Studies on the synthesis of liver proteins with particular reference to prothrombin

by

Pratul Goswami, M.B.B.S., D.Phil.(Med.)

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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INTRODUCTION

Morphological Structures in the Cell associated with protein synthesis

Studies of protein synthesis in subcellular systems is a problem not only to the biochemists but also to the morphologists and combined efforts of the biochemists and the morphologists have thrown new light on the problem. The liver contains and is capable of synthesizing many different types of proteins; because of the difficulty of isolating newly-formed protein, investigation has been restricted only to a few. Of the different proteins, plasma albumin is found in abundance and it has been now possible to isolate and to demonstrate definitely by immunological, electrophoretic and solubility tests that there is newly formed albumin in the liver. Studies of the incorporation of the labelled amino-acids have revealed that this protein is synthesized by a particular fraction of the liver cells rich in ribonucleic acid. Now evidence is accumulating to establish that the other fractions, namely the mitochondria and the nuclei, are also capable of synthesizing some proteins.

Many investigators are working both in multicellular and unicellular systems to explore the possibilities of the mechanism protein synthesis. It has been pointed out by Siekevitz (1960) that the nature of the purpose of the protein being synthesized in the two systems are different - in the unicellular system the newly-synthesized proteins are used in the growth of the existing cells and in the manufacture of the new cells; the life span of most of the unicellular system being very short, one can neglect the mechanism of replacement of the existing proteins in the cells. However, the life span of the multicellular system being considerably longer, one

might totally forget the production of proteins for new cells and the predominant feature of protein synthesis in these cells is replacement of proteins already in the cells. The difference between the multicellular and unicellular organism is not only functional but also morphological. In the bacterial cells there is no endoplasmic reticulum and on homogenization no microsomes are obtained (Palade - 1958). Pellets obtained from bacteria are much smaller and simpler and morphologically they correspond to free ribonucleoprotein particles of pancreas. In recent years the ribonucleoprotein particles separated from the microsomal membrane by using detergents are regarded as the site for protein synthesis (Cohn - 1959, Rendi & Hutlin - 1960, Korner - 1960) in which case a natural question arises about the sites of protein synthesis in the bacterial cells. Do the membrane-bound ribonucleoprotein particles and the free particles behave in different ways in the two different systems? Answers to this and similar types of questions have yet to be obtained.

It has been demonstrated that ribonucleoprotein-like particles are present in the nuclei (Frenster et al 1960), and these particles remain capable of active amino-acid incorporation into their constituent proteins (Frenster et al 1960). These observations suggest that there should be more integrated-morphological and biochemical study of these subcellular particles

The work of Porter et al (1945) and Claude and co-workers (1947) on the study of the microsomal particles led to the discovery of the "lace-like" network which Porter (1952, 1953) named the endoplasmic reticulum. A clearer and elaborate definition of the endoplasmic reticulum has been

given by Palade (1959) as an "extensive system of membrane bound vesicles, tubules and flat vesicles or cisternae interconnected into a more or less continuous network." The endoplasmic reticulum is present in many cells varying in total volumes, general disposition and detailed structure from one cell type to another (Palade - 1956) and it has been suggested that the endoplasmic reticulum is directly or indirectly associated with cell division. The variation of endoplasmic reticulum from cell to cell has been reviewed by Palade (1956). Hartmann (1953) demonstrated the existence of a double membranous layer around the nucleus and dense particles seemed to be attached to the outer nuclear membrane (Palade - 1955) in the splenic reticulocytes the outer nuclear membrane is continuous with the membrane of the endoplasmic reticulum (Watson - 1957) and Palade's observation of similar structures in the lymphocytes, granulocytes, macrophage parenchymatous liver cells and acinar-cells of pancreas led to the suggestion that the nuclear envelope is a local variation of the endoplasmic reticulum. Their hypothesis may be a clue in the study of the "messenger" ribonucleic acid.

The endoplasmic reticulum may be circular, oval, elongated or irregular shape with their smaller diameter as 50 - 100 mμ (Palade and Siekevitz - 1956). Small dense particles of 10 - 15 mμ in diameter are attached to the membrane of the reticulum and also scattered in the cytoplasmic matrix and these particles correlate better with cytoplasmic basophilia than any other known cellular component. These particles are arranged in double rows and circles at the surface of the membrane limiting profiles (Palade

and Siekevitz - 1956). The endoplasmic reticulum is bound by a thin 5 mμ membrane. Palade and Siekevitz (1956) have also shown that the same dense compact bodies represent a constituent of the microsomes. The biochemical composition of the microsomal and postmicrosomal fraction of rat pancreas and liver as studied by them have shown that the microsomes are characterised by high RNA/protein and phospholipid/protein ratios, the postmicrosomal fraction contains much less RNA and phospholipid protein without a reduction of the protein content in the same proportion. The microsomes consist almost exclusively of closed vesicles limited by a dense continuous membrane and are filled with a material of relatively low density. These vesicles are all derived from the rough-surfaced part of the endoplasmi reticulum. The origin of the smooth surfaced vesicles of the microsomes has not been definitely ascertained, but they might be derived from the smooth surfaced part of the reticulum or from another source (Palade - 1958).

Birbeck and Mercer (1961) recently studied the cytology of cells which synthesize protein and observed two types of cells viz.:- (i) retaining cells which are characterised by free particles with a few α-cytomembrane (granular reticulum) and here the synthesized protein appears freely throughout the cytoplasm and is not contained in the vesicles, (ii) the secretory cells which are characterised by abundant α-cytomembrane. The retaining cells might again be of two class:- in one group of cells the cytoplasm is filled with specific protein and during protein-synthesis the cells contain free particles, the cytoplasm becomes completely filled

with protein particles and then the particles disappear. In the second group of cells, protein synthesised is devoted to growth. Watson's hypothesis (1955) of the nuclear membrane being a local variation of the endoplasmic reticulum and nuclear ribonucleoprotein particles being transferred to the cytoplasm by means of the membrane is disputed by them because of the absence of this connection in the retaining cells. These structures can be related to chemical reactions occurring in protein synthesi

It is widely accepted to-day that a series of reactions is involved in the protein synthesis as shown below:-

(1) activation of the amino-acid:-

Amino acid + ATP + Enzyme $\longrightarrow$ AA AMP-Enzyme + PP.

(2) addition of nucleotide end group to s-RNA:-

RNA + ATP + CTP $\longrightarrow$ RNA-$P_{CP.CP.A}$ + PP.

(3) binding of amino-acids to this s-RNA:-

AA + ATP + RNA-$P_{CP.CP.A}$ $\longrightarrow$ RNA-$P_{CP.CP.A.}$ - AA + PP.

(4) transfer of amino-acids:-

RNA-$P_{CP.CP.A.}$ - AA + ATP + CTP + Ribonucleoprotein particle +

soluble fraction $\longrightarrow$ Peptides.

The main features of these series of reactions is that a coupled energy-yielding reaction is necessary to make the synthesis proceed (Hoagland - 1960 Deprivation of oxygen (Frantz et al - 1947) presence of respiratory inhibitor (Farber et al - 1951), or uncoupling agents prevent protein synthesis.

Changes in cell structure with diet

From the above series of reactions of protein synthesis the importance

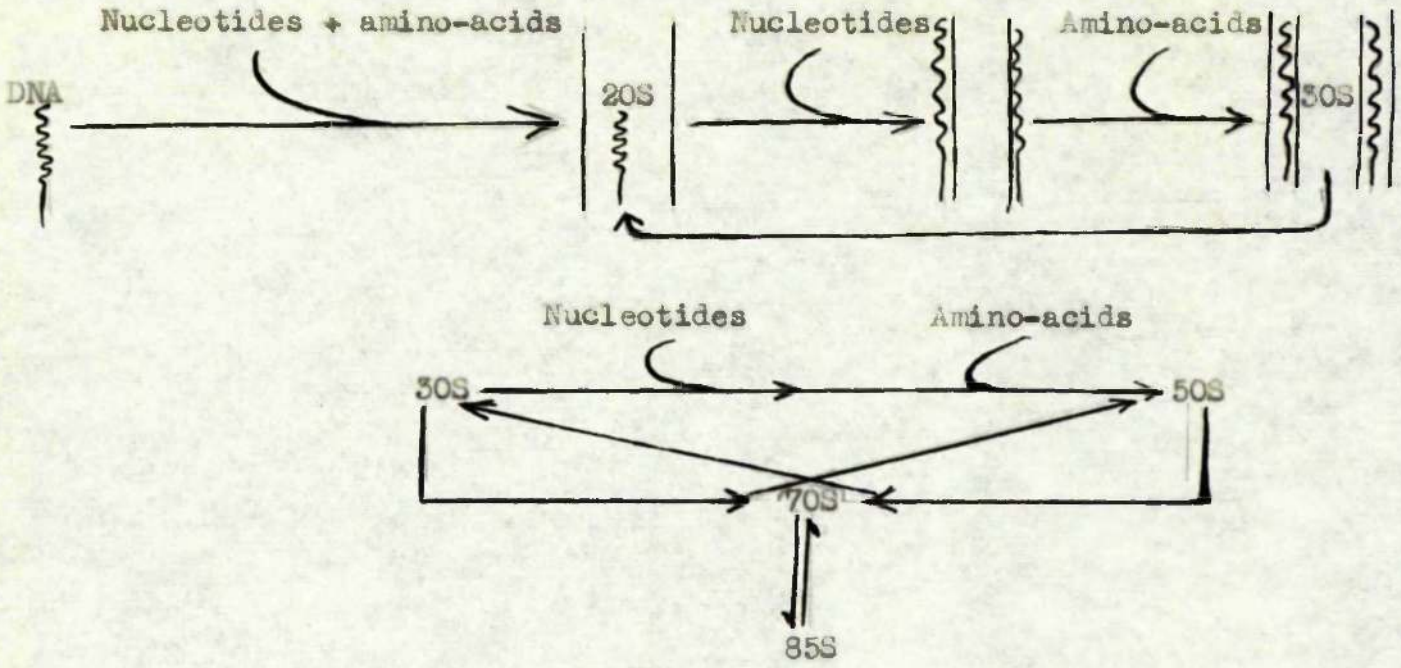
of ribonucleic acid in the process is evident. In the case of liver cells, the structure and RNA metabolism is influenced by diet. Munro and Clark (1959) observed a variation of ribonucleic acid content of the liver cell with variations in dietary protein and the change is essentially confined to the endoplasmic reticulum of the liver cells. Hence, they concluded that the supply of amino-acid for the reticulum is essential for synthesis of microsomal RNA. They have suggested that ribonucleic acid deposited during the formation of new reticulum arose most likely from the s-RNA. The changes in RNA content according to availability of exogenous amino-acids can be related to the morphological changes of the reticulum also. The effect of fasting was studied under electron microscope by Fawcett (1955) and Bernhard and Rouiller (1956) independently, and both observed a reduction of the reticulum; within a few hours after administration of diet containing a large amount of protein, regeneration of reticulum was observed. Porter and Bruni (1959) have also demonstrated structural changes in the hepatic endoplasmic reticulum with diet and suggest that alteration in morphological features rather than in biochemical mechanisms may lead to some changes in the normal process of protein synthesis.

