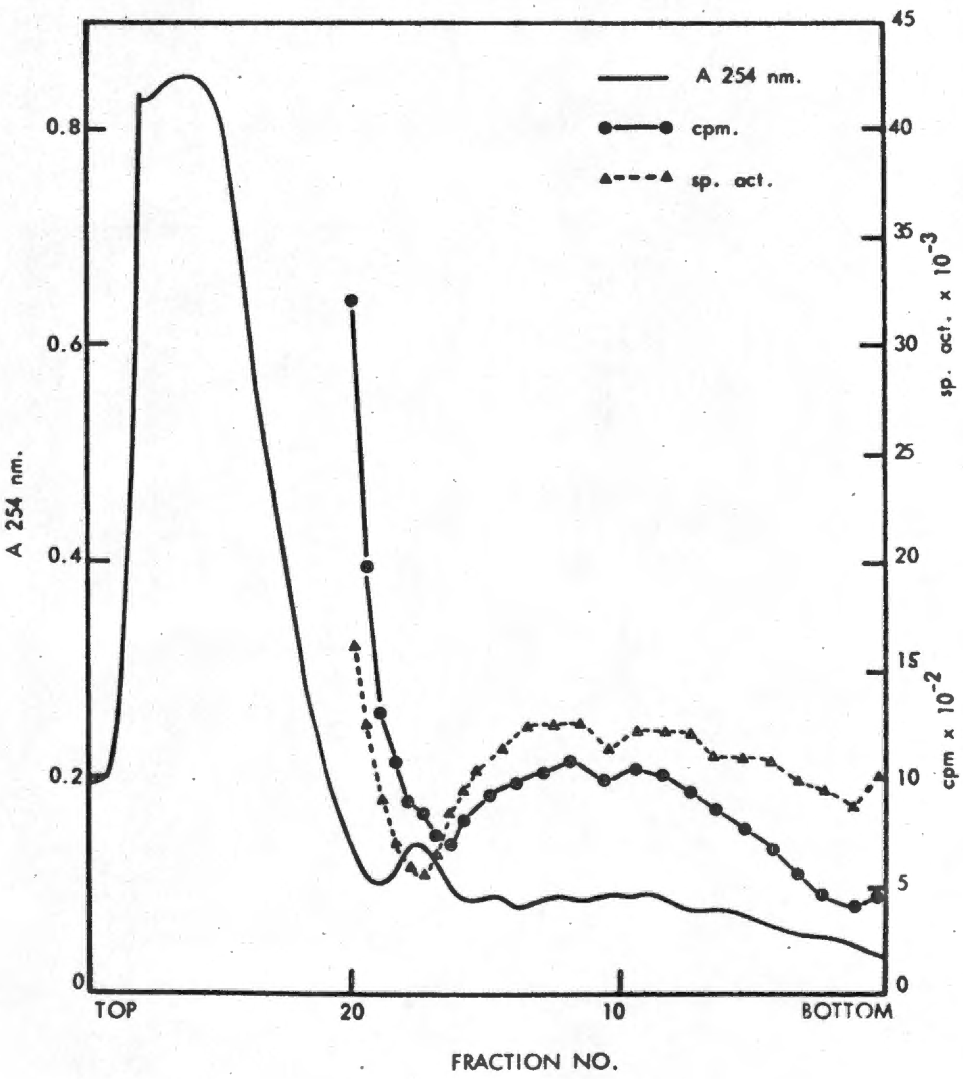


TABLE 10

Incorporation Gradients

Counts Released and in Pellets

	Preparation and Enzyme	Time (min)	Total	Counts per Minute		
				In Supernatant	% Released	Pellet
A	N/NpH5	20	93351	7808	8,4	
	Cd/NpH5	10	42828	4626	10,8	
	"	20	64370	7100	11,0	
B	Cd/CdpH5	2	10338	1694	16,4	2339
	"	10	56343	7389	13,1	5971
	"	15	68018	7187	10,5	
	"	30	79500*			6409
C	N/NpH5	15	69056	9631	14,0	2860
	"	30	90290	12203	13,5	2700
	"	60	94403	13088	13,9	2324
	Cd/CdpH5	15	54004	4971	9,2	4979
	"	30	73500*	5389	7,4	2616
	"	60	74000*	4801	6,5	1255

* Figures not available, estimated from incorporation graphs.

FIG. 32.

INCORPORATION GRADIENT 6 MIN.

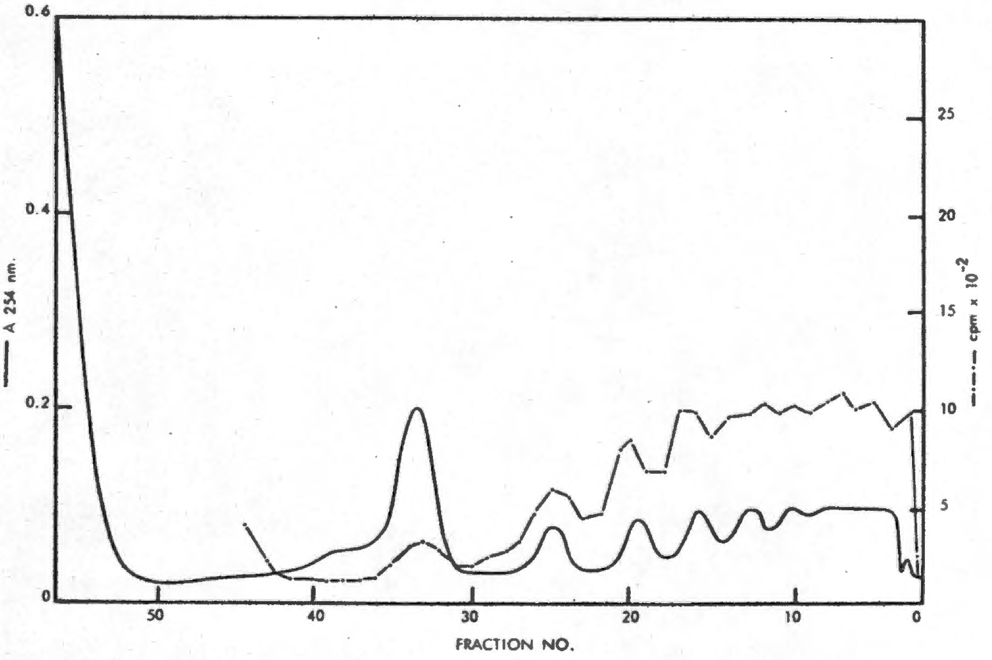
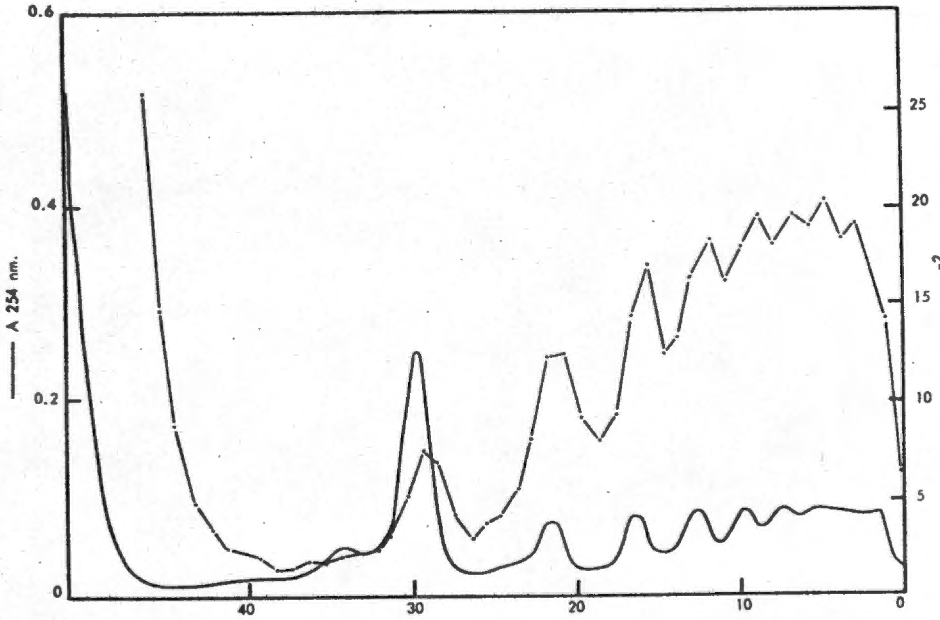


FIG. 33

INCORPORATION GRADIENT 15 MIN.



FRACTION NO.

pattern. These gradients illustrate incorporation after 6 and 15 min in an experiment using Cd ribosomes combined in the assay system with Cd pH5 enzymes. Counts released (Table 10B) were higher, 16%, after a short time interval but fell to 10% after 15 min.

The next figures (Figs. 34 - 39) compare, in a series of gradients, the complete normal system (N/NpH5) (a) with the cadmium-treated one (Cd/CdpH5) (b) at 2 min, 5 min, 10 min, 15 min, 30 min and 1 hr.

These were consecutive experiments with ribosomes prepared as far as possible under identical conditions. Comparison of the profiles of the ribosomes and the counts associated with them reveals the inhibition in the Cd system, which is particularly noticeable from 15 min to 1 hr, when an almost static situation is reached. It can be seen that this was not as a result of fission of the mRNA during incubation, as could arise by instability or nuclease action resulting in a reduced number of active polysomes. The monosome peak, particularly at 30 min and 1 hr is smaller than in the N, and the gradient profiles still show stable polysomes after 1 hr incubation. At this time, the N has a higher monosome peak and a low content of polysomes. The increased rate of incorporation in the N compared to the Cd is shown in the more extensive labelling,

FIG. 34a.

INCORPORATION GRADIENT. N/NpH5 2 MIN.

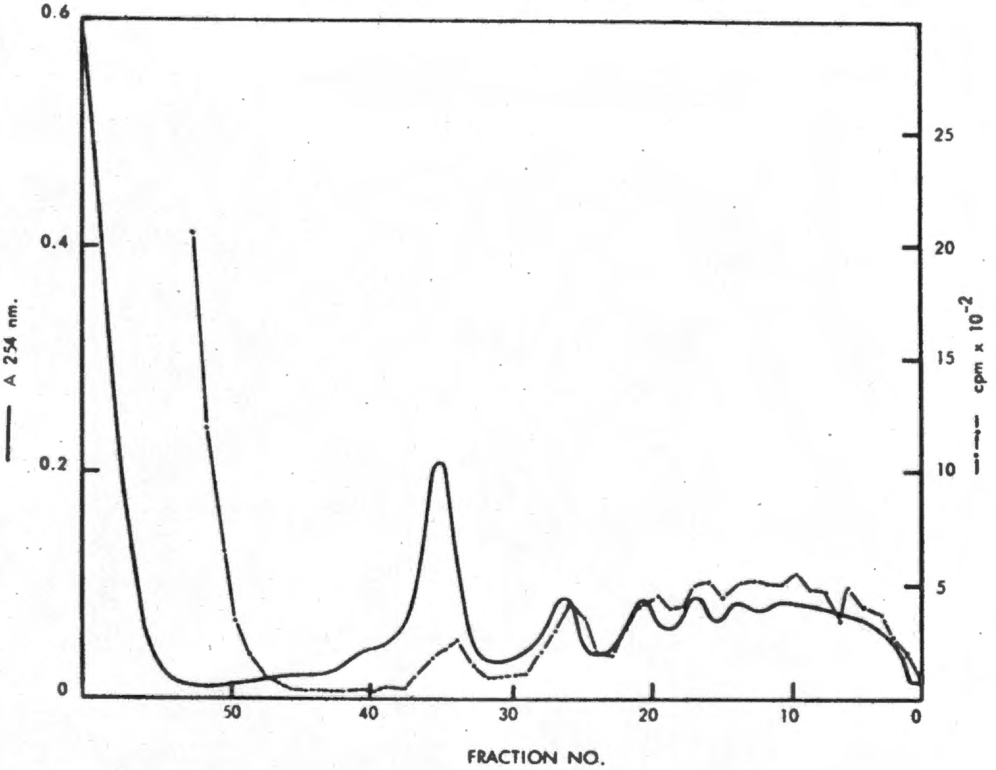


FIG. 34b

INCORPORATION GRADIENT. Cd/Cd pH5 2 MIN

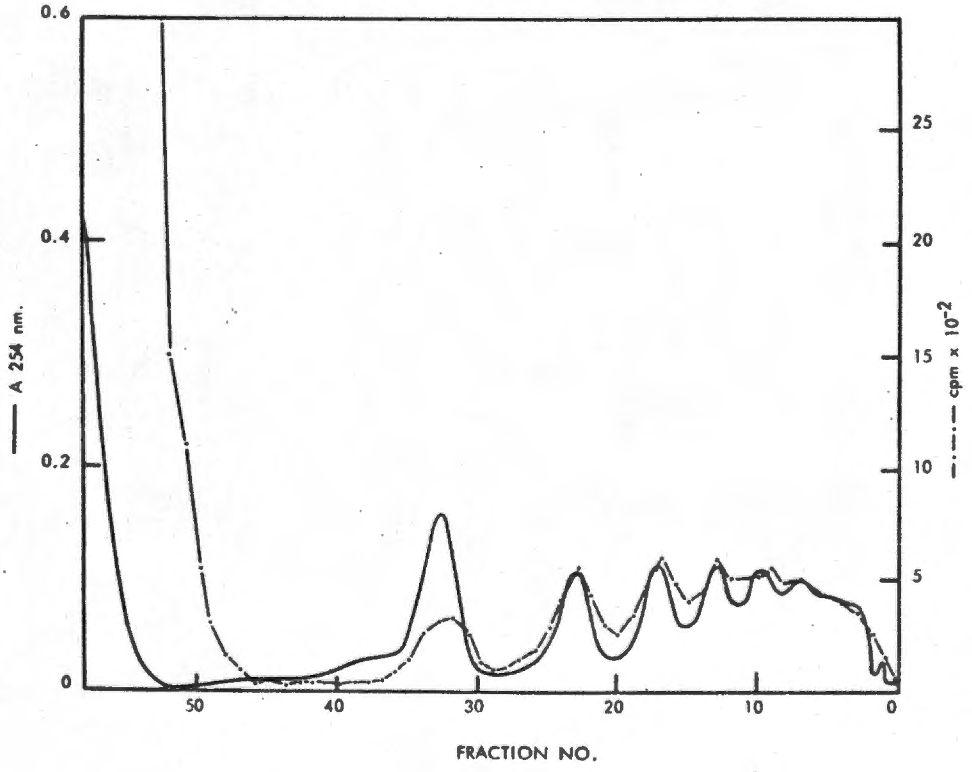


FIG. 35a.

INCORPORATION GRADIENT. N/NpH5 5 MIN.

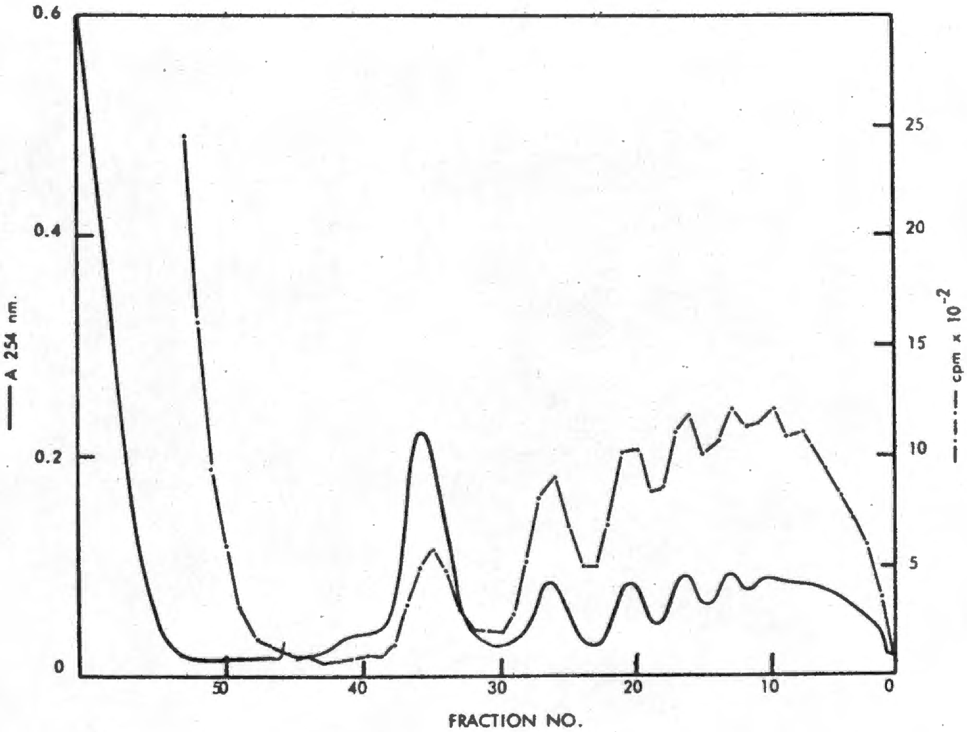


FIG. 35b.

INCORPORATION GRADIENT. Cd/CdpH5 5 MIN.

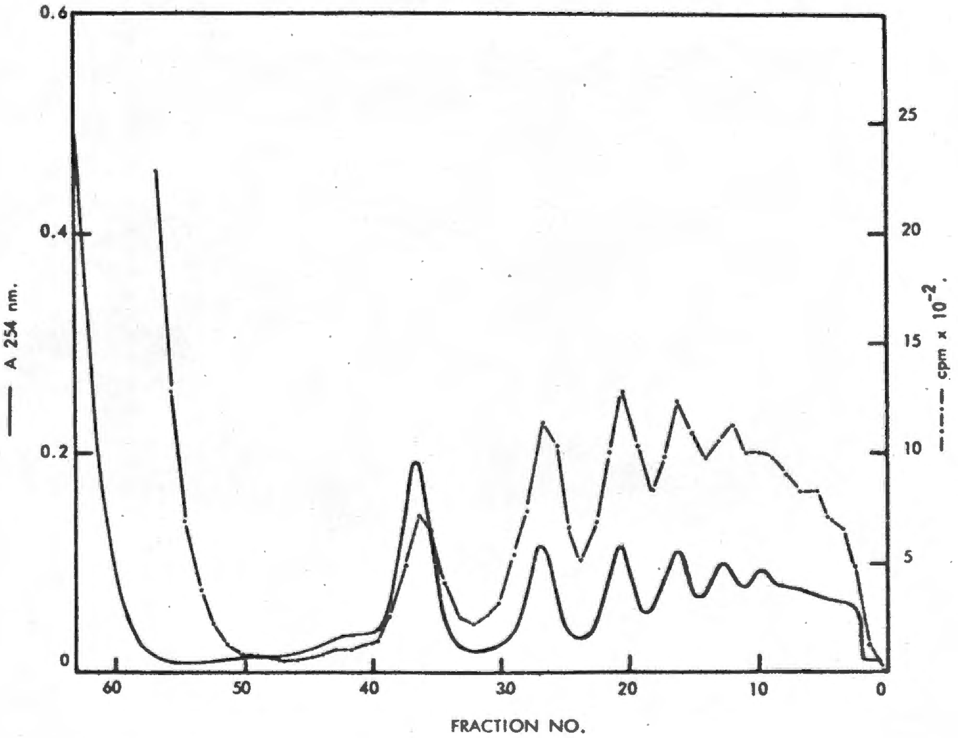


FIG. 36a.

INCORPORATION GRADIENT. N/NpH5 10 MIN.

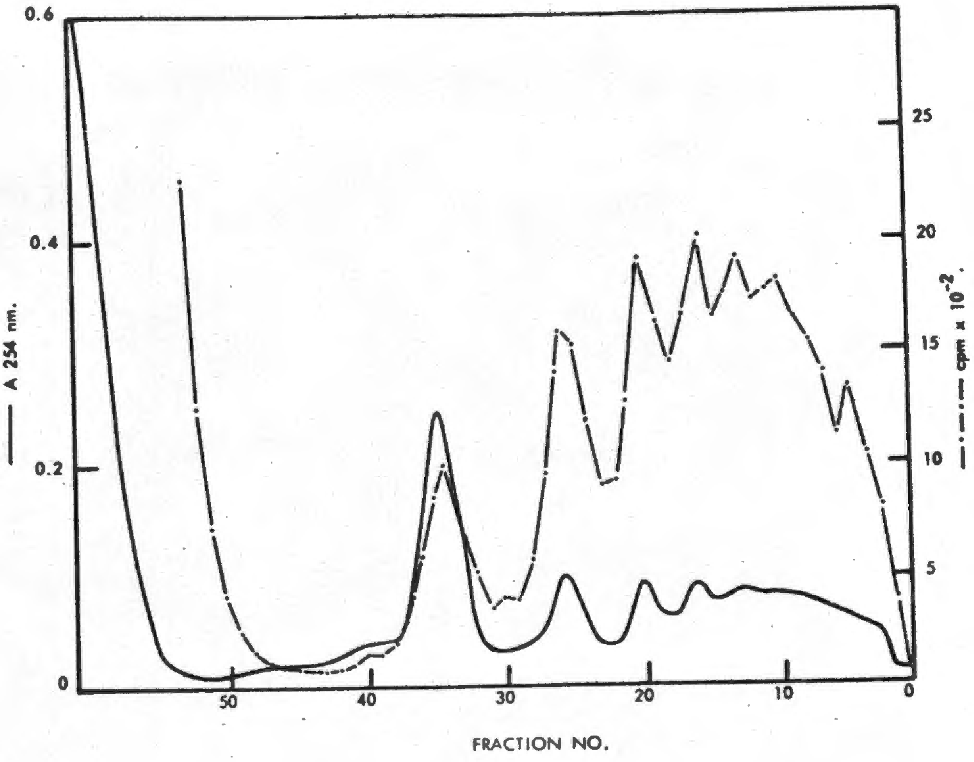


FIG. 36b.

INCORPORATION GRADIENT. Cd/Cd pH5 10 MIN.

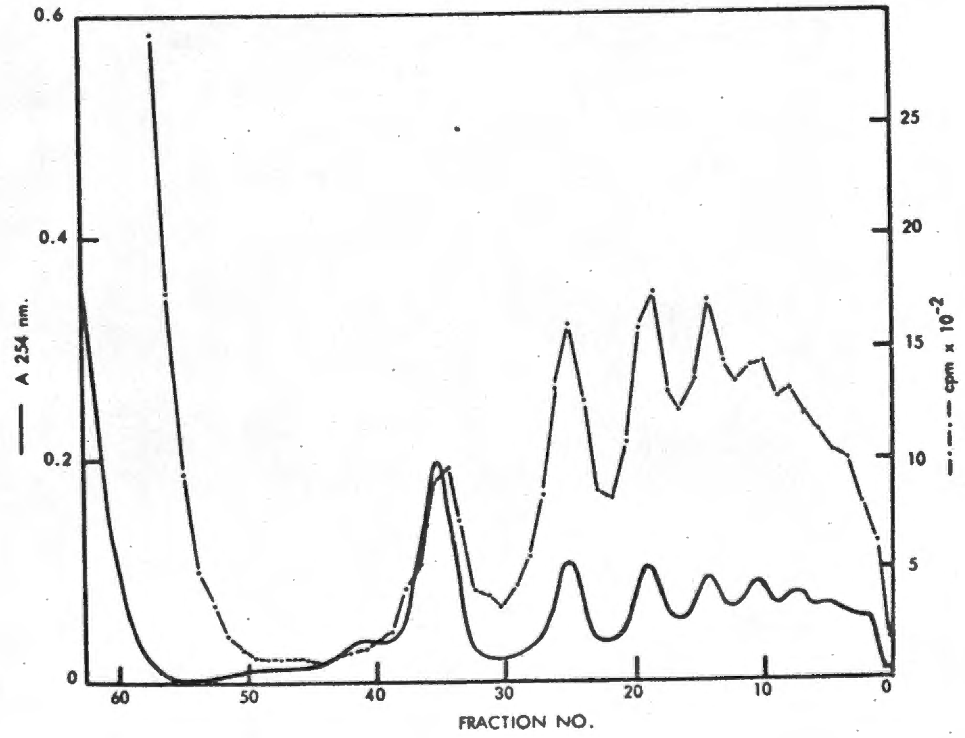


FIG. 37a.

INCORPORATION GRADIENT. N/NpH5 15 MIN.

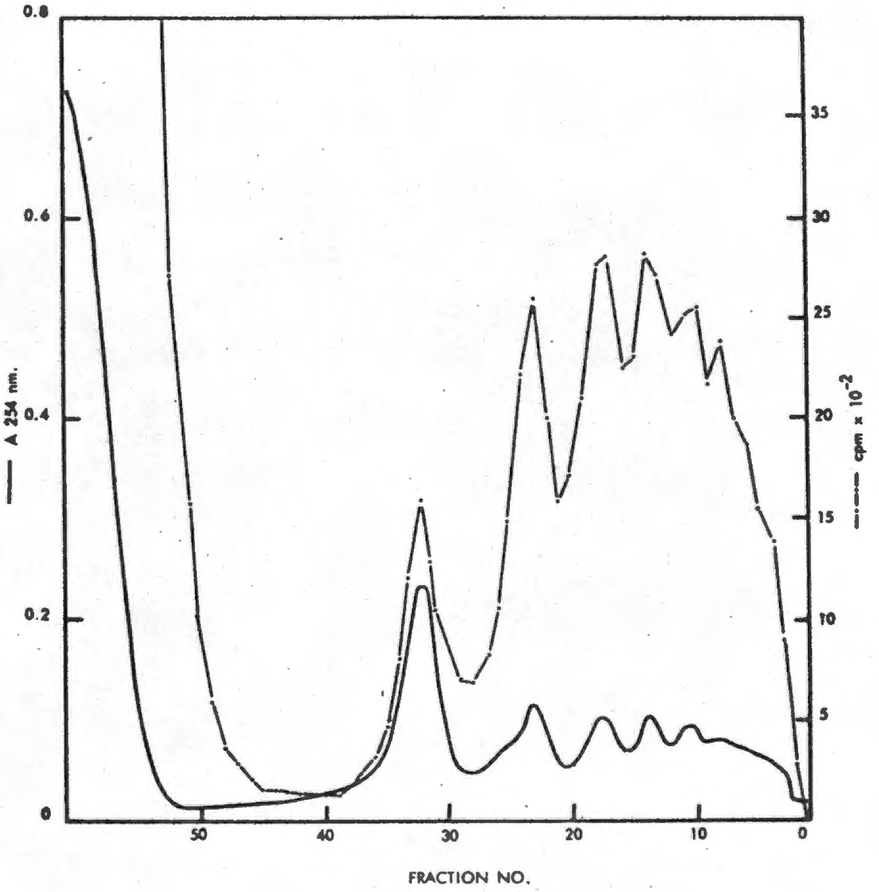


FIG. 37b.

INCORPORATION GRADIENT. Cd/CdpH5 15 MIN.

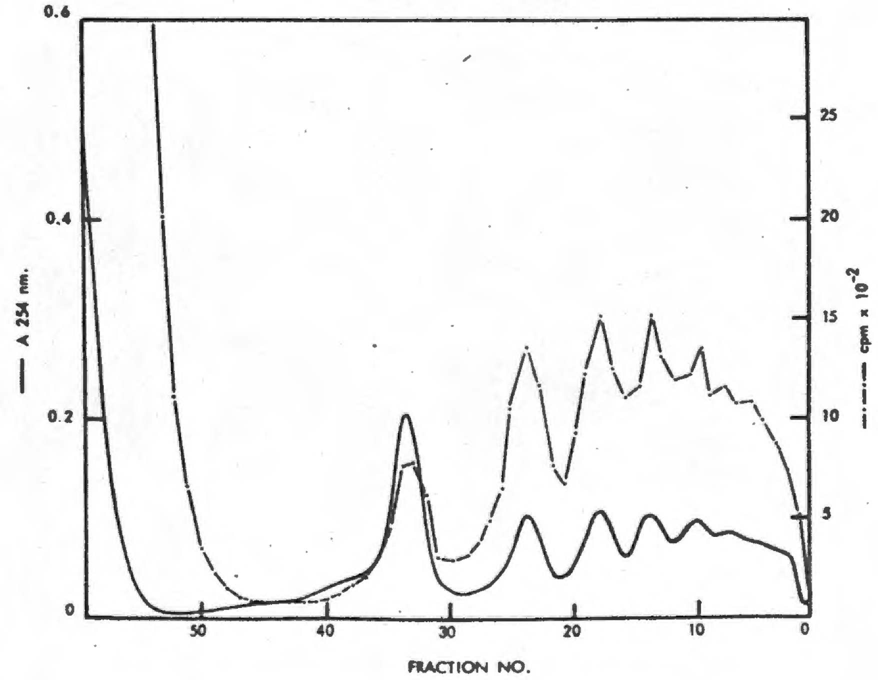


FIG. 38a.

INCORPORATION GRADIENT. N/NpH5 30 MIN.

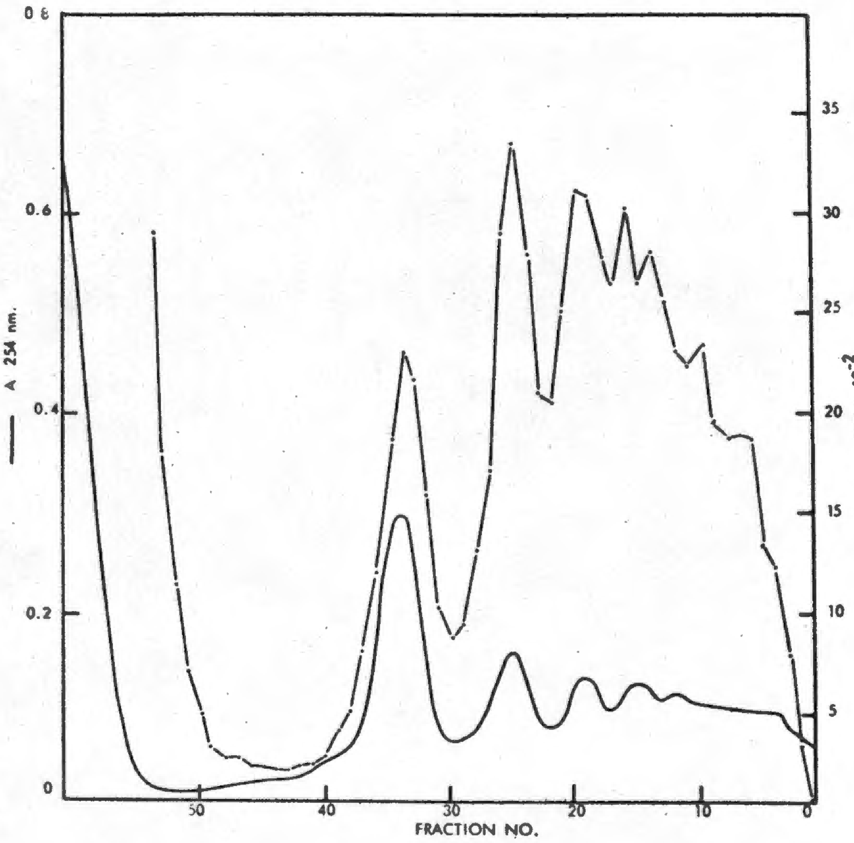


FIG. 38b.

INCORPORATION GRADIENT. Cd/CdpH5 30 min.

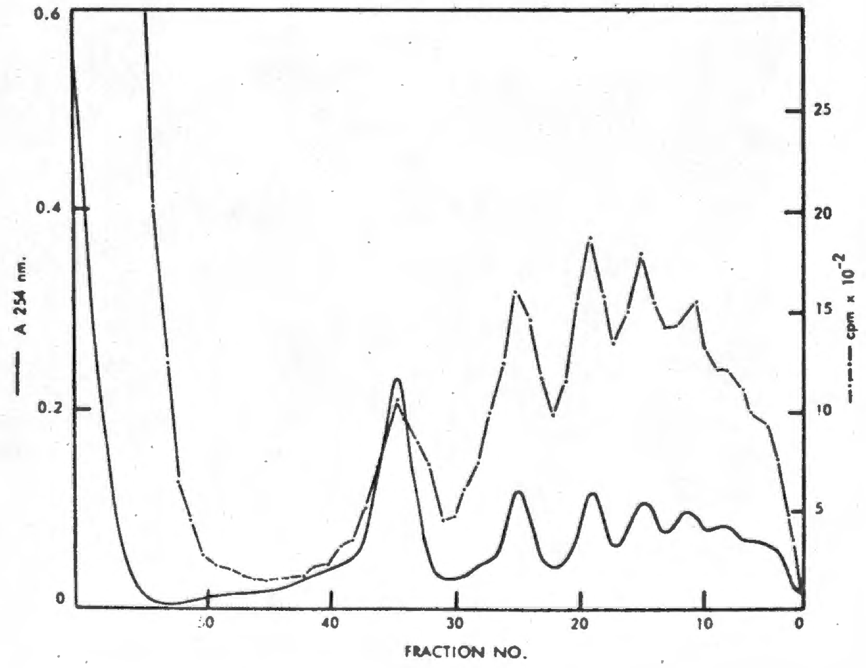


FIG. 39a.

INCORPORATION GRADIENT. N/NpH5 1 Hr.