Palade (1958) studied the structural variation of the pancreas of the guineapig in the starved and fed animals. In starved animals the zymogen granules accounted for approximately 40% of the trypsin-activatable proteolyt activity (mainly trypsinogen and chymotrypsinogen) of the cell and about 40% of its ribonuclease activity. The enzymatic activity of the microsomes in the starved animals is low in contrast to the fed animal which is in

agreement with the supposition that the endoplasmic reticulum is involved in the production of the digestive enzymes. In fed animals, proteolytic and ribonuclease activities are present mostly in the heavy microsomal subfraction than the parent microsomes, but in the starved animals no increase in this subfraction over the parent microsomes has been observed.

Ashikawa (1958) studied the ultracentrifugal characteristics of the microsomal particles from starving, nonproliferating and proliferating yeast cells and observed in the starved cells that the stability of the microsomes decreased and, at the same time, a rapidly spreading advance peak appeared, thus, an 80S component isolated from the log-phase and stationary-phase cell will give rise to 60S and 40S component, but in yeast cells starved for 72 hours the 80S component becomes further degraded. Purified RNP particles obtained from various sources show, that except in E. coli, many of the physical properties of the RNP particles of different sources are in good agreement. In pea seedling the 80S component dissociates to give 60, 41 and 26S units (Petermann et al - 1954). Similar components have been found in RNP particles from bacteria other than E. coli (Alexander - 1956) and animal sources (Petermann & co-workers - 1956). The RNP particles obtained from the rat liver shows that 83S component dissociates to 65, 50, 46 and 30S units. The dissociation of 83S units is dependent on magnesium concentration and pH (Hamilton and Petermann - 195 McQuillen (1961), studying the dissociation of 85S unit in the E. coli, has put forward a tentative scheme for ribosomal synthesis as follows:-

Tentative Scheme for Ribosome synthesis - (after McQuillen - 1961)



This tentative scheme of ribosomal synthesis may provide a clue to the mechanism carrying the genetic information and thus deoxyribonucleic acid may exert its ultimate control in the process of synthesis at this stage. The presence of RNP particles in the nuclei (Frenster et al - 1960), and in mitochondria (Rendi - 1959) supports this hypothesis.

In the present investigation some of the problems of protein synthesis have been studied. In Section I the synthesis of prothrombin by different fractions of the liver is described. In Section II, we consider whether this synthesis is any way related to ribonucleic acid and if the synthesis is energy-dependent. In Sections III and IV two types of RNA associated with microsomes have been studied and their probable relationship in transmitting genetic information and their response to dietary change have been studied.

SECTION I

Prothrombin formation

SECTION I

PROTHROMBIN FORMATION

Historical Introduction

The coagulation of blood is known from the days of Hippocrates, and both he and Aristotle tried to explain the phenomenon of coagulation on the basis of environmental changes like cooling, contact with air, etc. Their view was supported in the seventeen and eighteen century by Schroederv der Kolk, Bohn, Hoffman, Ludwig and others. That blood solidified on cooling was disproved by Hewson in 1772 on an experimental basis. Other physical causes like cessation of the natural flow of the blood were also contradicted by John Hunter, who showed that blood can remain stagnant in a vessel before coagulation occurs and drew attention to the fact that menstrual blood remains liquid. That the process of blood coagulation is a chemical one was supported by scholars like Galen who put forward the view that "the formation of a coagulum is the road to decay". Like Galen, Harvey also thought that the coagulation of blood took place due to decomposi tion and decay following the death and could not occur in the living blood. Home, Prevost and Dumas explained blood coagulation from the morphological point of view and thought that the investing membrane of the nuclei was damaged in the decomposing blood and was no longer capable of preventing the agglutation of the nuclei. Berzelius and Johnann Müller in the subsequent period did not give support to this hypothesis. Müller showed experimentally the existence of fibrin in solution in the plasma in his

famous experiment; though he did not explain the mechanism of conversion of the dissolved fibrin to the solid state. Virchow further modified the hypothesis of Müller and put forward the hypothesis that the fibrin exist in an isomeric liquid state in its precursor condition and termed it "fibrinogen". Prior to Virchow, Buchanan's observation with hydrocele fluid led him to postulate that two substances, namely (a) the liquid fibrin, which was a stable substance, and (b) a substance capable of converting the soluble fibrin into an insoluble one, were necessary for coagulation. Almost at the same time, Bruecke demonstrated that the vascular wall played a role in maintaining the blood in the liquid state; his observation is more important than his own interpretation of his own work. Denis isolated fibrinogen from blood by a salt precipitation method.

Following the hypothesis of Buchanan and Virchow, Alexander demonstrated the importance of blood cells in the mechanism of blood coagulation. His name was more famous for his original conception that blood coagulation is a process of fermentation, i.e., that the blood coagulation reaction is an enzymic one. He proposed the first theory of blood coagulation and stated that blood coagulation is a chemical combination of two substances and that the end product of the reaction is fibrin. Later, he modified his own theory and stated that a substance called "fibrin ferment" or "thrombin" was needed to produce coagulation and presented his argument regarding the enzymic nature of the fibrin ferment or thrombin. The fibrin ferment was prepared by him by the addition of alcohol to serum - a process later developed by Cohn for the fractionation of blood proteins. Alexander

Schmidt's theory can thus be regarded as the first classical theory of blood coagulation and part of his theory was supported by Hammarsten (1875); however, he disputed the part of Schmidt's theory which involved the role of "fibrinoplastic substance" (para-globulin) and he maintained that para-globulin was not essential for the coagulation. Schmidt first used the term "thrombin" to denote the substance previously known as the "fibrin ferment" and gave the name "prothrombin" to the precursor of thrombin. He thought that calcium salts activated the prothrombin to form thrombin, but Hammarsten differed from him. Hammarsten also thought that prothrombin which was considered to be a nucleoprotein in nature, was released from the formed elements into plasma after blood was shed, but Schmidt believed in the existence of prothrombin in the circulation blood and that it was activated by "zymoplastic agents" which are released from the leucocytes without calcium salts being necessary. This difference of opinions, as explained by Paul Morawitz, was due to the different substances identified as prothrombin. Fuld, Spiro and Morawitz, therefore, modified the classical theory of blood coagulation. "Fibrinogen, calcium salts, probably also thrombogen (modern term - prothrombin) exist in the plasma of circulating blood. Outside the vessels the formed elements specially the platelets, are disrupted by contact with the foreign surface and, therefore, yield thrombokinase into the plasma. Thrombokinase in conjunction with thromboge and calcium salts from thrombin". (Morawitz).

There was great controversy regarding the mechanism of conversion of fibrinogen to fibrin and Hammarsten thought that it was due to a process

of hydrolytic separation of fibrinogen into fibrin and his view was later supported by Schmierderberg (1897) and Haubner (1903) but not accepted by all and another school favoured the opinion of the intramolecular rearrangement of fibrinogen. The classical theory had also to face great criticism. Thus Wooldridge (1893) suggested that the formation of fibrin was the product of combination of two substances what were termed fibrinogen A and fibrinogen B. Nolf supported his view that thrombin was the product and not the cause of fibrin formation. In comparatively recent years factors like pro-serozyme, serozyme cytozyme (Bordet - 1920), disruption of protein complexes by foreign surface (Pickering - 1928) have been suggested as factors in the mechanism of fibrin formation.