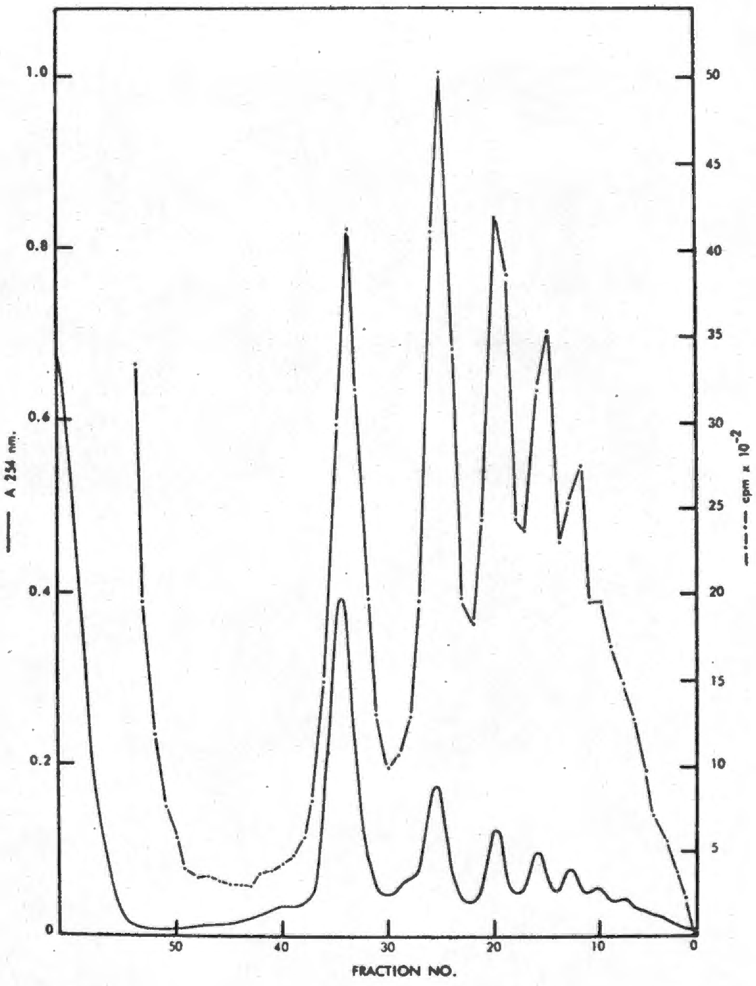
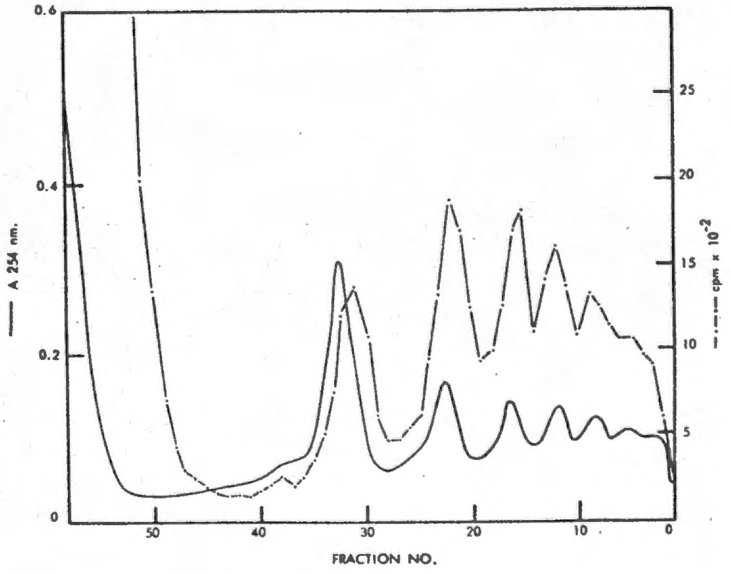


FIG. 39b.

INCORPORATION GRADIENT Cd/Cd pH5 1 Hr.



particularly after the longer time intervals, of the polysomes and oligosomes in the N system than in the Cd system. Counts precipitated from the supernatant in these two experiments (Table 10C) amounted to about 14% of the total number incorporated in the N and 7 - 9% in the Cd, showing slightly higher release of polypeptides from the ribosomes in the N system. The ratio of pH5 enzyme protein to ribosomal protein was about 10:1 which is a sufficient excess for polypeptide release (Hicks and Drysdale 1969), provided translation of mRNA is complete which may not be the case in vitro. The counts remaining in the gradient pellet provide some indication of the labelled heavy polysomes remaining in the system after incorporation. Data in Table 10 show a 2 - 3 fold increase in the Cd over the N in some cases, but the results are variable.

II 15. Cadmium Analysis of Subcellular Fractions

Analysis of enzyme preparations, used in incorporation experiments, for their content of Cd, was carried out by Atomic Absorption as before. Results compared with the protein content of the fractions are shown in Table II.

TABLE II

Preparation	Cd, μg/100 ml	Protein mg/ml	Cd, ng/mg Protein
N Cell Sap			
Fraction 3	3,0	38	0,8
" 4	2,4	25	1,0
" 5	3,6	19	1,9
" 6	3,8	16	2,4
" 7	2,6	15	1,7
Cd Cell Sap			
Fraction 3	211	40	53
" 4	85	24	35
" 5	44	20	22
" 6	38	13	29
NpH5 Enzyme	1	24	0,4
CdpH5 Enzyme	9,0	26	3,5

Preparations of ribosomes, available only in very small volume, were analysed by a method similar to that of Kubasik and Volosin (1973, 1974), developed in this laboratory (Ross and Kench 1973). This method employs a Varian Techtron Model 63 Carbon Rod Atomiser in conjunction with a Varian Techtron AA-5 Atomic Absorption Spectrophotometer with a strip chart recorder. Acid digestion was found unnecessary, and 5 µl samples were ashed and then atomised directly. The absorption peaks of the sample were corrected for non-atomic absorption and the Cd concentrations calculated from standard curves. Results are shown in Table 12.

TABLE 12

Ribosomal Preparation	Cd µg/100 ml	RNA mg/ml	Cd, ng/100 µg RNA
N	2,9	3,10	0,9
Cd	17,7	2,96	6,0
Cd	32,4	3,30	9,8
Cd	17,2	2,54	6,8

Taking an average figure of 7,5 ng Cd/100 µg RNA from the above table (12), 5A.U. (170 µg) of the ribosomal preparation when added to the 0,5 ml assay tube will result in a Cd content of the incorporation mixture of $2,5 \times 10^{-7}$M. The incorporation mixture contains $3,16 \times 10^{-6}$M Cd, if cell sap from cadmium-treated rats (3 mg protein) is used as an enzyme source. Total Cd in the system will then be $3,41 \times 10^{-6}$M.

II 16. Protein Synthesis Studies with Individual Amino Acids

Experiments to compare the incorporation of individual amino acids into polypeptides by the N and Cd systems were carried out during the course of this work. The amino acids which had been reported to be deficient in minialbumins were of particular interest, (Kench and Sutherland 1967), so lysine, low in minialbumins and tryptophan, absent from minialbumins, were the main ones chosen for study. The amino-acylation of tRNA had been also reported to be lowered in extent by Cd intoxication in the case of these two amino acids. (Sutherland and Kench 1971).

Conditions for incorporation were as described earlier substituting the individual amino acid under study for the ^{14}C hydrolysate mixture. For each assay tube, 0,5 μCi of the ^{14}C-labelled amino acids glycine and lysine was used. For the tritiated isotopes, leucine and tryptophan 1 μCi and 5 μCi were taken respectively. As tryptophan represents only about 1% or less of the total amino acids in protein, the tritiated isotope was used so that the high specific activity would result in incorporation of a maximum number of counts. In order to provide all the standard amino acids, mixtures were prepared to contain the following 20 amino acids:- alanine, arginine, aspartic acid, cysteine, cystine, glycine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. In each case the amino acid under study was omitted from the mixture. The remaining 19 amino acids were added at 5 n moles of each for a 0,5 ml assay tube (Huston et al 1970). To lower the blank values, the final TCA precipitates on millipore filters, after washing with 100 ml 5% TCA, were washed with 50 ml ethanol (Eker, Weidenmüller and Pihl 1970). The filters were then dried and counted as before.

In an early experiment, incorporation of ^{14}C glycine into TCA-precipitable counts was demonstrable at a low level but the results for ^{3}H tryptophan were indistinguishable from blank values. It was thought possible that addition of unlabelled tryptophan to the mixture in ^{14}C-labelled hydrolysate incorporation experiments might result in raised counts. This amino acid mixture lacks tryptophan, which is destroyed during acid hydrolysis. In the course of Expt. 10 (Second Series), tryptophan (5 n moles) was added to some assay tubes. No stimulation was found using either N or Cd ribosomal or enzyme preparations, counts being close to or lower than the level found without the additions.

In the next experiment (Table 13), ^{14}C glycine and ^{14}C lysine were compared using normal pH5 enzyme, two different Cd ribosomal preparations and a normal one. Each result in the table represents a single assay tube and the ribosomes added are reported as μg RNA. Counts incorporated into TCA-precipitable material are calculated as p moles of amino acid per mg ribosomal RNA for each tube. The ratios of incorporation ^{14}C glycine/ ^{14}C lysine for assay tubes to which the same quantity of ribosomes had been added are shown. Glycine incorporation is somewhat higher than lysine in both N and Cd preparations. There does not appear to be any significant difference between the N and Cd preparations in the respective values for ^{14}C glycine and ^{14}C lysine in this experiment.

Two recent experiments are reported in Tables 14 and 15. Here Cd pH5 enzyme was used with Cd ribosomes and these compared with the complete N system. In the first experiment (Table 14), ^{14}C lysine and ^{3}H tryptophan were compared. A high level of incorporation of ^{14}C lysine was found, while significant incorporation of ^{3}H tryptophan was observed at a low level for both N and Cd preparations. The second experiment (Table 15) compares ^{3}H leucine and ^{3}H tryptophan, and the pattern of results is similar. Poor counting efficiency probably accounts for the low figures for ^{3}H leucine as compared with ^{14}C lysine in the previous experiment. ^{3}H tryptophan figures are very similar. Ribosomal preparations (a) and (b) were from the same homogenate in each case. Those marked (a) were isolated from PMS withdrawn from the central region of the centrifuge tube (See Section _II_ 12. (a). The remaining PMS was used for the preparations marked (b). The latter could contain traces of degradative enzymes which would account for the drop in incorporation at high ribosomal levels observed in each instance.

No difference could be demonstrated between preparations from normal or from Cd-treated animals in these experiments.

TABLE 13

Ribosomes	^{14}C Glycine (108 mCi/m mole)			^{14}C Lysine (336 mCi/m mole)			Ratio
	cpm	µg RNA	p mole/ mg RNA	cpm	µg RNA	p mole/ mg RNA	^{14}C Glycine/ ^{14}C Lysine
				6694	95	95	
N	3442	126	114	7077	126	75	1,5
	4446	158	117	10558	158	90	1,3
Cd (1)	2360	170	58	5940	170	47	1,2
	2206	204	45	4832	204	32	1,4
Cd (2)	1266	77	69	3610	77	63	1,1

TABLE 14

Ribosomes	pH5 Enzyme	^{14}C Lysine (336 mCi/m mole) cpm	µg RNA	p mole/mg RNA
N	N	16864	105	226
		20691	139	200
Cd	Cd	18228	99	246
		20548	129	214
		22644	163	186

Ribosomes	pH5 Enzyme	^{3}H Tryptophan (3,1 Ci/m mole) cpm	µg RNA	p mole/mg RNA
N	N	2199	105	3
		3731	139	4
Cd	Cd	1061	99	2
		2211	99	3
		3807	129	4
		3497	129	4
		3674	163	3

TABLE 15

Ribosomes	pH5 Enzyme	^{3}H Leucine (250 mCi/m mole)		
		cpm	µg RNA	p moles/mg RNA
N (b)	N	2137	114	34
		2114	142	27
Cd (b)	Cd	2941	145	36
		2638	169	28

		^{3}H Tryptophan (3,1 Ci/m mole)		
		cpm	µg RNA	p moles/mg RNA
N (a)	N	2823	139	3
		3606	190	3
N (b)	N	2147	114	3
		1399	142	1,5
Cd (a)	Cd	2431	129	3
		2034	129	2
		1954	161	2
		3524	161	3
		3774	199	3
Cd (b)	Cd	3932	145	4
		1395	169	1,2

II 17. Estimation of Total Ribosomes and RNA

Results reported earlier (First Series of Experiments) showed higher recovery of RNA in ribosomal preparations from Cd-treated liver. In later preparations (Second Series) of purified ribosomes the difference between the two was no longer significant. Experiments were, therefore, carried out and some further determinations made concurrently with the preparation of polysomes for protein synthesis studies, in order to quantitate the RNA by analysis and the total ribonucleoprotein particles in the post mitochondrial supernatant from gradient profiles.

Blobel and Potter (1967), found sedimentation of ribosomes through 2 M sucrose to be complete after centrifugation for 24 hr (100,000 g_{av}). Results of some experiments following this method, as compared with the usual 4 hr sedimentation, are shown below. At the same time, centrifugation through 1 M sucrose for 4 hr was carried out to compare recoveries, but these preparations contained impurities, including ferritin, and the ribosomal pellets were difficult to redisperse in buffer. Results of RNA analysis are reported as mg RNA/g liver employing the standard method of Munro and Fleck (1966). (Table 16). Each preparation was from an individual liver. Recoveries were variable but indicated a slightly

TABLE 16

Ribosomal Preparation	RNA mg/g Liver 1 M Sucrose 4 hr	 2 M Sucrose 4 hr	 24 hr
N	0,82*	0,87*	1,01
N	1,29	1,02	1,30
Cd	0,83	0,66	1,11
Cd	0,63	0,63	0,82
Cd	1,01	0,80	1,02

* These values were obtained after centrifugation for 16 hr.

lower level in the case of the Cd preparations.

Some estimations of total ribosomes were made through quantitation of the area under the curve of gradient profiles by planimeter. After sedimentation of the polysomal preparation, the total supernatant was mixed and centrifuged for 3 hr at 100,000 g_{av} (Beckman Model L, 40 rotor, 39,000 rev/min) to obtain a post-polysome preparation of oligosomes. (Wunner, Bell and Munro 1966). Pellets were transferred to a small glass vessel in Medium A buffer and the preparation homogenized at low speed with the teflon pestle. It was then left to stand for 1 - 2 hr, clarified by centrifugation at 800 g_{av} and made to volume. Sucrose gradient centrifugation of aliquots of the preparation was performed in 15 - 30% sucrose as before (SW39L rotor). Profiles varied from different preparations but all showed a high proportion of monomers and dimers. Three profiles are shown in Figs. 40, 41 and 42. In Fig. 42, a Cd preparation, the high peak in the monomer region is caused partly by the absorption of the ferritin in the preparation.

Results from a pair (N and Cd) of consecutive preparations, each from two pooled livers, are given in Table 17. For the supernatants (Figs. 43 (N) and 44 (Cd) the area measured was corrected for ferritin as follows: the gradient was sampled at regular

FIG. 40.

SUCROSE GRADIENT. SUPERNATANT N.

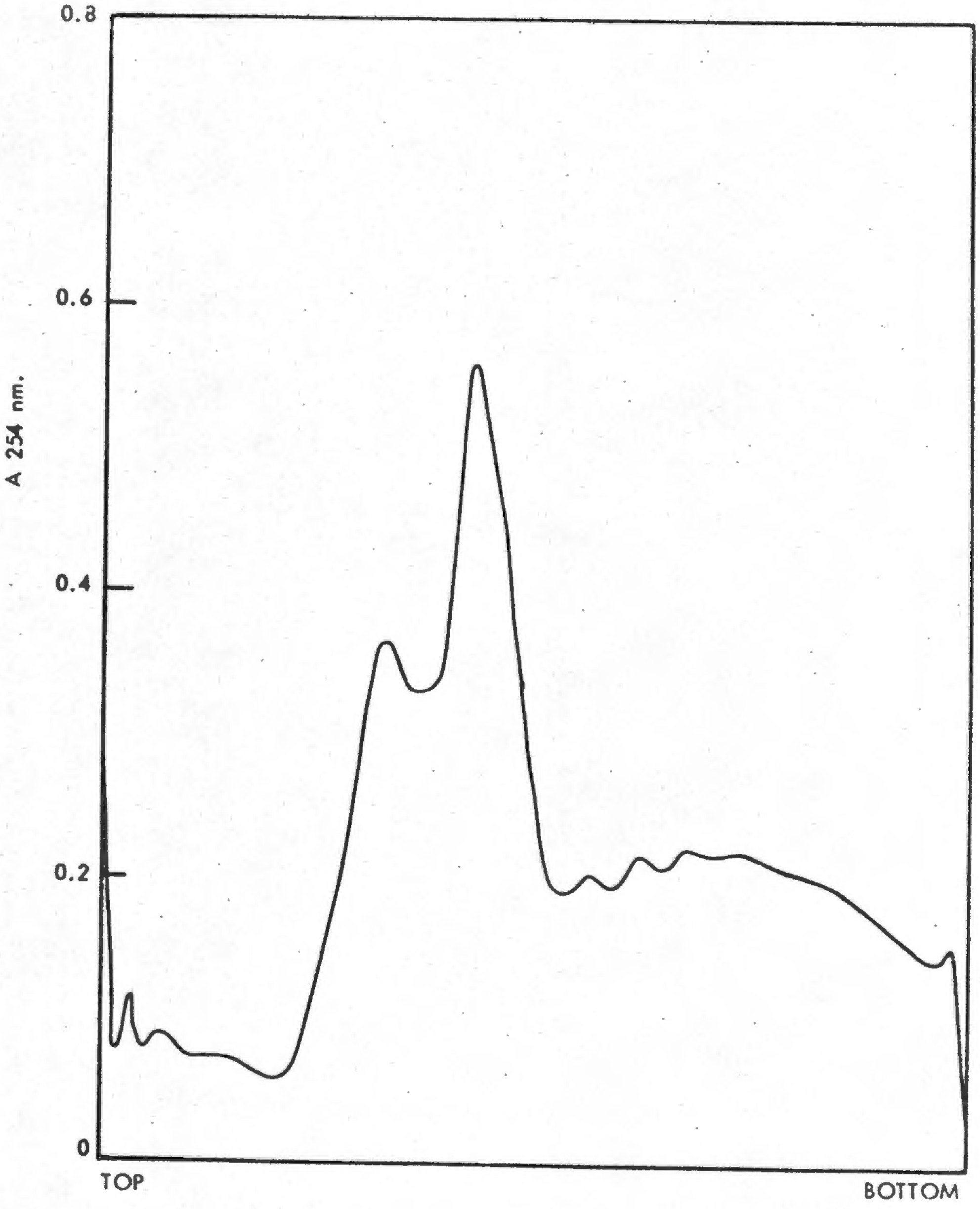


FIG. 41

SUCROSE GRADIENT. SUPERNATANT Cd.