It is Arnold Quick who has studied extensively the mechanism of blood coagulation from the biochemical aspect and a new era of blood coagulation theory started with him in 1935. He established the biochemical means for the estimation of prothrombin on the basis of the classical theory. His method of estimation of prothrombin was a major factor in the discovery of Vitamin K and revealed defective coagulation times in the different clinical conditions. Quick in his laboratory demonstrated that prothrombin is composed of two parts, namely a "labile component" or "component A" and a "stable component" or "component B". This discovery also established the discrepancy of prothrombin assay between his one stage method and a two stage method devised by others. The labile component of prothrombin is decomposed if blood is stored. Component B (or stable factor) becomes less in animal poisoned with dicumarol, in deficiency of Vitamin K, and is

destroyed by heating to 60°C and is removed completely by aluminium hydroxide and other absorbents from oxalated plasma but not from plasma in which calcium is not removed and the prothrombin complex has been allowed to remain intact. He believed that both components A and B are to a certain extent group-specific. He found that only a portion of prothrombin is in the active form in the plasma and the prothrombinogen, the inactive part of prothrombin of plasma is activated when the plasma comes in contact with rough surfaces like injured epithelium, glass surfaces, etc. Quick's labile factor was afterwards termed "accelerator globulin" by Ware and Seegers (1948), prothrombin accelerator by Fantle and Nancy (1948), thromboge by Nolf and Factor V by Owren (1947). Astrup renamed factor V "proacceleri and this term has been accepted by Owren. Owren has demonstrated that the presence of labile factor (Factor V or Proaccelerin), thromboplastin, calcium and prothrombin together form an intermediate product called Factor or accelerin before the formation of thrombin; prothrombin, Factor VI and calcium then form the thrombin which, reacting with fibrinogen, form the solid coagulum, fibrin. Maximal activity of Factor VI is dependent upon Factor V and Owren, therefore, suggested that Factor V is integrated with Factor VI wholly or partly.

Clinical and experimental evidence has shown that, in addition to the above mentioned factors, some other factors are necessary for coagulation with thromboplastin and calcium and one such factor has been called "prothrombin conversion factor" (Owen & Bollmen - 1948), "serum prothrombin conversion accelerator" (SPCA) (Alexander et al - 1949) and factor VII

(Koller, Loeliger and Duckert - 1951). The origin of this factor is thought to be "proconvertin", an inactive precursor in the plasma. Owen, therefore, renamed Factor VII "convertin" and this factor is reduced in quantity by dicumarol therapy. Alexander (1952) studied the biochemical and physical properties of this factor and found that it is relatively stable, absorbable by barium sulphate and barium carbonate, not precipitated from serum or plasma respectively at pH 5.0 - 5.2 or 5.7; it can accelerate the conversion of prothrombin to thrombin in the presence of thromboplastin, Ac-globulin and calcium, accelerate the coagulation of normal blood, heparinized blood, thrombocytopenic blood, higher in canine then in human serum, lower in bovine serum and it identical with convertin.

The above description of blood coagulation has been introduced with two main purposes in view, viz.: (1) to show that, whatever might be the mechanism of blood coagulation, prothrombin is essential, and (2) to make the reader familiar with the different terms used in connection with the study of prothrombin synthesis. Though Schmidt thought in his classical theory of blood coagulation that prothrombin is necessary in the first phase of coagulation, recent studies have shown that it is not only essential in the first phase of coagulation but also in the later phases.

Properties of prothrombin

Though prothrombin is such a vital protein in the body, its exact chemical nature is not yet definitely known because of difficulties in the isolation of this protein from the other proteins of blood; Seegers and

co-workers have been able to fractionate prothrombin to yield a fairly pure product. In recent years, electrophoretic separation, especially continuous curtain electrophoresis, has been used to isolate prothrombin from the other coagulation factors but how far the product is biologically pure has yet to be established. Seegers (1960) has fractionated the prothrombin factor in ion-exchange columns, and claimed that the product obtained by this method is quite pure.

The analysis of purified prothrombin has shown that the molecular weight of this protein is approximately 63,000 which is twice the size of the thrombin molecule. In the electrophoretic field prothrombin shows one major component consisting of 87 - 95% of the total prothrombin which moves along with alpha$_2$-globulin. Prothrombin is insoluble between pH 5.9 to 5.6, the isoelectric point being 4.8. Purified prothrombin goes into solution in 30% sodium citrate solution (Seegers & Ware - 1954). The apparent turnover of prothrombin is rapid and is nearly complete in 24 hours (Allen, Moulder, Emerson & Glatzer - 1949), a more recent and precise estimate being 20 hours; but the fate of prothrombin is not known though it has been assumed that the fibrin consumes the prothrombin for the maintenance of the integrity of the vascular wall. The prothrombin is retained on filtration through a 50% asbestos filter (Owren & Aas - 1951) and has antigenic properties (Halick & Seegers - 1956).

The analysis of dried prothrombin at first showed that it included 3.33% tryptophane, 4.58% tyrosine and 6% carbohydrate (Seegers, Loomis, Vandebelt - 1945, Seegers, McClaughry and Fahey - 1950). In more recent

Table 1

Composition of purified bovine prothrombin

	p.c. of N_2 Protein N_2	Amino acid per 100 Gm protein	Gms/moles/10^5Gm protein
Aspartic acid	7.15	10.01	75.30
Threonine	3.73	4.65	39.20
Serine	5.18	6.19	53.80
Glutamic acid	8.31	12.85	87.40
Proline	4.67	5.52	40.00
Glycine	5.69	4.49	59.80
Alanine	4.38	4.09	46.00
Valine	3.96	4.88	41.60
Methionine	1.24	1.94	13.00
Isoleucine	2.28	3.14	24.00
Leucine	5.52	7.60	58.00
Tyrosine	2.26	4.38	24.20
Phenylalanine	2.78	4.73	28.50
Histidine	3.40	1.84	11.90
Lysine	7.48	5.73	39.30
Arginine	17.90	8.19	47.00
Tryptophan	3.10	3.30	16.30
Cystine	2.50	3.14	26.30
Amide NH_3	6.49	-	(68.20)
N-acetylhexosamine	0.90	2.07	(9.40)
Hexose	-	6.50	(36.10)
Pentose	-	0.60	(2.50)
	98.83	105.84	739.60

(after Laki, Kominz, Symonds, Lorand & Seegers - 1953)

analyses, Laki, Kominz, Symonds, Lorand and Seegers (1954) have been able to isolate altogether 18 amino-acids and the composition of bovine prothrombin according to their analysis is tabulated in Table I. Though some of the properties of prothrombin resemble albumin, the amino-acid composition of two proteins is quite different. Thomas and Seegers (1960) have determined the terminal amino-acid of bovine prothrombin and thrombin and observed that irrespective of the procedure used for preparing prothrombin, the C-terminal amino-acid differs according to the procedure applied, though the N-terminal amino-acid is always alanine, as shown in Table II.

Table II

C and N-terminal amino-acids of Thrombin and Prothrombin

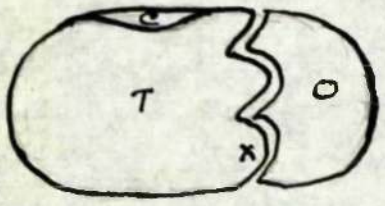
Preparation	N-terminal	C-terminal
Thrombin	Glutamic acid	Isoleucine
Prothrombin $Mg(OH)_2$ only	Alanine	Tyrosine Glycine
Prothrombin $Mg(OH)_2$ + IRC-50	Alanine	Tyrosine Glycine
Prothrombin $Mg(OH)_2$ + DEAE cellulose	Alanine	Serine

Bovine prothrombin, by ultracentrifugal analysis, is claimed to be homogeneous (Seegers - 1958), but Alexander (1958) obtained at least two components of purified human prothrombin on ultracentrifugal analysis, the lighter peak trailing behind and becoming evident during the second half of the run and comprising somewhat less than 10% of the total protein. The major peak had a sedimentation constant of 4.64 compared to Seeger's 4.84

The bovine prothrombin which is freed from proconvertin is also not homogeneous in his observations, suggesting difficulties in the preparation of purified prothrombin.

Activation of Prothrombin

The term "activation of prothrombin" is applied to the mechanism of formation of thrombin from prothrombin with molecular changes occurring inside the prothrombin proper. During the activation process, changes occur in prothrombin molecules in different ways (Penner & Seegers - 1956), and this formation of thrombin, as described in the blood coagulation theory, is nothing but the molecular rearrangement of prothrombin. The activators of prothrombin are found in three different anatomical compartments, namely (i) tissues (intra-cellular), (ii) plasma and (iii) Platelets. Waugh and others (1960) have explained the mechanism of activation of prothrombin from a hypothetical model of prothrombin as follows:-



where c is the carbohydrate subunit,

T & O protein subunit,

X is the active site for thrombin activity by T subunit.

In activation mechanism the subunit T might possibly require a specific enzymic reaction to produce X, but the removal of O may or may not be necessary for enzymic activation; alternatively subunit T may be thrombin whose active site is masked by specific combination with O. In the activation process, the subunit O is removed from its specific masking combination. This removal of subunit O, as suggested by them, might occur

partially or completely by enzymic action or by shifting O to unmask X, although O remains in the vicinity of X. The carbohydrate subunit C is unnecessary for activation. Because of molecular rearrangement, there is degradation of prothrombin to several derivatives, the properties of which have been exhaustively presented by Seegers and his co-workers in many papers. Waugh and others have been able to isolate at least two immunochemically distinct moieties present in the activated prothrombin. Because of degradation of prothrombin molecules, much of the carbohydrates and nitrogen becomes soluble in 7% TCA solution early during the first hour of activation. In the process of activation the N-terminal amino-acid of prothrombin changes to proline instead of the usual alanine (Seegers, Landaburn and Thomas - 1959).