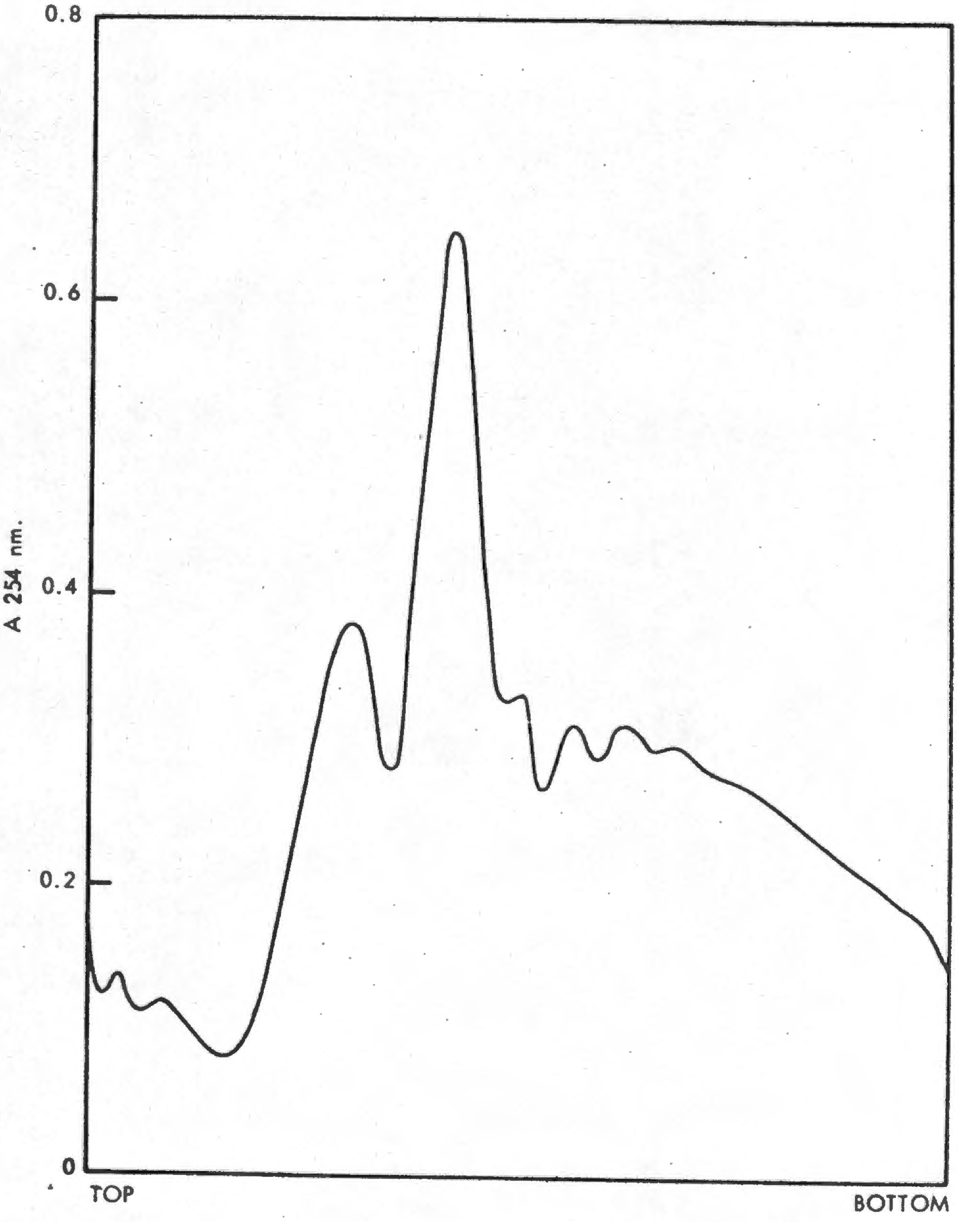


FIG. 42

SUCROSE GRADIENT SUPERNATANT Cd.

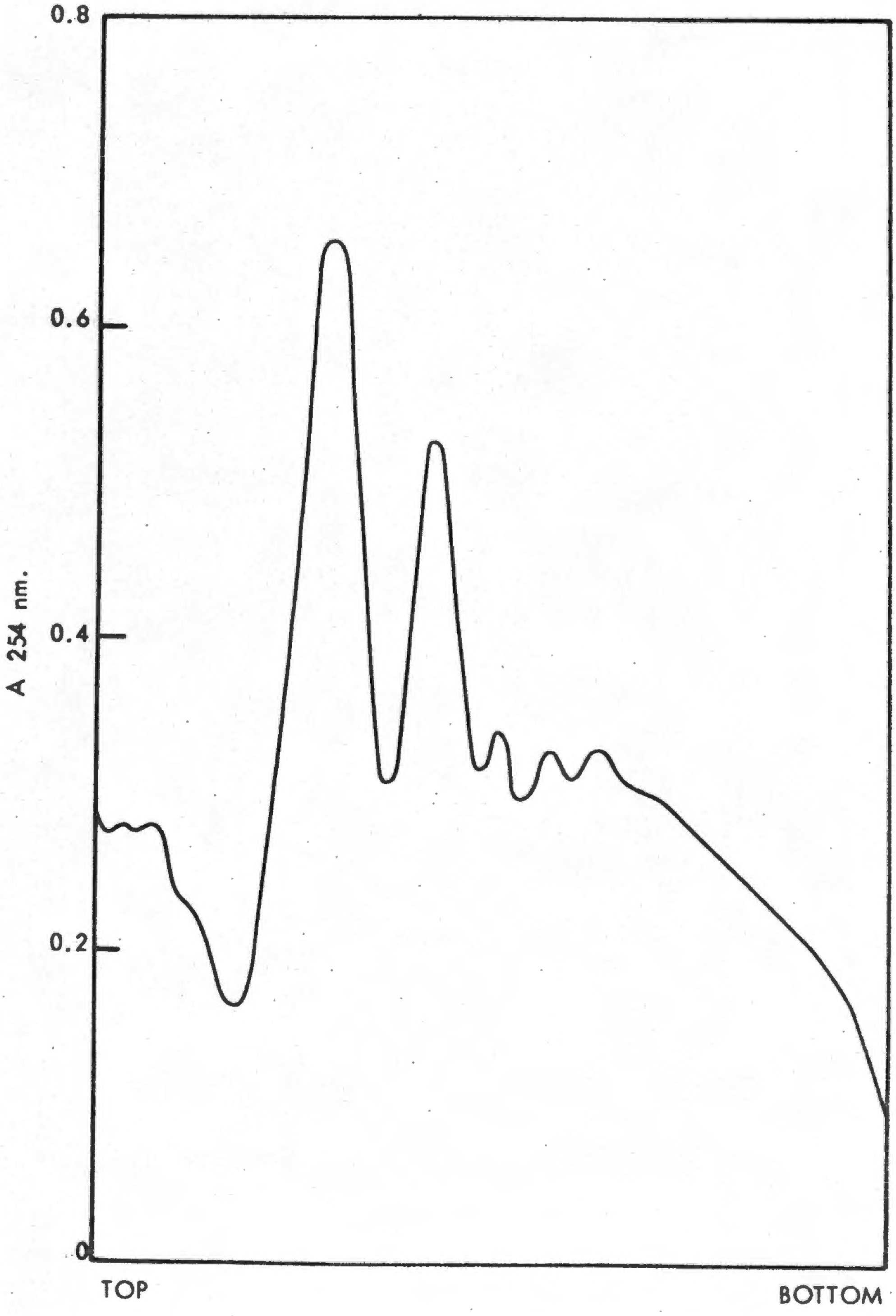


FIG. 43.

SUPERNATANT GRADIENT. SAMPLED. N.

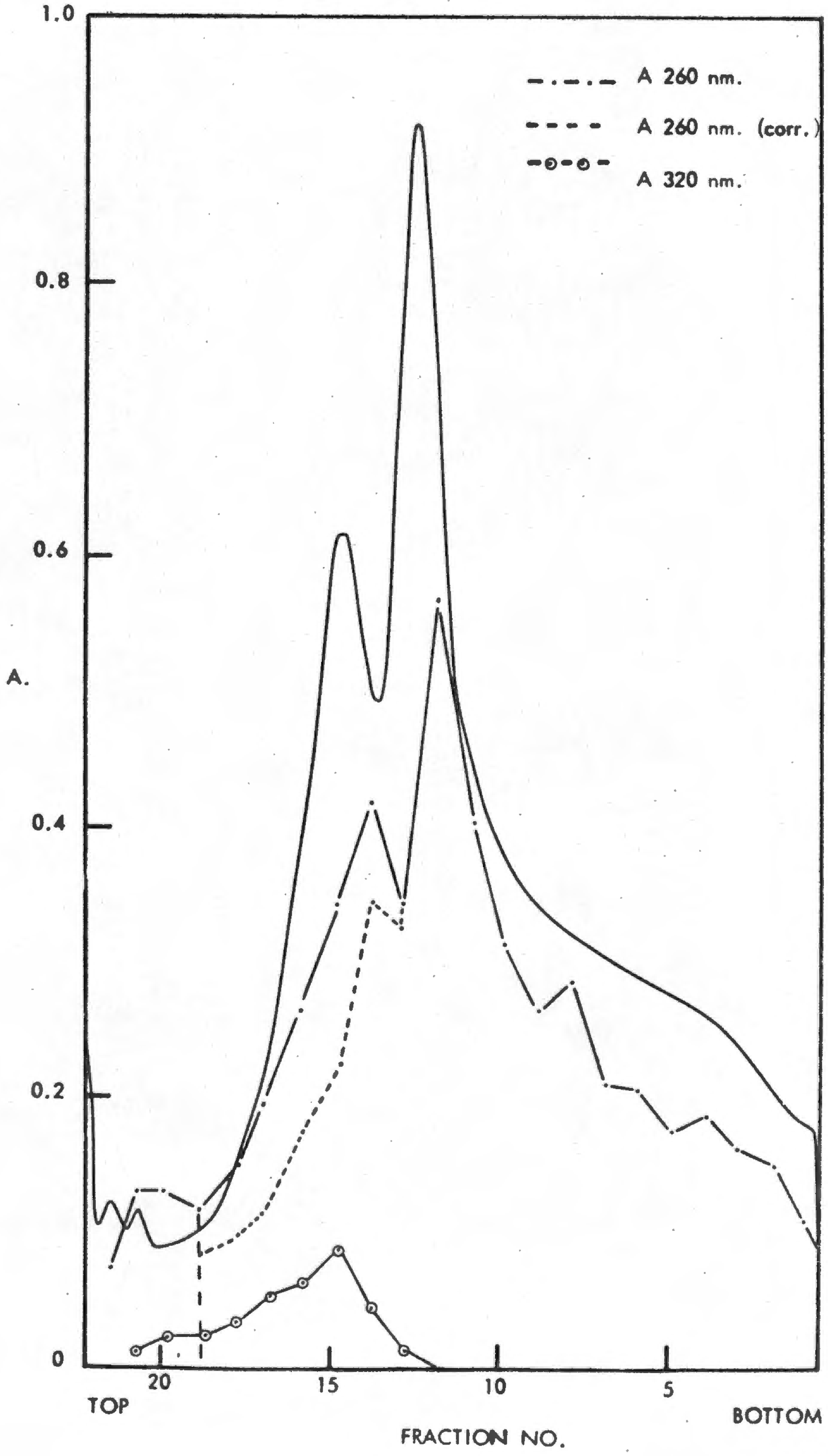


FIG. 44.

SUPERNATANT GRADIENT SAMPLED Cd.

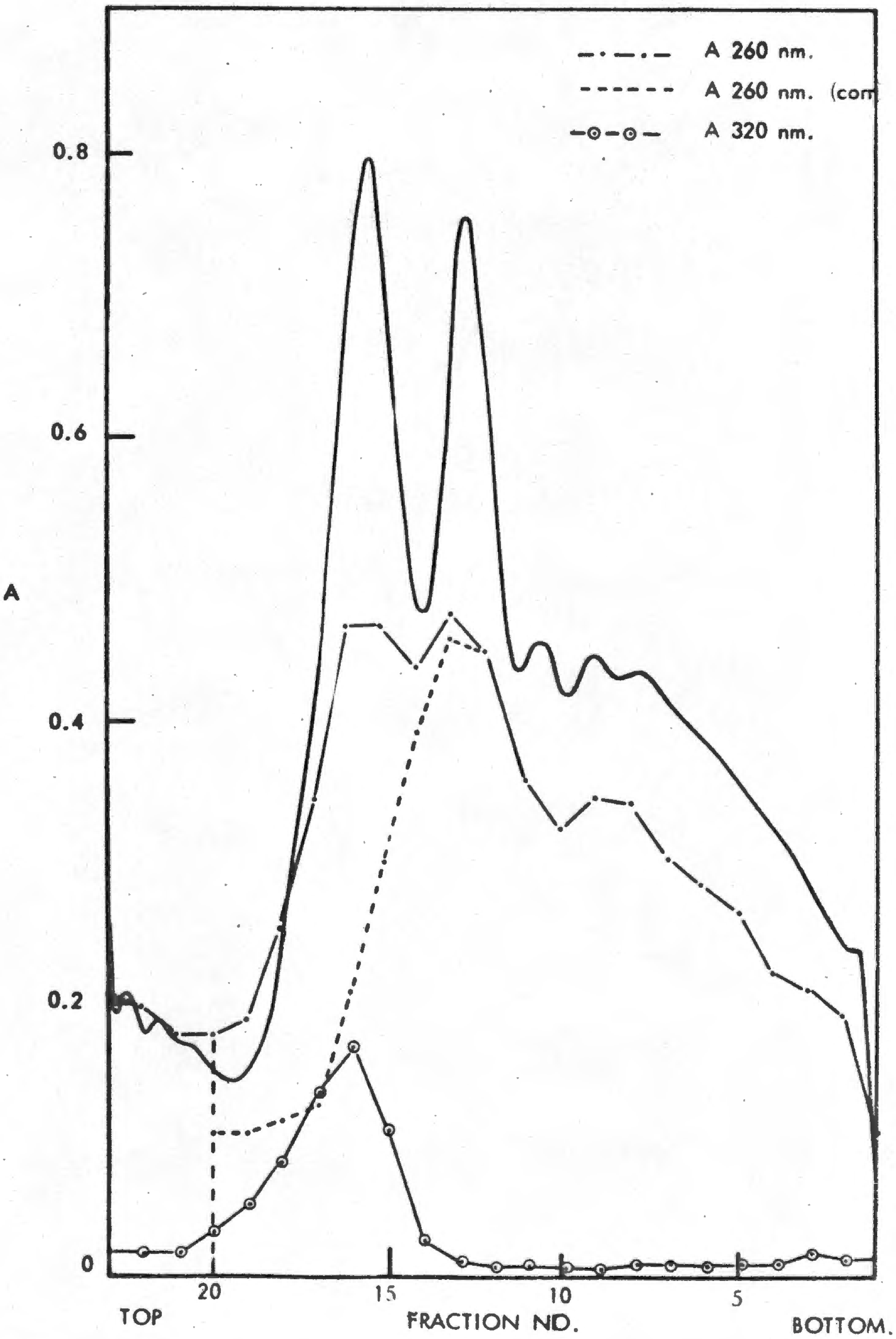


TABLE 17

Estimation of Total Ribonucleoprotein Particles from Sucrose Gradients

Ribosomal Preparation		Gradient Area cm^2	Ferritin Correction Factor	Total Preparation Area(Corr.) cm^2	Area cm^2/g Liver
N	Polysomes	39,6	-	672	262
	Supernatant	105,8	0,903	572	222
				Total:	484
Cd	Polysomes	61,9	-	866	274
	Supernatant	124,4	0,835	622	197
				Total:	471

intervals, the fractions diluted, and absorption at 260 $_{nm}$ and 320 $_{nm}$ measured. Graphs were drawn and the absorption (A320 $_{nm}$ x 1,59) subtracted from A260 $_{nm}$ to give a corrected curve. The reduction in area was used to calculate a factor for correction of the area of the original curve. The total area - polysomes + supernatant - is expressed as cm^2/g liver. Again a similar or slightly lower recovery was found for the Cd preparation.

During some later experiments when polysomes were rapidly sedimented in the 60 Ti rotor, time was available for recording profiles of the total ribonucleoprotein particles in the PMS, a method for comparison used by Brubaker, Lucier and Klein (1971) in studies on mercury intoxication. With the SW50.1 rotor, recording of gradient patterns could be completed within 4,5 hr of the start of the experiment. Samples (300 or 500 µl) of the PMS, to which DOC had been added, were layered over 500 µl. Medium A on 15 - 45% linear sucrose gradients and centrifuged at 234,000 g_{av} (Beckman L2-65B 50,000 rev./min SW50.1 rotor) for 30 min at 2°C. Gradients were eluted and A254 $_{nm}$ recorded as before (Fig. 45). Gradient profiles of the PMS were also recorded on 1 ml or 2 ml samples of the DOC-treated PMS centrifuged at 52,400 g_{av} (Beckman L2-65B 20,000 rev./min SW27.1 rotor) for 17 hr at 2°C on 15 - 45%

gradients. These profiles were similar to those obtained on the 50.1 rotor but resolution was improved and the monomer peak was better resolved from background absorption. A broad shoulder sedimenting more slowly than the monomer peak could represent ribosomal subunits in the preparation. Typical gradients are shown in Figs. 46 (2 ml) and 47 (1 ml). Variations in elution and in baseline due to background absorption made quantitation by measurement of areas inaccurate but, where comparison could be made, results were similar to those discussed above, N and Cd PMS had similar profiles.