Autoactivation of Prothrombin

It has been observed that, when purified prothrombin is stored at -20°C, the prothrombin loses some of its original properties (Seegers *et al* - 1955) but becomes capable of accelerating conversion of prothrombin to thrombin. This new property during storage is attributed to prothrombin proper (McClaughry and Seegers - 1952). This new derivative of prothrombin is called "autoprothrombin". This inactive prothrombin is also obtained by addition of calcium and platelet factor to the prothrombin solution, but no autoprothrombin is formed if platelet factor is pretreated with ether and thus its lipid content is removed, showing that platelet factor lipoprotein is important in the conversion of autoprothrombin to thrombin.

Studies of Prothrombin Synthesis

Liver diseases are associated with prolonged clotting time. This simple clinical observation led to the hypothesis that liver, the site of synthesis of other plasma proteins, is also the site for the formation of clotting factors. Toxic agents like chloroform and carbon tetrachloride, which have a direct effect on the liver cells, cause a fall of prothrombin level in the blood (Warner, Brinkhouse & Smith - 1936, Bollmen, Butt and Snell - 1940); even after 10 hours following chloroform anaesthesia, the prothrombin level decreases and reaches its minimum value within two to four days and this is reputed to be due to liver damage (Smith, Warner & Brinkhouse - 1937). The actual experimental study of the role of the liver in the formation of prothrombin - or rather of clotting factors as a whole - started in 1938 by Warner who found that after partial hepatectomy in rats there was a reduction of prothrombin level, the minimum level being on the sixth day after hepatectomy and on the tenth day of operation prothrombin level again returned to normal. Being encouraged by their results, Warren and Rhodes (1939) found that, after total hepatectomy on the dog, an immediate reduction of prothrombin level took place. The works of Lord and colleagues (1939, 1940), Munro, Hart and Munro (1945), Mann, Shonyo and Mann (1951), and of Alagille (1954, 1956) supported this view and it was definitely established that the liver is the site of origin of clotting factors.

Because of the fact that the prothrombin in the electrophoretic field moves with the globulin rather than the albumin, it has been assumed in recent years that, like globulin, prothrombin might also have both intra

and extra hepatic origin. The idea that the prothrombin might be of extra hepatic origin dated back to 1916 when Drinker and Drinker, after doing a perfusion through bone marrow, observed an increase in the activity of prothrombin and they concluded that bone marrow was the site of origin of prothrombin. In more recent times it has also been shown that neither the liver nor the bone marrow alone but the reticulo-endothelial system as a whole is the site for prothrombin synthesis (Heppich and Schmid - 1948, Schmid - 1951, Jurgen - 1952). There is a direct relationship between the reticulo-endothelial hyperplasia in bone marrow and prothrombin activity (Milletti and Di-Paolo - 1951), but Deutsch (1955) neither contradicting nor supporting their views, and pointed out that tissue factor inevitably contaminates the bone-marrow samples while puncturing and thus changes the stability of coagulation mixture and hence the conclusion that activity of plasma prothrombin is directly related to hyperplasia of bone marrow could not be accepted in the strictest sense. Slätas (1958), by blocking the reticulo-endothelial system concluded that blockage of this system markedly depressed the prothrombin and proconvertin restoring action of Vitamin K in dicumarol-treated rats. Liver is the chief organ of the reticulo-endothelial system of the body and so if reticulo-endothelial system be taken as the site for prothrombin synthesis, the liver in that case also is the major site of prothrombin synthesis.

Lasch and Roka (1953) demonstrated that the mitochondrial suspension obtained from the rat liver was capable of increasing its prothrombin content when the mitochondrial suspension was incubated in veronal buffer

with the sodium oxalate, and synkavit (Vitamin K) at 37°C at pH 7.9 (optimum) in an atmosphere of oxygen and carbon dioxide. The increase activity of prothrombin has been assayed by the usual method of Owren one-stage coagulation time. From their experimental data, they suggested that proconvertin (Factor VII) is formed by the liver cells and before being released into the general circulation it is converted to prothrombin in the liver and prothrombin thus formed is then circulated in the blood and a reversible process, i.e. conversion of prothrombin into Factor VII, takes place in the peripheral blood. Alkjaersig and Seegers (1955), contrar to the hypothesis of Lasch and Roka, observed no additional formation of prothrombin when mitochondrial suspension is added to a purified prothrombin solution but they observed that autoprothrombin, whether derived from purified prothrombin or from serum, can be converted to prothrombin by means of liver mitochondria. Barnhart (1957) observed that, by addition of cathepsin or a mitochondrial suspension at pH 7.9, the activity of the inactive prothrombin was regained and that both substances under similar conditions behaved almost in the identical way. She discarded the theory of an oxidative mechanism located in the mitochondria as the process for reactivation of the inactive prothrombin and suggested that any of the following mechanism might occur, namely (a) exposure of a group substance which had been covered, (b) folding of the molecule, (c) a polymerisation reaction with or without transamidation, (d) a substitution reaction and (e) cleavage or hytrolysis of inactive prothrombin to thrombin might be responsible for the activation mechanism. The assessment of prothrombin

activity was done by both the group of workers by the modified two-stage methods of coagulation of blood. Whether it is one-stage or modified-two-stage method which is used, all the techniques in main do study a series of reactions rather than absolute concentrations of substances and hence the coagulation time is a compound of various phenomena including different preliminary reactions such as contact with foreign surfaces, formation of thromboplastin, conversion of prothrombin to thrombin and finally clotting (Biggs - 1955). It may be mentioned here that when purified prothrombin is incubated with 25 p.c. sodium citrate, activation occurs (Seegers et al - 1950) and they proposed that activation of prothrombin thus involved a stepwise proteolytic degradation through a cycle of prothrombin derivatives. Barnhart (1960) recently observed that the regeneration of prothrombin-like activity by cathepsin B and by mitochondria was unable to form a clot of thrombin and so she concluded that most likely a substance with a smaller molecule is regenerated; her suggestion was based on the finding of prothrombin derivative-I of Seegers and Alkjaersig (1956), as evidence of auto-activation. Pool and Robinson (1959) studied the synthesis of coagulation factors by liver slices using the conditions of Peters and Anfinsen (1950) and were able to demonstrate the synthesis of Factor VII (precursor of prothrombin) in the tissues until a maximum was reached. This did not occur in liver homogenates. Because of the importance of this plasma prote and at the same time uncertainty of its origin the aim of the present study has been to investigate the site of its synthesis in the subcellular structu and also to explore the mechanism of synthesis of this important plasma protein, follows conditions used to study the synthesis of other plasma proteins.

MATERIAL AND METHODS

Measurement of Prothrombin

There are two methods for the measurement of prothrombin - the one stage method initially described by Quick (1935) and the two stage method of Warner, Brinkhouse and Smith (1926). In the two stage method, thrombin is formed from prothrombin and at invervals the formed thrombin is transferred to a standard solution of fibrinogen and the time taken for the firm clot to form is recorded; the minimum time for firm clot formation is taken when there is maximum amount of thrombin formation, i.e. when all the prothrombin is converted to thrombin. The one stage method measures the effects of the amount of thrombin formed, the speed of its formation and the reactivity of the fibrinogen. The advantage of the one stage method over the two stage method is not its accuracy; in the latter it may be necessary to measure the clotting time 5 to 6 times for a single coagulation time whereas in the one stage method a single measurement is sufficient. As in the two stage method, when prothrombin is measured by the one stage method with different dilution of prothrombin, a hyperbolic curve is obtained which can be converted to a straight line by plotting the reciprocal of the prothrombin concentration against the clotting time.

The original one stage method has been modified by Owren. Even then the effect of heparin, and anti-thrombin present in the plasma could not be overcome and so Allington (1958) introduced a second dilution and thus the effect of heparin in coagulation is greatly reduced. To carry out Allington's one stage method for the measurement of the prothrombin the

following reagents are necessary:-

(1) Owren's buffer-pH 7.33. This is prepared by dissolving 5.878 gm. of sodium diethyl barbiturate and 7.335 gm. of sodium chloride in distilled water to which 215 ml. of 0.1 N HCl added and the final volume is made up to 1000 ml. with distilled water.

(2) Citrate solution. This is prepared by dissolving 31.3 gm. trisodium citrate (dehydrated) in distilled water from which 240 ml. is diluted further to 1000 ml. with distilled water to give 25.6 mM trisodium citrate.

(3) First diluting solution is made by diluting 100 ml. of 3.13% trisodium citrate with 0.9% sodium chloride and the final volume is made up to 700 ml.

(4) Second diluting fluid is made with 200 ml. of Owren's buffer, 200 ml. of 25.6 mM trisodium citrate and 600 ml. of 0.9% sodium chloride.

(5) Preparation of Fibrinogen from Bovine plasma. For the removal of prothrombin by adsorption, the bovine plasma is treated with citrate-washed barium sulphate. The citrate-washed barium sulphate is prepared according to Biggs (1957) by suspending and resuspending the barium sulphate in 0.005 M trisodium citrate. The barium sulphate is thus prepared to get rid of the fine particles of barium sulphate which could not be sedimented at 2000 r.p.m.