Finally, estimations of total RNA by analysis are reported as mg RNA/g liver in Table 18. Results were variable but the mean values for the PMS, either by direct analysis or from RNA content of polysomal preparations and supernatants, did not vary significantly between N and Cd-treated liver. Only an average of 40% of the RNA in the PMS was recovered in the total ribosomal preparations. Values for liver homogenates exhibited more variation but again N and Cd averaged similar RNA content. Losses in the nuclear and mitochondrial pellet were high, only about 40% being recovered in the PMS. The few data available for RNA content of the pellet show higher values in the case of the Cd-treated liver. It is possibly here that differential losses of RNA between N and Cd preparations may have occurred. Cd-treated liver is more difficult to homogenize than normal and insufficient fragmentation of endoplasmic reticulum would result in large pieces sedimenting in the pellet. Double centrifugation in the preparation of the PMS and washing of the nuclear pellet in the First Series of Experiments increased the recovery of RNA in the ribosomal preparations. Variable losses occurring from centrifugation of the homogenate, in the following DOC treatment of the PMS and up to the final separation of the ribosomes from the remaining supernatant, would probably account for the difference between the RNA content of N and Cd-treated preparations in the First Series of Experiments.

FIG. 45

PMS GRADIENT PROFILE SW 50.1 ROTOR.

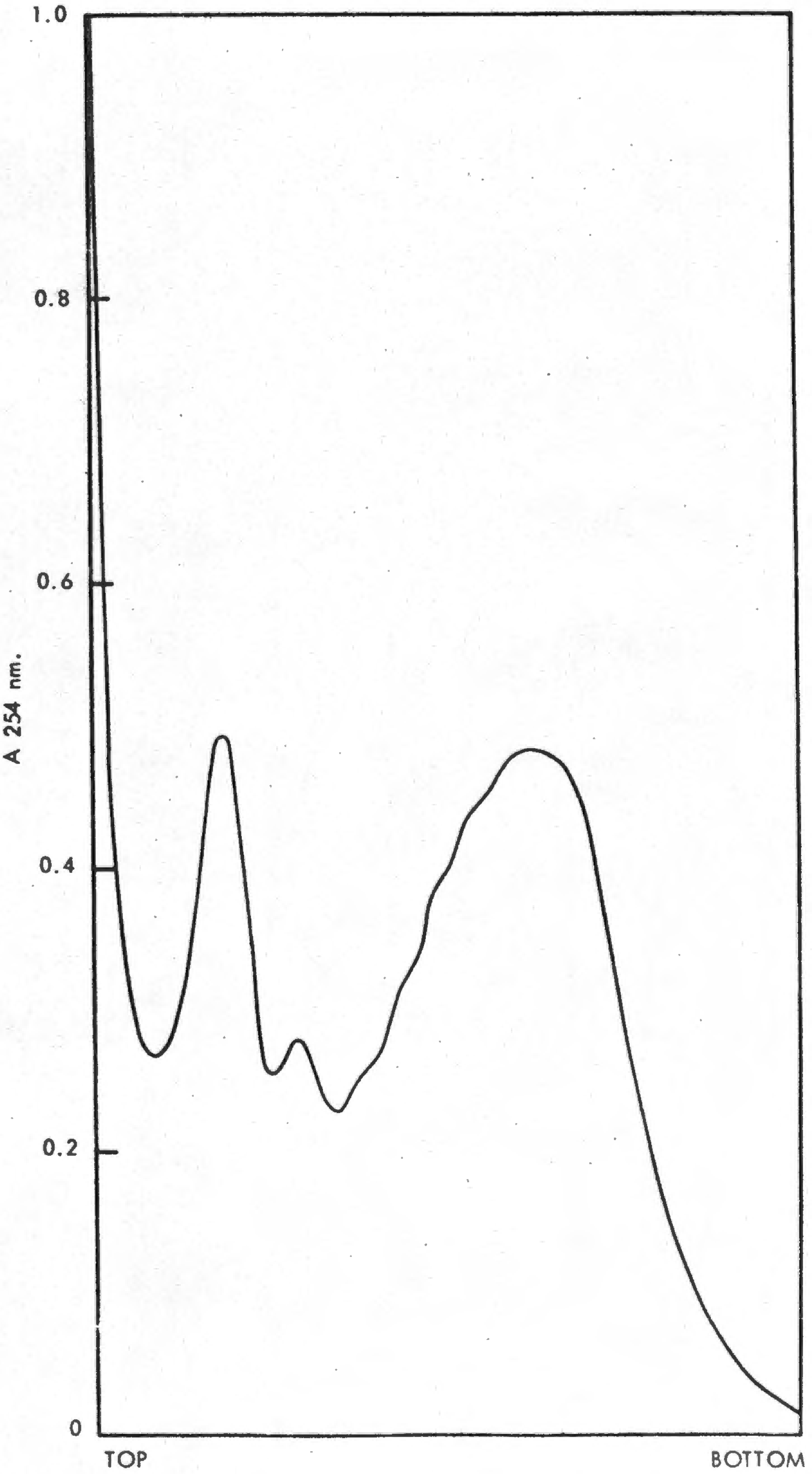


FIG. 46

PMS GRADIENT PROFILE 27.1 ROTOR 2 ml. SAMPLE.

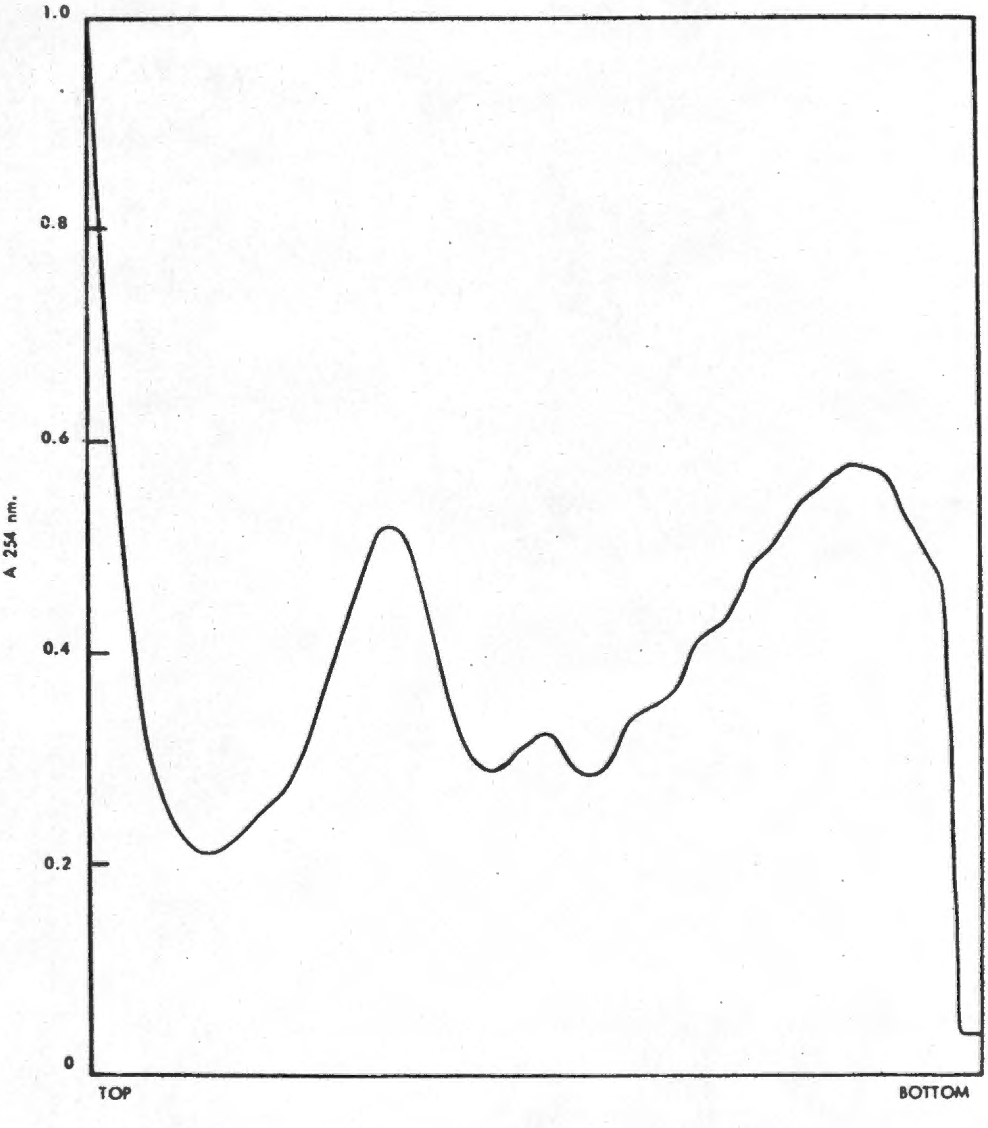


FIG. 47.

PMS GRADIENT PROFILE 27.1 ROTOR 1 ml. SAMPLE.

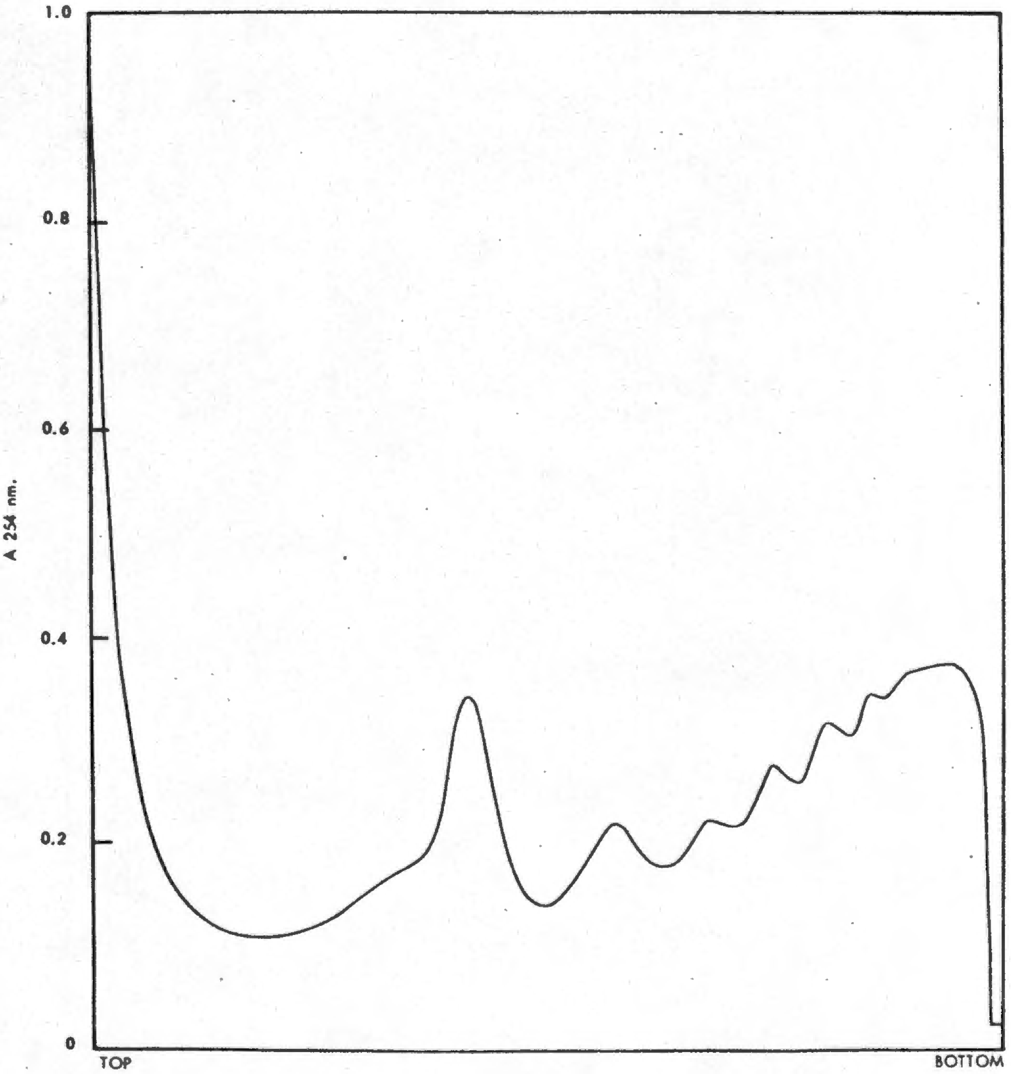


TABLE 18

RNA mg/g Liver

Preparation		Homogenate	PMS	Pellet (Nuclei, + Mitochondria)	Ribosomes + Supernatant
Cell Sap	N	6,40	2,23		
	Cd	6,06	2,19		
Ribosomes	N				1,83
	Cd				1,40
	N	6,60	2,66		0,92
	Cd	5,13	2,47		1,16
	N	-	2,70	4,38	1,61
	Cd	-	3,42	5,08	1,71
	N	8,27	2,78	4,78	1,03
	Cd	7,77	2,51	6,48	0,90
	N	6,58	2,84	4,43	1,00
	Cd	8,13	2,94	5,88	1,32
Mean	N	6,96	2,64	4,53	1,27
±s.d.		0,87	0,24	0,21	0,41
Mean	Cd	6,77	2,70	5,81	1,29
±s.d.		1,41	0,48	0,70	0,29

II 18. Separation of RNA from Polysomes

To compare the constituent RNA molecules of polysomes prepared from normal and from Cd-treated rats, phenol treatment was employed to remove the protein, and the isolated RNA was separated by polyacrylamide gel electrophoresis. This is a technique which provides high resolution separation of RNA of different molecular sizes. Small RNA molecules obtained by digestion of reticulocytic ribosomes by means of pancreatic ribonuclease have been separated in this way by Gould (1966). Loening (1967), developed the method for high molecular weight RNA and separated the RNA from several sources, including reticulocyte polysomes, by this method. Further studies with gels of different concentrations enabled Loening (1969), to measure molecular weights of different RNA species. It has been demonstrated by Harley, White and Rees (1973), Loening (1969) and Groot, Aaij and Borst (1970), however, that conformational effects can have a marked influence on electrophoretic mobility, and calculations of molecular weight must take into account the structural class to which the RNA belongs and the extent to which it is folded into double-helical regions.

II 18. (a) Phenol Extraction

Phenol is an efficient deproteinizing agent and is used, in conjunction with 8-hydroxyquinoline, which improves RNA yield and inhibits ribonuclease, to remove the proteins from RNA (Kirby 1968). RNA is sensitive to ribonucleases even in trace amounts so extraction procedures must be carried out at low temperature and in the presence of nuclease inhibitors. The clay, bentonite, is an efficient inhibitor as is sodium dodecyl sulphate (SDS), a detergent which inhibits nucleases without interaction with nucleic acids and is also a powerful protein denaturant. EDTA is added to the extraction buffer as a chelating agent to remove multivalent cations which cause RNA aggregation, which also occurs if the monovalent cation concentration rises above 0,01 M.

In the procedure followed, polysomes (50 A.U.) prepared as before from normal and Cd-treated liver were dispersed in a sodium acetate buffer containing NaCl 50 mM, EDTA 0,1 mM, sodium acetate pH 5,2 10 mM and SDS 1%. Bentonite, 0,1% was added to the buffer just before use. An equal volume of phenol, water saturated, and containing 1% 8-hydroxyquinoline was added, and the mixture shaken thoroughly, cooled, and centrifuged at 3,000 rev./min for 10 min. The upper, aqueous phase, containing the RNA, was carefully aspirated without disturbing the denatured protein at the phenol interface. This solution was then extracted twice with chloroform saturated with water, the chloroform separated by centrifugation and discarded. To the final aqueous phase 2,5 volumes of ethanol containing 4% potassium acetate were added, and the suspension of RNA left overnight at -10°C.

II 18. (b) Polyacrylamide Gel Electrophoresis

The principle of the method and the procedures have been described by Richards, Coll and Gratzer (1965), Gould (1966), and Loening (1967). Gels are prepared by polymerization of acrylamide and cross-linked with a small proportion of N,N'-methylene bisacrylamide. The gel acts as a molecular sieve through which RNA molecules travel distances, under a constant applied voltage, determined by their molecular size and shape. Immediately after electrophoresis gels are fixed in 15% acetic acid to prevent diffusion of the zones. The use of purified acrylamide to diminish background absorption enables gels to be scanned in the ultraviolet spectrophotometer, when bands at 260 $_{nm}$ can be recorded, these being a measure of the concentration of the different RNA species. Gels can also be stained with Toluidine Blue for visualisation of the bands, or sliced, if the RNA has been labelled for counting of the radioactivity of the bands.