The ox blood was collected in the slaughter house with 3.5% potassium oxalate as the anticoagulant as suggested originally, but we have observed that by using potassium oxalate in aqueous solution the blood is hemolysed and so the calculated amount of potassium oxalate in the proportion of 1:9 is used in the powder form. Plasma is collected by centrifuging at 2000 r.p.m. at 0°C and to each 100 ml. of plasma 2 gm. of citrate-washed barium sulphate as prepared above is added, the barium sulphate plasma suspension is gently agitated for 15 to 20 minutes, barium sulphate is removed by centrifuging at 2000 r.p.m. at 0°C. A second adsorption with barium sulphate is performed and then the plasma is tested by the one stage method by addition of a mixture in equal parts of adsorbed plasma, brain extract and calcium chloride. If the clotting takes place before 30 minutes, that shows the plasma is not free from prothrombin and a third treatment with barium sulphate is done and the plasma is tested again. The adsorbed plasma is then stored in small quantities in universal containers with metallic screw caps at -12°C.

(6) Preparation of thromboplastin. Thromboplastin is prepared from fresh human brain collected from the hospital. The brain is freed of all blood vessels and membranes and mixed up with 1500 ml. of 0.9% sodium chloride and an

emulsion is made with an Atomix blender. The emulsion is then centrifuged at 2000 r.p.m. at 0°C for ½ hour and the supernatant is collected. The supernatant with varying dilutions, namely 1:1, 1:2, 1:4, 1:8 with 0.9% sodium chloride, is tested for the shortest clotting time by the one stage method and the supernatant then diluted to that dilution which gives the shortest clotting time. Some authors have recorded delayed clotting with 1:1 dilutions, but in our preparations it has been observed that 1:1 dilution gives the shortest clotting time, as shown in Table III.

Table III

Clotting time with various dilutions of the thromboplastin

Dilution	Clotting time
1:1	10 seconds
1:2	20 seconds
1:4	22 seconds
1:8	28 seconds

Finally Owren's buffer of 10% volume is added to the diluted thromboplastin and stored under the same conditions as the prothrombin-free plasma.

It must be mentioned here that the fibrinogen solution and the thromboplastin preparation is never used after two months' storage and

for daily requirement appropriate amounts of both are taken out from the frozen state. The amount that is left after each day's work is not used but is discarded.

Apparatus for measuring prothrombin: In the other fields of biochemistry laboratory methods have been extensively developed; for the measurement of coagulation there is no precise instrument to determine the end point of the clotting. People working in this field used to tilt the tube this and that way and the period is thus recorded when after tilting the liquid does not move, indicating formation of a firm clot. To overcome this difficulty we have devised a simple arrangement as shown in Fig. 1. To an ordinary reagent bottle of about 3 litre capacity (E) is fitted one T-tube and a second open-ended glass tube. The stem of the T-tube goes almost to the bottom of the reagent bottle which is nearly half-filled with water. One limb of the T-tube is connected to a hand bellows and the other limb to a syringe needle by means of rubber tubing. The tip of the needle is slightly bent upwards like a fish-hook. By adjusting the screws on both sides of the T-tube a constant pressure can be maintained inside the bottle (E). The other parts of the apparatus are (i) a chromatographic tank filled up with water, the temperature of water is kept at 37°C with the thermostat (b), (ii) a table lamp (D) behind the chromatographic tank, (iii) a wooden bar running across the tank and fitted with a thermometer (B) and some terry-clips for holding small tubes. The tube (C) with 0.5 ml. of plasma, 0.5 ml. of thromboplastin and 0.5 ml. of fibrinogen is incubated for 3 minutes to raise its temperature to that

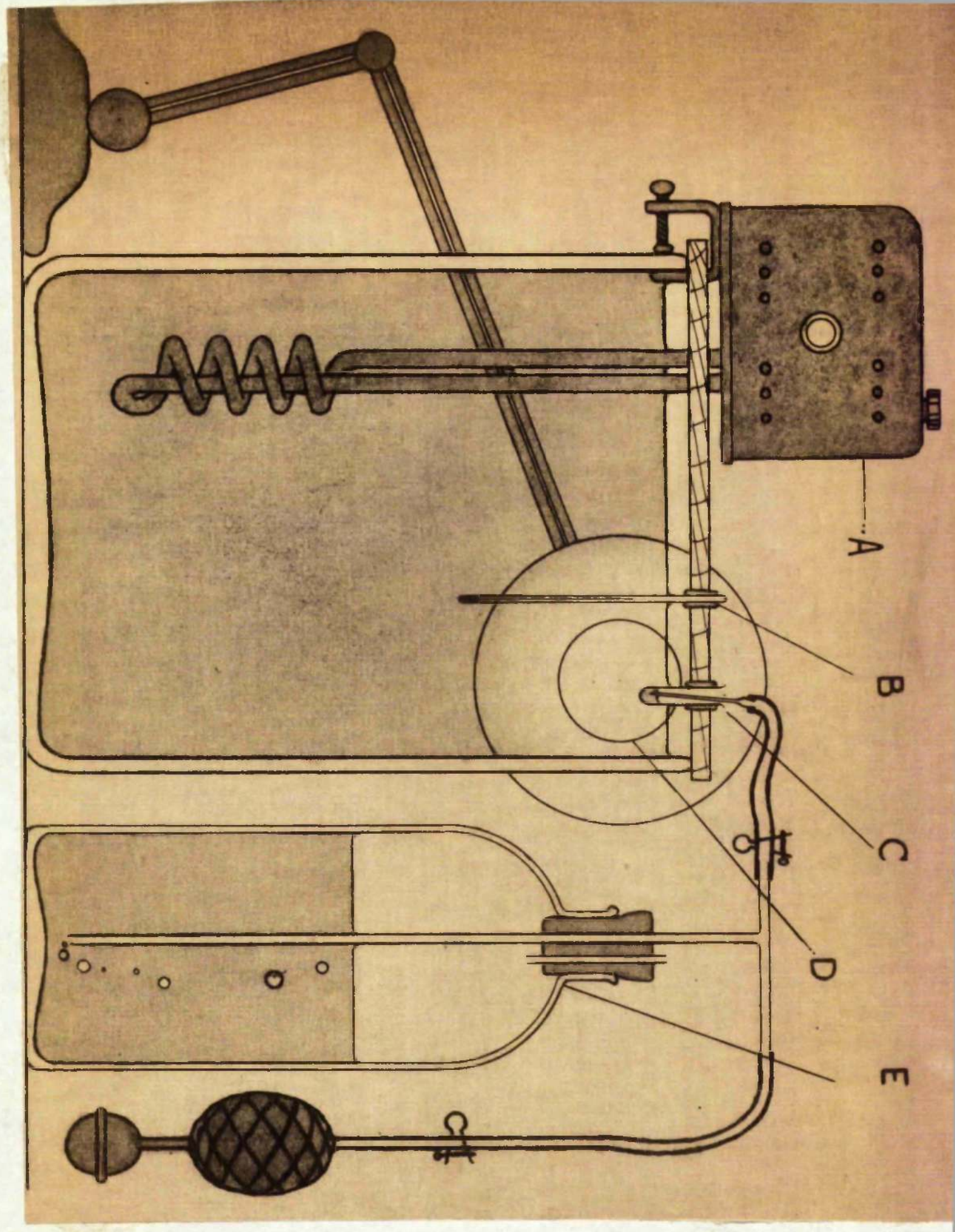


Fig. I

Apparatus for measuring prothrombin concentration

of the bath. While waiting during the incubating period, a constant pressure in the bottle is produced by pressing on the hand bellows so that immediately after addition of 0.5 ml. of 1/30 M calcium chloride at the end of the period, the needle can be put into the tube (C). The air bubbles come through the needle at a constant rate. When the process of coagulation reaches its end point the air bubble is arrested by the firm coagulation. The time taken from the addition of calcium chloride to the arrest of air bubble is the coagulation time. This device gives such a precise end point that one can judge the time to the fraction of a second. The air bubble not only gives the end point but it also helps in mixing the samples in the tube. The reproducibility of the method is seen from the results tabulated in Table IV.

Table IV

Reproducibility of coagulation time by one stage method with varying strengths of prothrombin.

Strength	Coagulation time in Sec.
100%	35, 35, 35½, 36
50%	48, 48, 47
20%	71, 72
10%	90, 90

The 100% prothrombin is made by diluting the plasma in the first diluting fluid only and the subsequent strength of prothrombin is prepared by diluting the 100% prothrombin in varying proportion of second diluting fluid.

This device was originally used for our study in one stage method only but it can be used for the two-stage measurement of prothrombin. After we developed this device a description for an automatic time recorder was published by Toohey and Cook (1960). The main principle behind their device is to some extent similar to ours - when there is firm clot formation the continuity of the electric circuit is broken and thus the automatic time recorder stops.

Dilution curve: For the measurement of prothrombin unit in terms of per cent of normal strength a dilution curve as shown in Fig. 2 is prepared with different strength of prothrombin with 1/30 M calcium chloride. From this dilution curve using the formula

$$y = bx + a$$

the prothrombin unit in the suspension of the different fractions of liver cells is calculated. Like Allington we have also found that 1/30 M calcium chloride is the optimum strength and hence calcium chloride of that strength has been used throughout this investigation.