For these experiments gels were run in a continuous buffer system containing tris-HCl pH 7,5 30 mM, NaH_2PO_4 30 mM, EDTA 1 mM and 10% glycerol (to facilitate slicing). SDS was omitted from this running buffer as it interferes with staining but 1% SDS was added to the dissolving buffer. This consisted of running buffer diluted 5 times to accelerate initial migration with 15% glycerol for rapid settling on the gel. Gels were prepared from a 12% stock solution of acrylamide (12 g) containing bisacrylamide (0,6 g), both reagents being recrystallised, and made to 100 ml in water. For a 2,6% gel, 8,67 ml of this solution was mixed with electrophoresis buffer (4 ml) and glycerol (4 ml). Polymerization was started by adding ammonium persulphate (10%, 0,34 ml) and NNN'N'-tetramethylethylenediamine (10%, 0,34 ml) as catalyst, the whole being brought to 40 ml. Solutions were degassed before use to remove oxygen which inhibits polymerization. Gels were poured in cylindrical tubes 120 mm long and 7 mm in diameter, and allowed to gel overnight at 4°C. They were cleared for 30 min at 125V before running.

A portion of the RNA suspension was centrifuged at 14,000 g_{av} (Beckman Model J-21, 13,000 rev./min JA-20 rotor) for 15 min at 2°C. The supernatant was poured off, the pellet drained, a small volume of dissolving and layering buffer added, the A260 $_{nm}$ measured and aliquots containing 40 - 50 µg RNA layered on each gel. Electrophoresis was carried out at 125V for 3 hr in an apparatus cooled with tap water. The length of the gels was measured and they were extruded into buffer for scanning at 260 $_{nm}$ in a Gilford Recording Spectrophotometer (Model 2400). They were fixed in 15% acetic acid and stained with Toluidine Blue (2% in 15% acetic acid), destained in the same solvent and photographed (Plate I). Scans recorded are shown in Figs. 48 (N) and 49 (Cd). The typical peaks for 18S RNA (A) and 28S RNA (B) are evident, separated by minor bands. The patterns and relative proportions of the bands are similar for the N and Cd preparations. Low MW species are not retained on gels of this strength.

FIG. 48.

RIBOSOMAL RNA 2.6% GEL SCAN N

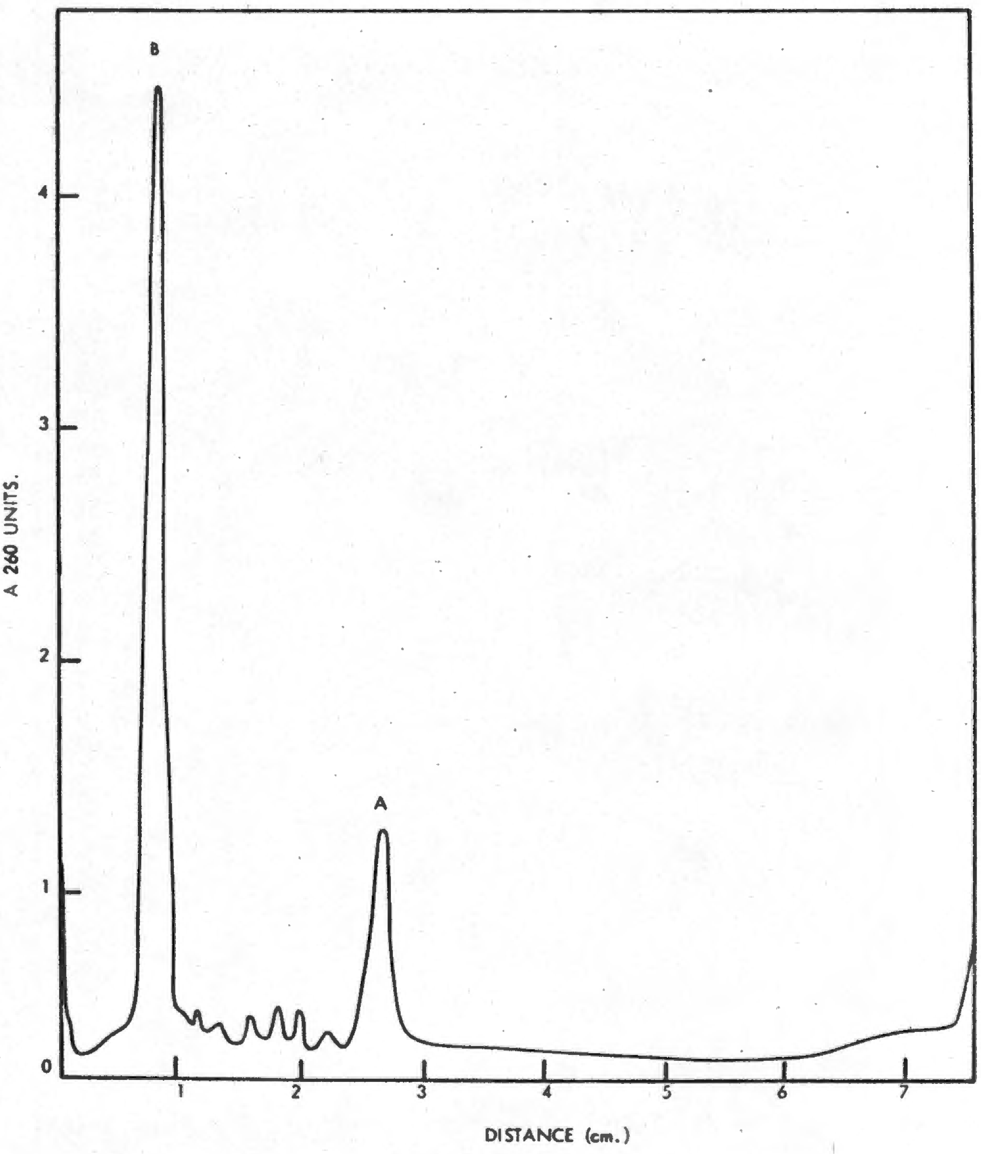


FIG. 49.

RIBOSOMAL RNA 2.6% GEL SCAN Cd.

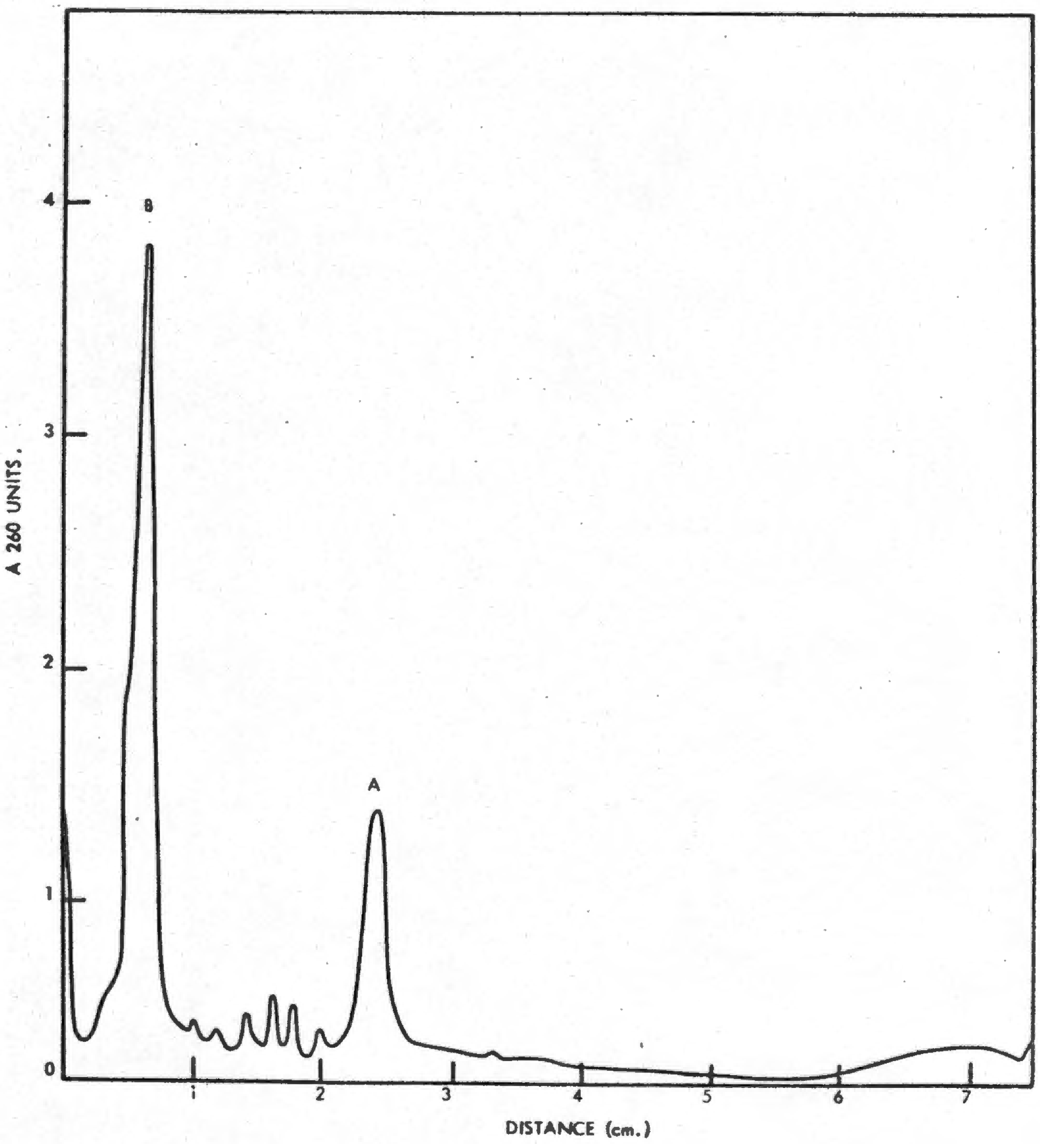
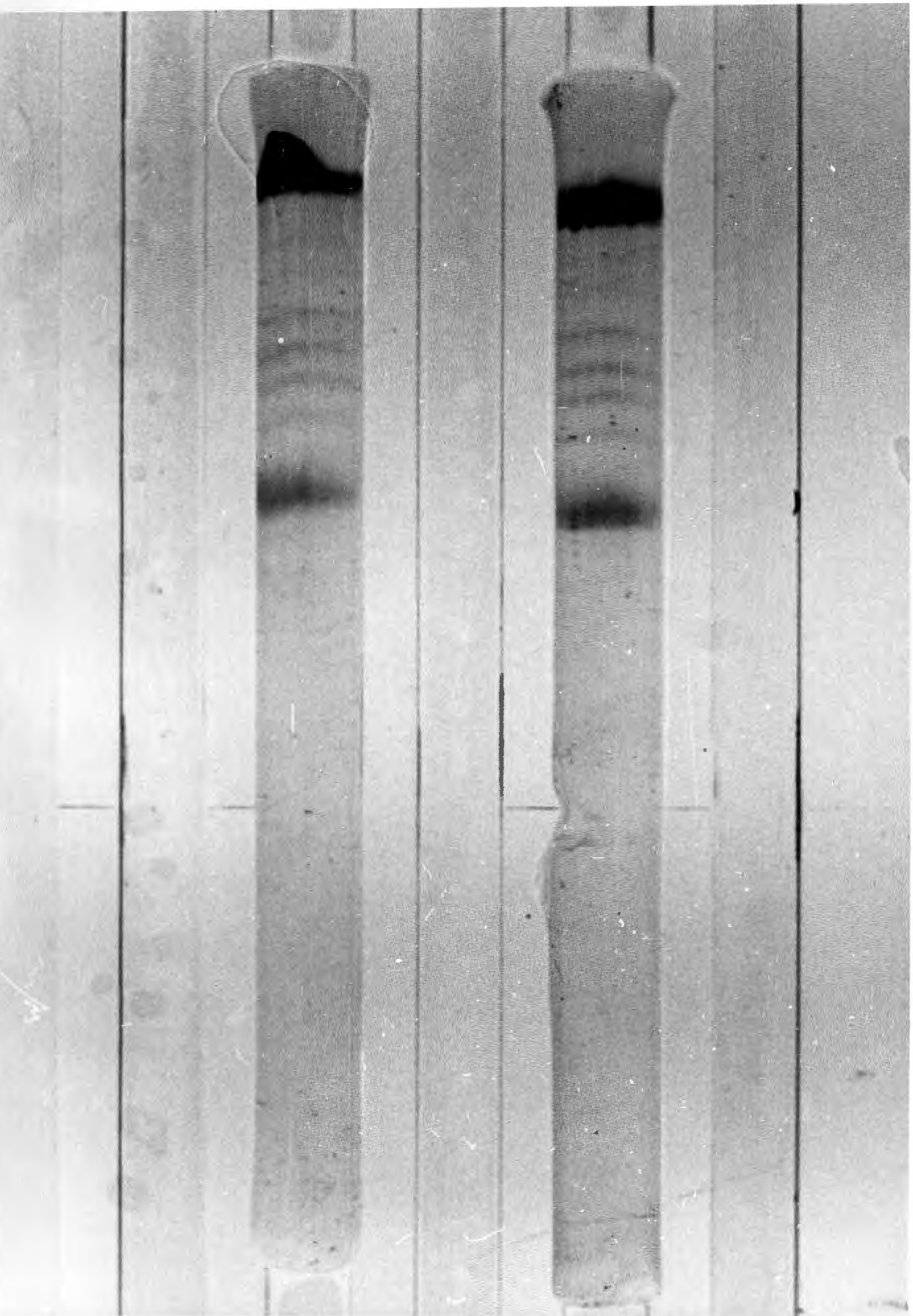


PLATE I

POLYACRYLAMIDE ELECTROPHORESIS OF RIBOSOMAL RNA

Left hand gel - Cd preparation

Right hand gel - N preparation



II 19. Tissue Culture Experiments

Cells in tissue culture, the HeLa cell line CCL2, have recently become available in this laboratory and some experiments have been carried out to study the effect of exposure of these cells to varying concentrations of Cd. Cultured cells offer the advantage of a simpler mammalian system in which immediate effects can be observed morphologically and the Cd concentration accurately controlled. The HeLa line is a human cell line from a carcinoma of the cervix which is widely used, it was originally isolated and described by Gey, Coffman and Kubicek (1952).

Cells were grown in Eagle's minimal essential medium supplied with 10% (v/v) calf serum, glutamine and antibiotics, and buffered with 2% (w/v) $NaHCO_3$. Monolayer cultures were grown on cover slips in test tubes and used one day after subculture. They were healthy and semiconfluent at the start of the experiment as judged by phase-contrast microscopy. Cd was added as $CdCl_2$ in aqueous solution. The medium was poured off and replaced with 2 ml fresh medium to which 100 µl of $CdCl_2$ solution of the requisite concentration was added to give final concentrations of $10^{-4}M-10^{-8}M$. The control culture was treated in the same way, 100 µl water being added instead of the $CdCl_2$.

Photomicrographs of the monolayers are shown in Plates 2 - 6.

After 6 hr, in the culture in which the Cd concentration was 5×10^{-4}M a cytotoxic effect was seen (Plates 3 and 4). Marked morphological changes and nuclear and cytoplasmic degenerative effects could be discerned. Cultures in which the Cd concentrations were 5×10^{-5}M - 5×10^{-8}M showed normal growth.

At 24 hr the cells treated with 5×10^{-5}M Cd now showed extensive morphological changes. There were no mitoses, cells were rounding-up and falling off the cover slip (Plate 5), and possible nucleolar changes could be seen (Plate 6). Lower concentrations of Cd, 5×10^{-6}M - 5×10^{-8}M did not affect cell growth over a 24 hr period and the cultures appeared similar to the control.

The control (Plate 2) had the typical appearance of a confluent healthy monolayer with several mitoses.

PHASE CONTRAST PHOTOMICROGRAPHS
of HeLa CELLS.
MAGNIFICATION X 400

Control
PLATE 2

Cells treated with Cd (5×10^{-4}M)
for 6 hr
PLATES 3 AND 4

PLATE 2

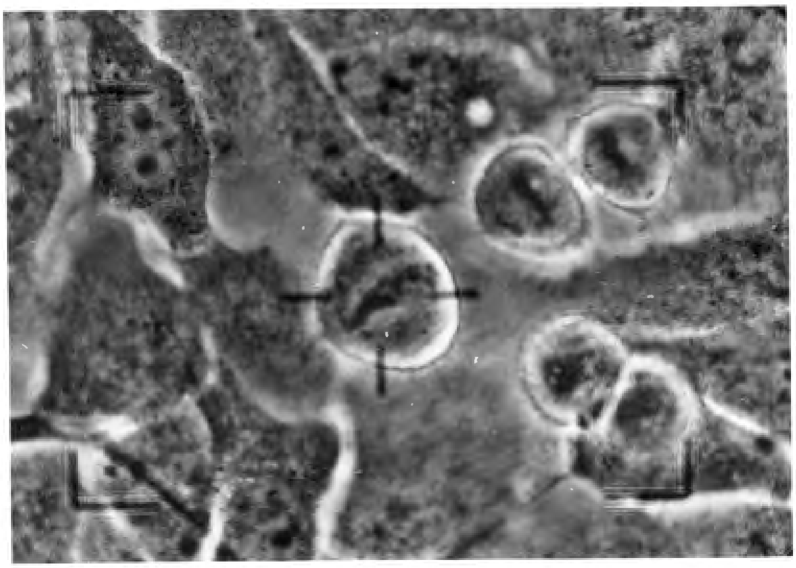


PLATE 3

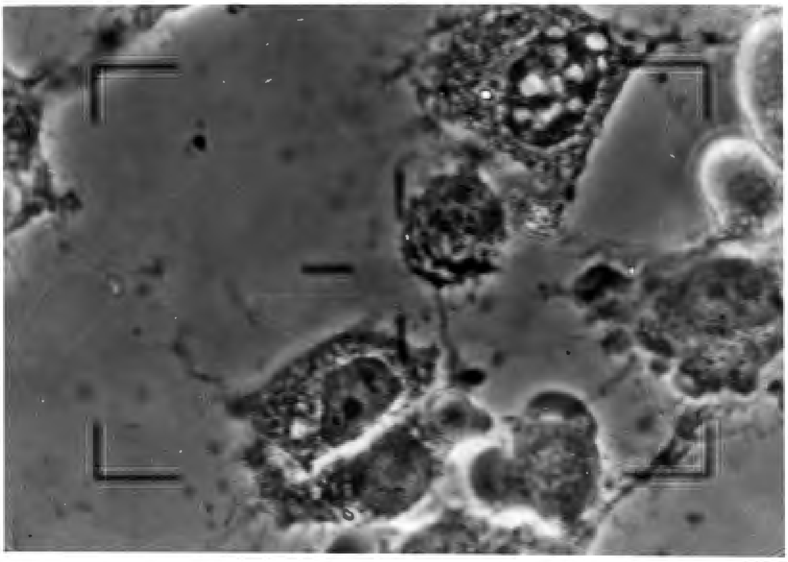
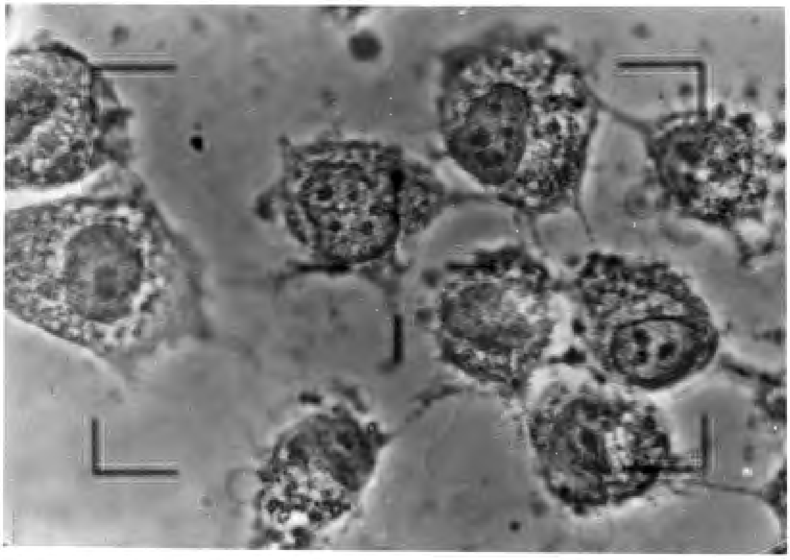


PLATE 4



PHASE CONTRAST PHOTOMICROGRAPHS
OF HeLa CELLS.
MAGNIFICATION X 400

Cells treated with Cd (5 x 10^{-5}M)
for 24 hr
PLATES 5 AND 6

PLATE 5

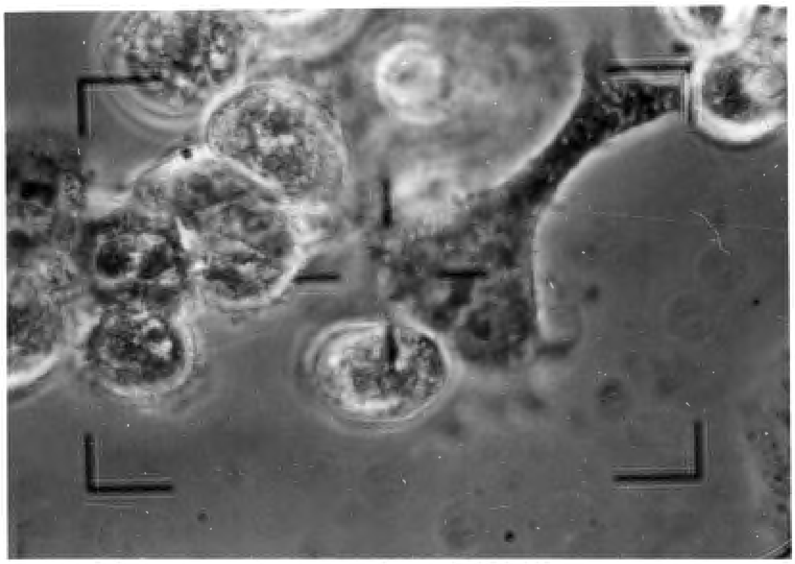
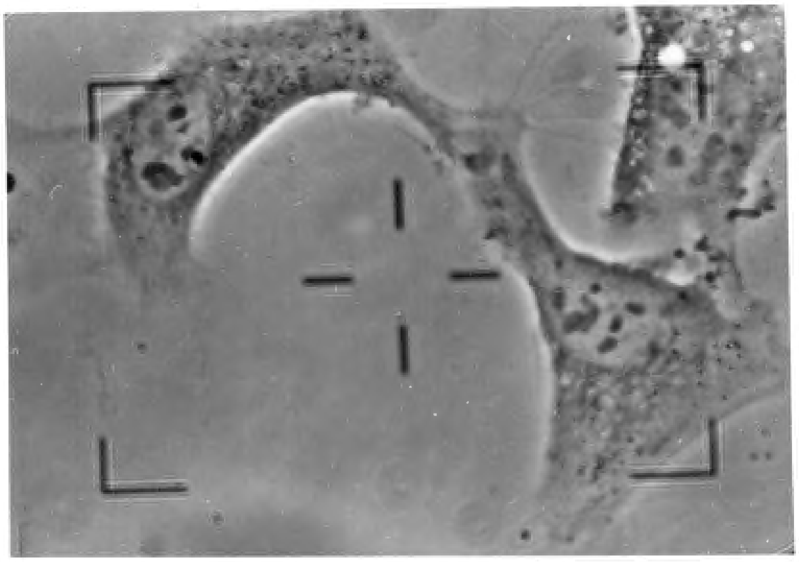


PLATE 6



PART III

DISCUSSION

The regime adopted throughout this investigation for intoxication of rats was based on previous animal studies in this laboratory (Kench and Sutherland 1966), and the same as that carried out during experiments demonstrating the induction of methallothionein by Cd. (Diamond and Kench 1974c). During the course of intoxication the amount of metallothionein progressively increased and at the same time its Cd content rose steadily, reaching a maximum of 2 - 2,5 µg/100 µg purified protein. A high proportion of the Cd was bound to this protein and separation of proteins by Sephadex chromatography demonstrated relatively little binding to other protein fractions. In the experiments reported here, Cd in liver tissue was found to be 0,1 mg/g (10^{-3}M) and presumably most of this is present in a bound form. The figure for Cd in the cell sap enzyme was 5 ng/100 µg protein and metallothionein would be responsible for binding of Cd in this preparation. The pH5 enzyme was observed to contain only one tenth of this Cd level (0,4 ng/100 µg protein) so Cd, and possibly also metallothionein, must be removed in the supernatant during the double precipitation at pH 5,2. The remaining trace amount of Cd still produced an inhibitory effect. The ribosomes retained Cd at the higher level (7 ng/100 µg RNA) and metallothionein is unlikely to be present in these preparations. Purification by sedimentation through sucrose would be expected to remove contaminating proteins and unbound Cd, that remaining being presumably bound to the protein or RNA.

Studies of metal binding lead to this conclusion. Cd has been placed among metals that bind to nucleotide bases and destabilise DNA by Eichhorn and Shin (1968) and Hg could be included with these metals. Hg complexes with nucleotides mainly by bonding to N atoms which bind it more strongly than oxygen but both types of binding have been demonstrated as well as interaction with SH groups. In these respects Cd would be similar to Hg. Recent data from nonlethal Hg intoxication studies (10^{-3}M in drinking water for 1 month) indicated *in vivo* binding of Hg to be preferentially to protein, as isolated DNA was found to be unchanged by several criteria (Bryan, Guy and Hardy 1974). The possibility of Cd binding to sites both on protein and RNA must therefore remain, and exposed regions of RNA on the ribosomal surface demonstrated by Hartman et al (1970) may be available for interaction with Cd.

The relative enlargement of the liver and general loss in weight as compared with control animals are typical features in these intoxication studies. Inhibition of growth in the rat by Cd and other metals, including Zn, Cu and Pb, has been reported by Everett and Holley (1961). When compounds containing high levels of these individual metals were included in the diet significant inhibition at 10^{-4}M concentration was noted of the *in vitro* incorporation of ^{14}C leucine into protein.

Even though histologically the normal and Cd-treated livers were similar, there was a marked difference in ease of homogenization, still noticeable to a small extent after use of the tissue press, and this complicated the isolation of truly comparative preparations. It was also noticed that degradative enzymes, probably both proteases and nucleases, were more readily released from the Cd-treated liver into the preparations, indicating fragility of cellular constituents. Such problems as possible rupture of nuclei, variable fragmentation of endoplasmic reticulum and subsequent losses in pellets during centrifugation remained a confusing issue throughout the studies, particularly with regard to RNA recovery. Cd interaction with ER membranes through binding to SH groups could influence release of ribosomes by DOC. Separation of ribosomes free in the cytoplasm for comparison with membrane-bound ribosomes, without the use of detergent, (Bloemendal et al 1967), would make an interesting comparative study in this regard. Differential recovery of ribosomes from membranes by deoxycholate can therefore also be a complicating factor. The significance of these variables has been discussed by Blobel and Potter (1967). For the preparation of active ribosomes it is necessary to strike a balance between quantitative recovery on the one hand and degree of purification on the other. This was demonstrated in the First and Second Series of Experiments. The higher recovery of ribosomes in the First Series was offset by the contamination by proteases and nucleases resulting in isolation of preparations of only low activity in protein synthesis. The Cd-treated ribosomes had a lower protein content, more variable 260 $_{nm}$/280 $_{nm}$ ratios and in some cases contained more oligosomes. Preparations from normal animals also had a low proportion of polyribosomes in these experiments.

Improved preparations in the Second Series, purified by sedimentation through 2 M sucrose, yielded ribosomes which were more closely comparable and similar in protein content and ultraviolet spectral characteristics. Improved sampling procedures for sucrose gradients made comparison of polysome profiles possible. It was at first thought that the distribution of sizes of polysomes from Cd-treated rats might show some difference from the normal pattern. It soon became clear, however, that there was considerable variation in profiles even in pairs of strictly comparable experiments both in normal and Cd-treated animal preparations. Again the pattern for the treated preparations was more variable and a lower content of polysomes was usually noted. Normal preparations also contained a low proportion of polysomes compared to literature reports (Wettstein et al 1963). The main characteristics of this series of preparations were the small but constant proportion of monomers and the high content of dimers. A prominent dimer peak has been frequently reported as a characteristic feature of disturbances in metabolism in studies on protein synthesis in the rat, associated with a lower proportion of polysomes. An abnormally high dimer concentration was found in profiles of ribosomes isolated from various types of hepatomas by Webb, Blobel, Potter and Morris (1965). A similar high dimer concentration was then found to be a feature of preparations from rats treated with CCl_4 or actinomycin D (Webb and Potter 1966). This disaggregation caused by actinomycin D was found to be reversed by subsequent administration of hydrocortisone (Enwonwu and Munro 1971), demonstrating that changes in synthesis of RNA do not cause the change in profile observed. A similar effect was recorded in the case of diabetic rats by Pilkis and Korner (1971), who found that treatment with insulin reversed the dissociation. This effect had also been reported both in diabetic rats before and after insulin treatment, and in fasted animals, when disaggregation of polysomes was reversed by feeding with glucose. (Wittman, Lee and Miller 1969). Induced hypothermia caused the same changes in rat brain polyribosomes (Raghupathy, Peterson and Ko 1971). These authors found the disaggregation to be prevented by previous treatment of the rats with cycloheximide, which blocks protein synthesis by interacting with the ribosome.

The question as to whether this type of dimer was present in vivo in these various abnormal conditions or appeared during isolation procedures was resolved by the work of Reader and Stanners (1967) who presented evidence that the dimer was an artefact not associated through mRNA and formed at low temperature during isolation procedures. As a result of further studies Stanners et al (1971) and Elicieri (1972) found that whereas monomers from mouse-hamster hybrid cells contained only mouse-type 28S RNA, dimers had only hamster-type. They were thus able to demonstrate that dimerisation takes place on the 28S RNA and therefore through the 60S subunit.

In some of these studies on polysome gradients high monomer peaks were also observed. Brewer et al (1969) found that in hypophysectomized rats profiles of isolated ribosomes exhibited a high proportion of oligosomes but that after treatment of such rats with growth hormone the profiles reverted to the normal pattern. They compared protein synthesizing capacity with polysome profiles and also investigated the ribonuclease content of the preparations. The "active" ribonuclease in the preparations was assayed and then the total ribonuclease assessed in the presence of p-chloromercuribenzoate which inactivates any ribonuclease inhibitor which may be present. The total and the "active" ribonuclease could be related to the profiles recorded. Those with a high polysome content had lower total nucleases and no active fraction. Increases in levels of total and "active" ribonucleases in the preparations were associated with shifts to smaller size classes, and when ribonuclease was highly active mainly monomers were isolated.

The experiment comparing sedimentation of ribosomes through 1,5 M and 2 M sucrose would be seen to illustrate this variation in ribonuclease activity. In both cases the preparations clearly contained nucleases as on warming to 37°C most of the polysomes disaggregated to oligosomes and monomers accumulated. (Fig. 13). This is similar to the mild treatment by crystalline ribonuclease illustrated in Fig. 9.

Dimers not attached by mRNA are stable at 0°C and resistant to low level ribonuclease activity, but on warming they dissociate to monomers. Apparently tn the 2 M sucrose preparations the ribonuclease present is not sufficiently active to break down the dimers to monomers even after long storage at 0°C. Sedimentation through 1,5 M sucrose resulted in contamination of the preparations with a more active form of ribonuclease to which the dimers were no longer resistant. It would thus seem that if ribonuclease activity is restricted below a certain level polysomes are degraded to dimers which are then completely resistant to further attack even after long exposure provided the temperature remains at 0°C. N and Cd ribosome preparations were not found to differ on storage at 0°C in this respect, usually a high proportion of dimers accumulated, but both monomers and dimers increased in some preparations.

The small but constant proportion of monosomes isolated in the second series preparations would presumably be ribosomes attached to mRNA. Such ribosomes are protected by the mRNA, or polypeptide, or both, and are resistant to dimerisation. They could either be ribosomes attached to varying lengths of mRNA and polypeptide, i.e. programmed ribosomes, or they could have resulted from the fragmentation of polysomes by ribonuclease. These have been called complexed ribosomes (Davis 1971). Such single ribosomes can be distinguished from free or run-off ribosomes, which do not have attached mRNA or polypeptide, by their greater resistance to dissociation into subunits. Lawford (1969) demonstrated that incubation of liver ribosomes with puromycin, which freed them from attached mRNA and peptidyl-tRNA, resulted in their dissociation into active subunits when the Mg^{2+} concentration was lowered to 1 mM. Programmed liver ribosomes only dissociated in 10^{-5}M Mg^{2+} and then remained inactive when the Mg^{2+} was restored. This can be explained by the bridging effect of the peptidyl tRNA, which is attached by the tRNA to the messenger on the small subunit and to the peptidyl transferase on the large subunit. This greater ease of dissociation in the case of the free ribosomes has been demonstrated in the case of 70S ribosomes by Beller and Davis (1971). They substituted Na^+ for K^+ in sucrose gradients of a mixed population of ribosomes, those carrying nascent polypeptide being pulse-labelled with ^{14}C amino acids. The Na^+ caused dissociation of the unlabelled free ribosomes into subunits, but the labelled monosomes remained intact. Stanners and Becker (1971) made use of the difference between free and programmed ribosomes to separate them and estimate their relative abundance in cultured cells during the phases of growth. They subjected ribosome extracts from cultured hamster fibroblast cells to mild ribonuclease treatment (15 min at 0°C with crystalline ribonuclease). This treatment degraded polysomes to single ribosomes. Subsequent sucrose gradient analysis separated the complexed ribosomes, which did not dimerise, from the free, which had dimerised, and so the fraction of each could be estimated. In this way they demonstrated that in stationary phase only half the number of ribosomes were present compared to exponential phase and furthermore that only two thirds of these were attached to mRNA, whereas in exponential phase nearly all were bound to mRNA in polysomes. Formation of polysomes is therefore controlled by physiological mechanisms directly related to the protein synthesizing capacity of the cell. Apparently even mild treatment at 0°C with highly active crystalline ribonuclease cleaves mRNA in such a way that the single ribosomes produced cannot dimerise, unlike the less active form contaminating ribosomal preparations.

The gradient profiles of the preparations of total ribosomes separated from the supernatant after sedimentation of the polysomes showed mainly oligosomes. Both monomer and dimer peaks were high, ferritin contributing largely to the ultraviolet absorbance of the monomer peak. The increase in ferritin content of the Cd-treated liver is apparent when Figs. 43 and 44 are compared.

A significant accumulation of ferritin has been reported by Kwan, Webb and Morris (1968) in the host liver of rats bearing large hepatomas; they also found isolated ribosomes to consist mainly of oligosomes.