Estimation of Protein: Protein of the different fractions in suspensions have been determined after Lowry et al (1951) by diluting the mitochondrial the microsomal (heavy and light) suspensions 1:40 and cell sap 1:100.

Prothrombin Activity: In this study the prothrombin unit has been expressed as follows in relation to protein:-

Units of prothrombin in 0.5 ml. suspension calculated from the dilution curve

mgm of protein per 0.5 ml. of the suspension.

Animals: Male albino rats weighing between 200 - 250 gm. of the Departmental

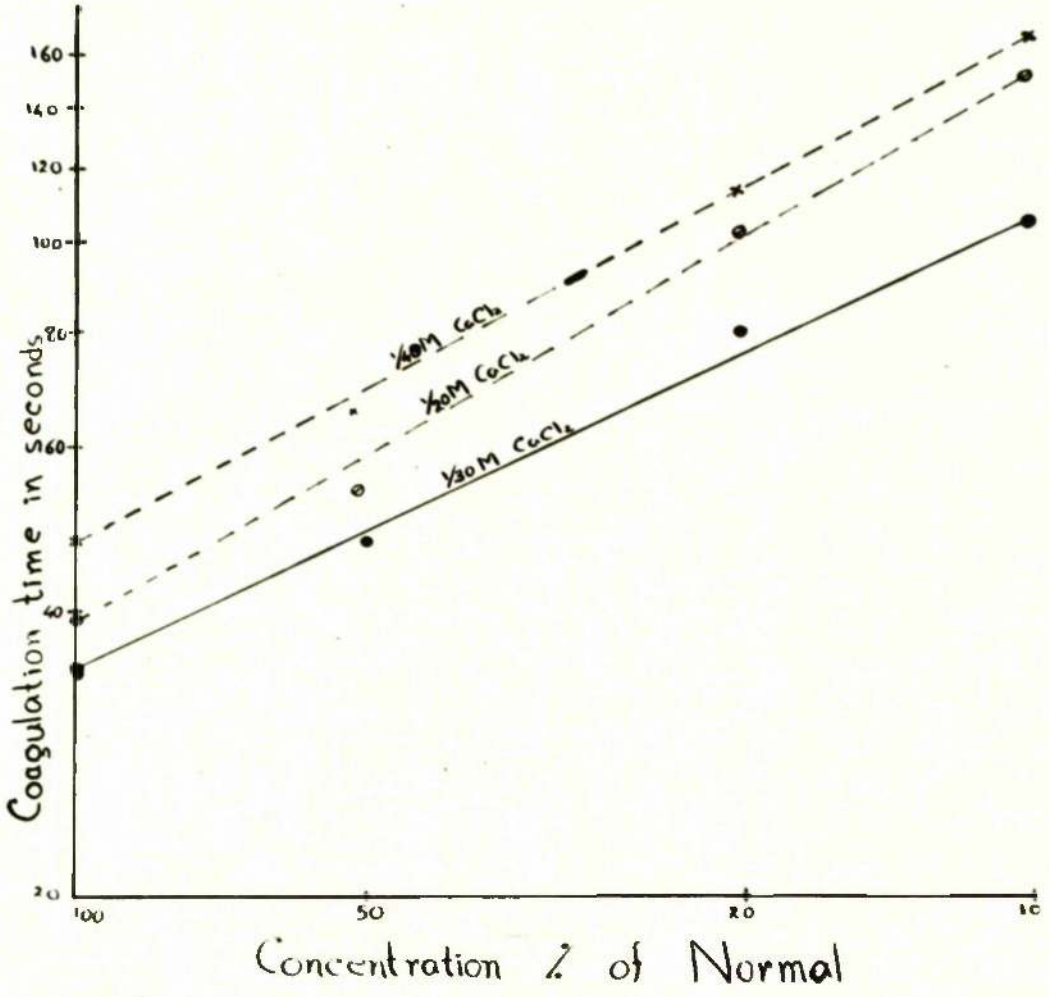


Fig. II

Dilution curve showing the concentration % of normal. The effect of different concentrations of calcium chloride has been also demonstrated.

stock has been used throughout this investigation. The rats were fed on the usual stock diet.

Preparation of Homogenate and isolation of Fractions: The rat is killed by striking it on the head, the liver is exposed immediately and removed from the body and washed with ice cold 0.25 M sucrose solution, then put in between two sheets of filter paper and thus the sucrose solution is removed from the liver. The liver is then weighed and transferred to a beaker in ice. It is then chopped with ice cold 0.25 M sucrose solution and finally homogenised with 0.25 M sucrose solution, using a total volume of 2.5 times the wet weight of liver, in a Potter-Elvenhjem homogenizer equipped with a plastic pestle, by 8 - 10 up and down strokes. The homogenization is carried out in ice.

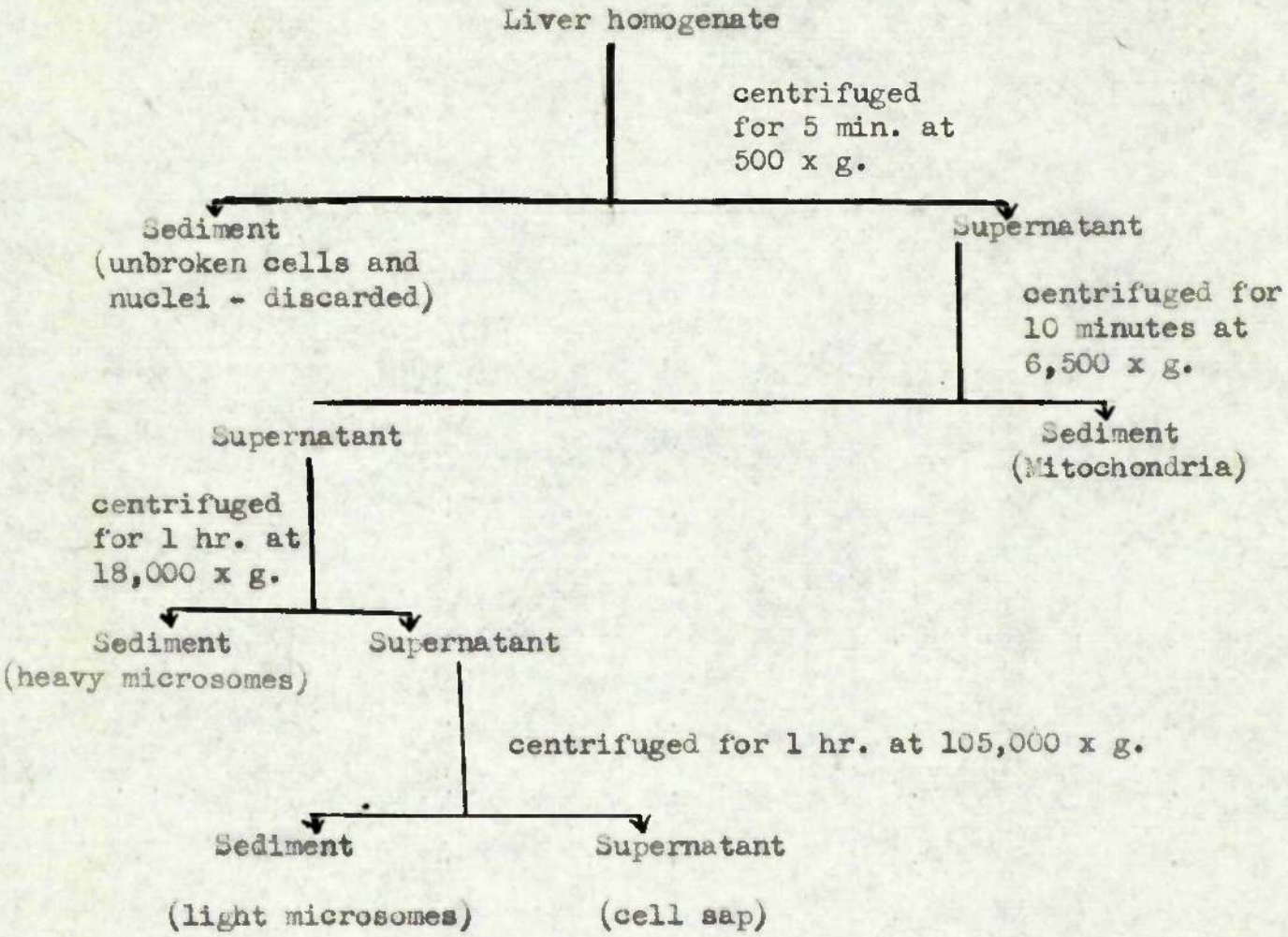
The homogenate is centrifuged and different fractions are isolated as shown in the flow sheet(Fig. 3).

All centrifugations have been carried out at $0^{\circ}C$, the final one being performed in a preparative Spinco and the rest in the MSE refrigerated centrifuge. We have separated the cytoplasm into (a) mitochondria (6,500 g x 10 min), (b) a heavy microsomes fraction (18,000 g x 1 hour), (c) a light microsome fraction (105,000 g x 1 hour) and (d) cell sap, the supernatant remaining from the last spin. The heavy microsomes deposit at the bottom of the centrifuge tube as bright reddish sediment covered with fluffy layer which have also been included as the heavy microsomes.

Preparation of medium: The medium for the incubation of the different fractions is composed of:-

Fig. 3

Scheme for preparation of different fractions of liver cell.

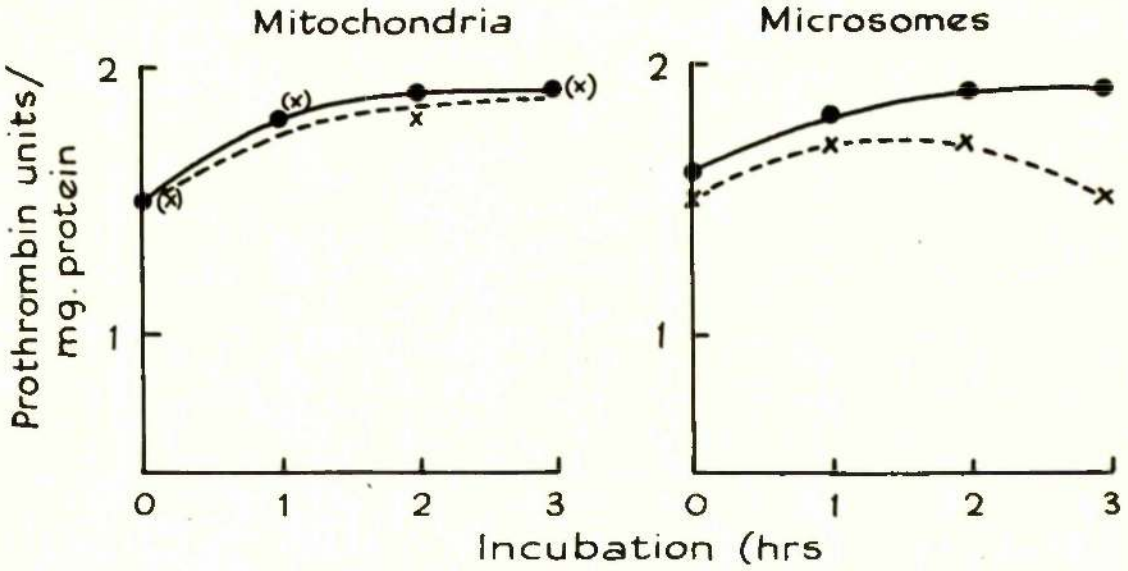


NaCl - 0.127 M, kcl - 0.047 M, KH_2Cl - 0.0012 M, Mg SO ($7H_2O$) - 0.0012 M, $NaHCO_3$ - 0.025 M, pH 6.9.

RESULTS

First we studied conditions which eventually led to evidence of prothrombin formation.

Selection of medium for incubation: Lasch & Roka (1953) incubated their mitochondrial suspension in veronal buffer and obtained activity at an optimum pH of 7.9. To minimise the effect of calcium ions present in liver they added sodium oxalate to the incubation media, though later Bernhart observed that the presence of calcium in traces in the incubating media accelerated the initial reaction in prothrombin regeneration by mitochondria. Pool & Robinson (1959) on the other hand incubated liver slices in Peters & Anfinsen (1950) medium and observed the formation of proconvertin in an alkaline medium. We incubated liver slices in Peters' medium and the subcellular particles both in Peters' medium over a different pH range and in veronal buffer (pH 7.9) and observed that, when the liver slices were incubated in Peters(medium at 37°C in an atmosphere of O_2:CO_2 (95:5%) with constant shaking for periods up to 3 hours, there was practically no increase in prothrombin activity. Subcellular particles in veronal buffer behaved in the same way, as seen in Fig. 4; but when these particles were incubated in Peters' medium there was some increase in prothrombin activity in the first hour, then the activity dropped due to a change of pH of the medium. The results obtained from a pH range of 7 - 8.5 has been tabulated in Table V. We then tried with Krebs-Ringers



Figs. IV and V

Mitochondria and microsomes of rat liver incubated in Peters and Anfinsen's (1950) media, and in veronal buffer (pH 7.9)

Peters and Anfinsen's media.

veronal buffer.

Table V

Effect of varying pH of the incubating media (Peters & Anfinsen)

Prothrombin Activity in Unit

Incubation Period in Hours

pH	0	1	2	3
7.0	1.9	1.5	1.4	1.4
7.5	1.3	1.3	1.9	-
8.0	1.0	1.4	1.3	1.1
8.5	1.7	1.4	1.3	1.0

(The heavy microsomal suspension was incubated with Peters and Anfinsen medium up to a period of 3 hours in an atmosphere of O_2:CO_2 at 37°C with constant shaking).

bicarbonate solution with the omission of calcium chloride, as it has been pointed out that the presence of calcium chloride in the medium is necessary only for the utilization of glucose (Peters - 1950), moreover, calcium chloride interferes with the estimation of prothrombin by starting coagulatio before the calcium is added in its estimation. At pH 7.6 there is some increase in activity of prothrombin up to 2 hours of incubation as shown in Fig. VI. From the figure it is clear that in the pH range between 6.5 - 7.0 the increase activity of prothrombin is sustained for a longer period and so the fractions of the liver have been incubated in the modified Krebs-Ringer's bicarbonate solution at a pH 6.9 in the conical flasks in an atmosphere of O_2:CO_2 (95:5%). The freshly prepared particles were suspended in a solution of 0.25 M sucrose solution (volume 1.5 x weight of the wet liver taken). To obtain a uniform suspension, the particles in the sucrose solution are transferred to the homogeniser tube and at a very low speed the pestle is moved up and down 2 - 3 times. To the suspension one modified Krebs-Ringer's bicarbonate medium (volume 1 x original weight of wet liver) has been added. Into each incubation flask, a total volume of 4 ml. of the final suspension was taken.

Reaction: Lasch and Roka (1953) observed that pH 7.9 was the optimum pH for prothrombin formation when they incubated mitochondria for activation of prothrombin. Alkjaersig and Seegers (1955) also maintained the same pH for the formation of prothrombin for autoprothrombin with the addition of mitochondria. Barnhart (1957) demonstrated that at pH 7.9 there was activity regeneration of prothrombin but with lowering of pH the rate of

prothrombin formation increased and he maintained that the optimum pH at which to study prothrombin formation is 7.5; below pH 7.0 there is loss of some activity of prothrombin. Our samples have been incubated at pH 6.9 and we have, as pointed out before, observed that between pH 6.5 to 7.0 the formation of prothrombin activity lasted for a longer time (Fig. VI). The former group of workers have incubated the samples for a period of 1 hour only and we have also noticed that for that period of incubation pH 7.5 is best, but for the longer period of incubation a pH range between 6.5 to 7.0 is the best; at pH 6.0 there is only a very slight increase in activity and it has been observed that an extreme of either acid or alkaline reaction clumping of the particles occurs after 1 hour incubation period.

Activity associated with different fractions: Having found activity with the modified Krebs medium, we examined different cell fractions. From Table VI it is evident that in the first hour of incubation there was no significant increase in activity of prothrombin in the mitochondrial suspension, but some increase in activity was observed at the second hour. From the very beginning of incubation the heavy and the light microsomal suspension showed synthesis of prothrombin, the effect being more marked in the case of heavy microsomes and after two hours' incubation period the synthesis is more or less a steady one. In the cell sap, prothrombin concentration declines during incubation.

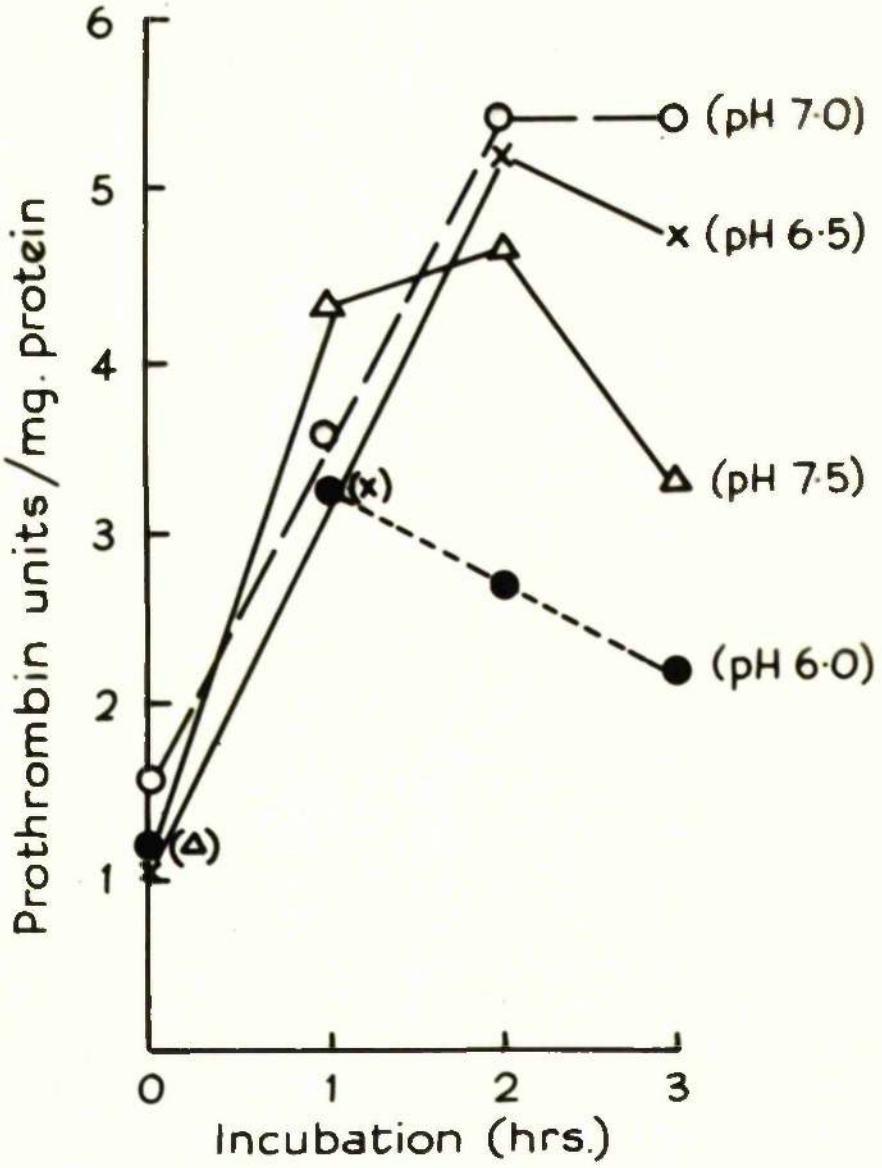


Fig. VI

Effect of incubation of heavy microsomes in different pH of modified Krebs-Ringers bicarbonate solution on prothrombin formation.