Direct recordings in later experiments of gradient profiles of the total ribonucleoprotein particles in the PMS probably bear the closest relation of all the gradients to the in vivo distribution in the cell. The prominent monomer peak would be due partly to contaminating ferritin. Subunits presumably contributed to the shoulder on this peak (Fig. 46). The dimer peak was low, as the presence of ribonuclease inhibitor in the cytosol (Blobel and Potter 1966), and the short time interval before start of centrifugation would help to prevent breakdown of the polysomes. There were present as a high proportion of the whole in both normal and Cd-treated supernatants.

It is possible that an increased number of monomers could have been present in these studies as a consequence of removal of the food from the rats overnight before the start of the experiments. Norman et al (1972) found this to be the case in a careful study of ribosomal distribution in mouse liver preparations. Ribosomes isolated from mouse liver do not dimerise, so monomer peaks could be studied directly. They noted a 10% increase in free (run-off) ribosomes when animals were starved overnight. Uncertainties due to ribonuclease activation were prevented by using double labelling techniques and mixing homogenates from the fed and starved animals. Difficulties of preparing ribosomes from fed animals due to the presence of glycogen were obviated by treatment of the PMS with amylase, which degraded the glycogen, according to the method developed by Gamulin, Gray and Norman (1972).

The protein-synthesizing capacity of a ribosomal preparation is directly related to the content of polysomes and this has been demonstrated by Wettstein et al (1963) and many other workers. True comparison could therefore only be made when profiles of isolated preparations used in these experiments were similar and contained a high proportion of polysomes. This is illustrated in the relationship shown between profiles and incorporation of amino acids in Experiments 10 and 11. In Experiment 10, considering the results using normal pH5 enzyme, the level of incorporation (cpm) in the N preparation which, in this case, had fewer polysomes fell below the Cd at about 150 μg RNA per 0,5 μCi assay tube although at lower and higher ribosome levels less difference was noted. A considerable fall-off in incorporation levels in the case of the Cd preparation is apparent in Experiment 11 where the polysome content is low. Experiment 12 demonstrates the difference between more closely comparable N and Cd preparations with similar profiles. Here the Cd preparation fell about 10% below the N, cpm incorporated rising steeply in a similar fashion in both cases as added ribosomes (estimated by RNA analysis) were increased from 100 µg to 200 µg RNA. In these three experiments, specific activities dropped slightly as the amount of added ribosomes was increased in the assay tube. Whether this is due to saturation with ribosomes or instability in the system is not clear. An amount of ribosomes equivalent to 150 - 170 µg RNA was chosen as a suitable level for later studies.

The pH5 enzyme provides a source of amino acid activating enzymes and transfer factors which are concentrated in the fraction of the total cytosol proteins precipitated at this pH. This relatively stable enzyme concentrate was first prepared by Hoagland, Keller and Zamecnik (1956) and has been extensively used in general studies of incorporation of amino acids into protein.

In the three experiments discussed above the effects of Cd intoxication on the activity of isolated pH5 enzyme used in the protein synthesizing system was investigated. It was seen to exhibit a generally lower incorporating activity, the extent of inhibition when compared to the N varying with the individual preparation. The very small content of Cd found in the pH5 enzyme preparation by analysis and the fact that lowered activity was found to occur in the N as well as the Cd system, would point to some inhibitory effect on these enzymes not directly caused by the final Cd concentration in the assay tube. This inhibition could have taken place in vivo or by destabilisation of labile proteins during isolation procedures.

The improvements in technique enabled more truly comparative ribosomes from N and Cd-intoxicated animals to be prepared in the third series of experiments. The main changes which had been introduced were, standardised homogenization, speedy preparation of the PMS and sedimentation of the ribosomes and protection with GSH throughout the procedure. The N and Cd ribosomes prepared in this way both had similar profiles high in polysomes and activity of ribonuclease was evidently low as a considerable proportion of polysomes remained after warming a preparation to 37°C for 15 min (Fig. 20).

In the time-course incorporation experiments on these improved N and Cd preparations, a consistent difference between the two now became apparent. The steady rise in incorporation of amino acids observed in the N for up to 20 min continuing actively for 30 min contrasted with the lowered rate in the case of the Cd. This was apparent after 5 min and resulted in incorporation of only 75% of the counts recorded for the N at equivalent time intervals, and in a levelling off between 15 and 30 min. Only pH5 enzyme from normal rats was used in these experiments so ribosomes could be directly compared. From analysis of ribosome preparations the Cd present in the assay tube from treated ribosomes was $2,5 \times 10^{-7}$M. Inhibition therefore occurred at a very low Cd level.

The pH5 enzyme is possibly deficient in some factors essential for sustaining protein synthesis in an in vitro system, particularly those involved in initiation and release. An excess of pH5 enzyme has been shown to be essential for complete translation of the message and release of polypeptides into the supernatant by Hicks and Drysdale (1969). A high ratio of pH5 enzyme to polyribosomes was used in these studies but polypeptides released into the supernatant did not rise above 16% (Table 8). Estimation of these proteins was carried out by precipitation from gradient supernatants by TCA, so small size polypeptides not precipitated under these conditions would not be included in the estimates.

Whole cell sap prepared from the cytosol would be expected to provide essential factors in a balanced proportion. Filtration through Sephadex provides a rapid method for removal of amino acids (Baliga et al 1968, Huston et al 1970). When cell sap prepared in this way was substituted for pH5 enzyme in the assay system, however, activity was comparable at 2 min, and twice that found for pH5 enzyme in the N, but incubation for longer periods resulted in a rapid fall-off in incorporation levels, the Cd averaging 65% of the N. Both N and Cd preparations were equally unstable in the protein synthesis assay system and lost activity rapidly on storage at -80°C. Passage through Sephadex was found by Eker et al (1970) to activate latent ribonuclease of rat liver polysomes and so cause their disaggregation. It would therefore seem possible that a similar activation of degradative enzymes could take place during Sephadex treatment of cell sap.

Again in these experiments, incorporation levels of Cd ribosomes were lower than the normal and Cd cell sap was less active causing similar low levels of incorporation with both N and Cd ribosomes. The higher content of Cd of the cell sap resulted in a final concentration of 3×10^{-6}M Cd in the assay tube. It is possible that this may have contributed to the equally low activity of N and Cd ribosomes in this case. Optimum requirements for cell sap and Mg^{2+} in the assay system were the same for both N and Cd preparations. In these later experiments with Mg^{2+} when incorporation had fallen to a low level it was noted that the Cd cell sap had retained slightly more activity than the N. This could indicate some stabilisation of protein by the presence of the metal and prevention of further degradation. Such an effect could have contributed to the stimulation of enzyme activity observed at low Cd concentration (10^{-5}M) in the studies on tryptophan oxygenase (Diamond et al 1973). Ulmer and Vallee (1971) have reported hydrogen-tritium exchange measurements which show proteins to be stabilised by metals.

The incorporation gradients illustrate the labelling of the individual polysome peaks and the relatively low activity of the monomer. In the later stages of incorporation, counts increased in the monomer region, but this could have been due to random breakdown of polysomes after incubation for long periods of time. Extension of the results of the time-course experiments with pH5 enzyme by sucrose gradient analysis at different time intervals between 2 min and 1 hr using the complete N and Cd systems enabled a specific effect of Cd on ribosome function to be noted. In the N system cpm recorded for individual polysome peaks continued to rise up to 1 hr, but in the Cd system the situation remained almost static after 15 min. Profiles in the Cd system also remained very little changed, so fission of mRNA was not the cause of reduced incorporating ability. There must therefore be a blockage of translation on the ribosome resulting in a slower rate of elongation of the polypeptide chain. Individual steps in translation affected by Cd could be further investigated by the use of drugs. Incubation with puromycin was used to release nascent chains from reticulocyte polyribosomes by Jacobs-Lorena, Colombo and Baglioni (1970). Further protein synthesis was prevented by the immediate addition of cycloheximide or sparsomycin. The polypeptides released were then isolated for further study. A rat liver system has been used by Baliga, Pronczuk and Munro (1969) to study the specific effects of cycloheximide on elongation and polysome aggregation.

The diminished rate of reaction consistently observed would suggest inhibition of enzymes. This could be a primary effect of bound Cd or a secondary one resulting from disturbances in metabolism or of some part of ribosomal function. Cd available *in vivo* for attachment to incorporating enzymes and ribosomes in these studies is limited by the high binding capacity of the induced metallothionein. Addition of Cd to ribosome preparations *in vitro* would enable direct effects and greater changes to be observed.

Experiments comparing the incorporation of individual amino acids, including lysine and tryptophan, did not demonstrate any difference between N and Cd-treated incorporation systems. It is possible that some difference would become apparent if higher incorporation levels were achieved by refinement of methods, and use of purified enzymes and specifically labelled tRNA, as employed for example by Pilkis and Korner (1971) in their investigations of the effect of diabetes on protein synthesis in isolated rat liver ribosomes. The availability of tryptophan in the cell *in vivo* could also be lowered by the enhanced activity of tryptophan oxygenase demonstrated in Cd intoxication by Kench and Gubb (1970). Albumin forms only a part of the total protein synthesized in the liver, so a specific effect on albumin synthesis could well escape detection in a general protein synthesis study.

Estimations of total RNA and ribonucleoprotein particles carried out during these experiments did not show an increase in total ribosomes at this chronic stage of intoxication. This finding, together with the lowered level of protein synthesis demonstrated, would result in a general inhibition of protein synthesis. Stimulation of protein synthesis has been observed in short-term studies (1 - 2 days) of Hg intoxication by Brubaker et al (1971). Hawtrey and Nourse (1966) found increased labelling of rat liver polysomes to result after a similar period of time from a single injection of the carcinogen 4-dimethylamino-3'-methylazobenzene. Investigation of the effects of a single Cd injection would provide an interesting comparison with these results.

Induction of synthesis of nuclear RNA in rat liver by Cd, Mn and Zn has been reported by Weser and Hübner (1970) following a single injection of a salt of the metal at concentrations of 10^{-4} to 10^{-6} moles per kg rat. Mn,(1 mM $MnCl_2$) has also been found to interfere with in vitro processing of preribosomal particles by the nucleolar enzyme using the HeLa cell system (Mirault and Scherrer 1971). Some 18S RNA was produced but very little 28S, the 32S being cleaved to other products with different sedimentation constants.

Isolated rRNA from Cd-treated rat liver did not show any major difference from the normal on separation by polyacrylamide gel electrophoresis. More detailed studies of a similar nature after exposure of ribosomes to Cd might reveal changes resulting from Cd binding. Spectrophotometric methods, particularly CD, following earlier studies by McPhie and Gratzer (1966) and Watson and Kidson (1969) would provide information on induced conformational changes or destabilisation effects in RNA such as those already reported for polynucleotides by interaction with metals by Eichhorn and Shin (1968).

The levels of Cd which produced a cytotoxic effect on cultured HeLa cells in the above studies (10^{-4}M-10^{-5}M) were comparable with those recorded in the whole rat liver and preparations from it. More detailed studies of both immediate and delayed effects produced by Cd at specific concentrations can therefore be carried out to compare with the in vivo changes in the rat liver. HeLa cells have been used in studies on disaggregation of polysomes and inhibition of protein synthesis produced by aflatoxin by Harley, Rees and Cohen (1969).

Another aspect of Cd intoxication which could be clarified in the simpler cell culture system is the distribution of RNA. Quantitation of RNA can be accurately carried out in such a system by introduction of labelled precursors such as uridine and subsequent estimation of the class of RNA under study. Darnell (1968) reviews studies of RNA metabolism in mammals carried out mainly in HeLa cells, and the main pathways of RNA processing have been elucidated in this way.

By further cell culture studies, and more detailed investigation of protein synthesis on the ribosome, it is hoped that many of the present uncertainties as to the location and mode of action of cadmium in protein metabolism will be resolved. All investigations to date are consistent in underlining the extreme potency of cadmium in biological systems.

PART IV

SUMMARY AND CONCLUSIONS

1. Chronic Cd intoxication was induced in rats by intraperitoneal injection of $CdCl_2$ over a 2 - 3 week period, and a level of 10^{-3}M Cd in the liver was produced.
2. Rats lost weight compared with control animals and the liver was relatively enlarged.
3. Cd-treated liver was similar to the normal histologically but was more resistant to homogenization.
4. Degradative enzymes, proteases and nucleases were more active in preparations from treated liver, indicating fragility of cellular organelles and constituents.
5. Sedimentation through 1,5 M sucrose resulted in a higher recovery of ribosomes from Cd-treated liver with a lower protein content than the normal.
6. A higher content of oligosomes than the normal was sometimes observed on sucrose gradient analysis of Cd 1,5 M sucrose ribosome preparations.
7. Treatment of a ribosome preparation with crystalline ribonuclease and sucrose gradient analysis resulted in a shift from polysomes and oligosomes resulting in a high peak in the monosome region of the gradient.
8. An increased content of ferritin was observed in the PMS from Cd-treated rat liver.
9. Recoveries of N and Cd ribosomes sedimented through 2 M sucrose were similar and their protein content did not differ significantly.
10. Resolution of sucrose gradients of these preparations into individual peaks demonstrated a low proportion of

monomers in all cases but a higher and more variable content of dimers and fewer polysomes in Cd preparations.

11. The course of disaggregation to monomers and dimers of polysome preparations when kept at 0°C pointed to more active ribonuclease in the 1,5 M sucrose preparations than in the 2 M.
12. The level of incorporation of amino acids into protein was found to be dependent on the polysome content of the ribosomal preparation and was lower in both N and Cd when a high proportion of oligosomes was present.
13. When gradient profiles were similar in both N and Cd ribosome preparations a lowered level of amino acid incorporation was observed in the Cd preparation, using normal incorporating pH5 enzyme.
14. Substitution of pH5 enzyme prepared from Cd-treated rat liver caused a decrease in incorporation efficiency of both N and Cd ribosomal preparations.
15. When preparative methods were closely controlled, ribosome preparations from both N and Cd-treated liver were seen to have similar polysome content, ratio of RNA to protein and spectral characteristics.
16. Ribosomes prepared in this way from Cd-treated liver exhibited a diminished rate of incorporation of amino acids into protein, and cpm incorporated levelled off at 75% of the normal value after 30 min. Normal pH5 enzyme was used for these time-course studies.
17. The pH5 enzyme was prepared by precipitation from the

cytosol by lowering the pH to 5,2, washing, and resuspension in buffer. For comparison, cell sap incorporating enzymes were prepared from whole cell sap by gel filtration through Sephadex G10 to remove amino acids.

18. The N cell sap had comparable activity in the assay system up to 2 min with N ribosomes, but the rate declined rapidly and counts after 30 min were only 60% of those incorporated with pH5 enzyme. Cd ribosomes in this system only reached 65% of the N level. Substitution of Cd cell sap resulted in this same low level of incorporation for both N and Cd ribosomes.

19. Optimum requirements for both cell sap and Mg^{2+} were the same for both N and Cd ribosomes.

20. Cd content of ribosomes prepared from Cd-treated rats was 7 ng Cd/100 ug RNA. Cd pH5 enzyme had 0,4 ng/100 ug protein and Cd cell sap had 5 ng/100 ug protein. The incorporation assay system therefore had $2,5 \times 10^{-7}$M Cd using pH5 enzyme and 3×10^{-6}M Cd when cell sap was added.

21. Cell sap preparations were more unstable than pH5 enzyme, losing activity rapidly at -80°C.

22. By sucrose gradient analysis during the course of incorporation of amino acids preferential labelling of the polysome over the monosome region was observed.

23. Gradients at time intervals from 2 - 30 min after start of incorporation demonstrated the continued rise in cpm

associated with ribosome peaks in the N ribosome/NpH5 system up to 1 hr, at which time few polysomes remained. Labelling became almost static after 15 min in the Cd ribosome/Cd pH5 system although a significant proportion of polysomes was still present.

24. No difference between N and Cd systems was observed in the incorporation of the individual amino acids tryptophan, lysine and leucine.
25. Total RNA content of homogenates and preparations of total ribosomes was the same or slightly lower in the case of Cd-intoxicated animals.
26. Isolated RNA from N and Cd ribosomes had the same pattern on polyacrylamide gel electrophoresis.
27. Cd levels of 10^{-4}-10^{-5}M had a cytotoxic effect on HeLa cells in culture.

The main conclusions to be drawn from this work are:

Intoxication producing a level of 10^{-3}M Cd in the liver resulted in inhibition of incorporation of amino acids into protein in vitro by both isolated ribosomes and incorporation enzymes. No increase in the level of total RNA in the liver was detected. These findings, together with the adverse effect on the growth of the rat which was observed would point to a lowered efficiency in protein synthesis in vivo.

The specific defect demonstrated on the ribosome was not caused by fission of the messenger but appeared to result from inhibition of elongation. If this was a primary effect it was caused by binding of Cd to ribosomes in an active form at a low level (7 ng/100 ug RNA).

Lines planned for further investigation:

Further studies on protein synthesis, particularly elongation, could be carried out with more highly purified ribosomes and enzymes.

The use of drugs such as puromycin and cycloheximide would enable the effect of Cd on specific steps in translation to be clarified.

Direct interaction of Cd could be observed by additions at various levels <u>in vitro</u> to the assay system.

Immediate effects could also be observed after a single Cd injection, when long-term disturbances in metabolism could not have taken place.

Further tissue culture studies could elucidate the influence of both low and high levels of Cd at the cellular level. More detailed information on RNA processing and ribosomal RNA in cells exposed to Cd would be obtained.

Spectrophotometric studies on Cd binding, particularly to ribosomal RNA, are planned.

PART V

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