Table VI

Synthesis of Prothrombin by the different fractions of the liver cell incubated in 0.25 M sucrose + modified Krebs-Ringers bicarbonate solution at pH 6.9 at 37°C with constant shaking.

Samples	Prothrombin Units/mgm of Protein Incubation Period in hours			
	0	1	2	3
Mitochondria				
Mean	3.37	3.76	4.66	3.68
S.D.	1.10	1.15	0.60	0.94
St. error	0.64	0.68	0.35	0.55
Heavy Microsomes				
Mean	3.70	5.07	8.10	8.90
S.D.	0.47	0.21	0.87	1.00
St. error	0.28	0.12	0.51	0.59
Light Microsomes				
Mean	2.77	3.80	4.48	4.47
S.D.	0.76	0.82	0.57	0.64
St. error	0.45	0.48	0.33	0.37
Cell Sap				
Mean	1.00	0.77	0.73	0.70
S.D.	0.55	0.47	0.44	0.44
St. error	0.32	0.28	0.26	0.26

(The result is the average of 3 experiments with a single rat in each).

Effect of incubation in nitrogen: For the synthesis of proteins in vitro by liver slices, an atmosphere of oxygen is essential. Lasch and Roka (1953) incubated mitochondria both in O_2:CO_2 and N_2:CO_2 atmosphere and found that in both instances there was an increase of prothrombin activity, though synthesis was more pronounced in O_2:CO_2 atmosphere. The effect of two different atmospheres has been tried by us. In one set of conical flasks, the suspension of heavy microsomes was taken under the same condition as described before and gassed with O_2:CO_2 for about ½ minute, and the other set were gassed with N_2:CO_2 for the same period of time; all the flasks were then incubated at 37°C with constant shaking, and prothrombin measured at different time intervals. The results are tabulated in Table VII. The results showed that, up to the end of the second hour of incubation there is no significant difference between the two samples but there is a highly significant difference between the two samples when are incubated for a 3-hour period. Stefanini (1949) studied the effect of ultrafiltration and carbon dioxide on the antithrombin activity of fresh and stored human plasma, and noticed that, when thrombin plasma mixture is incubated in an atmosphere of CO_2, O_2 and N_2, the clotting times in the first two minutes in all three samples were almost equal, whereas during the third minute of incubation the clotting time in the N_2 atmosphere was almost double that of the other two. This delayed effect of N_2 might explain the difference of prothrombin content in the last hour of incubation between the two different atmospheres. It might also be due to effects of oxygen lack on microsome integrity.

Table VII

Effect of oxygen and nitrogen atmosphere on the synthesis of prothrombin. Conditions for incubation have been described in the text (mean data from three experiments).

Sample	Prothrombin Units/mgm of Protein			
	Incubation Period in Hours			
	0	1	2	3
O_2:CO_2				
Mean	2.70	5.55	6.35	6.65
S.D.	0.52	0.64	0.16	0.07
St. error	0.30	0.37	0.09	0.04
N_2:CO_2				
Mean	3.55	4.95	5.70	5.90
S.D.	0.35	0.35	0.71	0.14
St. error	0.21	0.21	0.41	0.08

Effect of DNP: Pool and Robinson (1959) observed in liver slices that with the addition of dinitrophenol to the incubation medium there was inhibition of the synthesis of prothrombin and Factor VII and suggested that the mechanism of prothrombin synthesis by the liver slice might involve oxidative phosphorylation. It is known that the mitochondrial particles are associated with enzymes that promote oxidative phosphorylation and that dinitrophenol inhibits the process.

To test the action of dinitrophenol on the prothrombin activity associated with microsomes of the liver we designed the following experiment. The same heavy microsomal suspension was taken in two sets of conical flasks. To one set we added 0.2 ml. of 0.25 M sucrose solution, the pH of which had been previously adjusted to 6.9 and this was regarded as the control series. To the other set 0.2 ml. of dinitrophenol (3×10^{-4} M, pH 6.9) was added and incubated as before and the amount of prothrombin units measure at different time intervals. The results are shown in Figure VII. It is clear that there is no significant difference of prothrombin synthesis in both the groups up to the end of 2 hours incubation period, but again at the third hour the microsomal suspension incubated with the dinitrophenol showed a marked inhibition of prothrombin synthesis, the cause of which cannot be explained.

Effect of disintegration of the microsomal particles: Douglas and Munro (1958) have shown that there is an apparent increase of the amylase content of the different cell fractions of the pigeon pancreas following treatment with ballotini beads, the marked and significant increase being in the

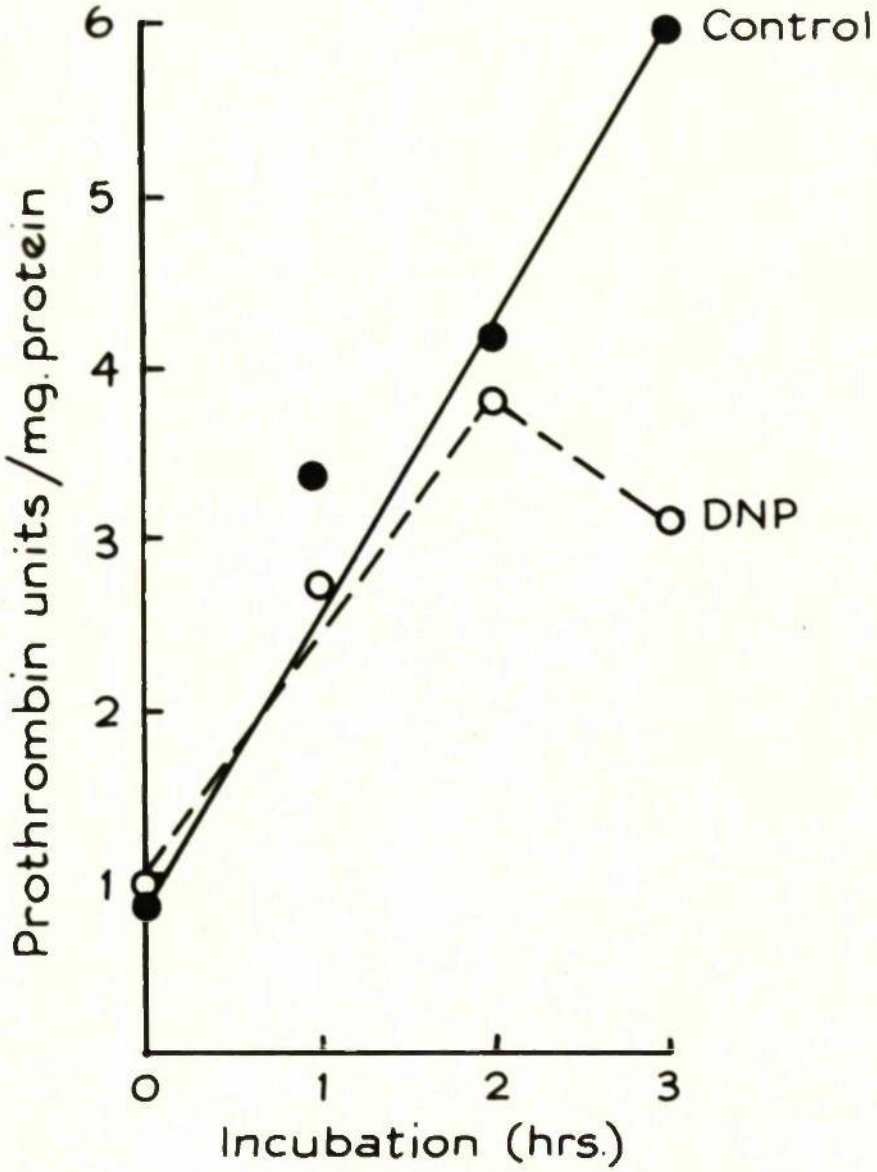


Fig. VII

Effect of dinitrophenol:- 0.2 ml. of dinitrophenol (3×10^{-4} M, pH 6.9) was added to the system and incubated under the same experimental conditions as the control.

control
+ dinitrophenol

microsomal (heavy microsomes) fractions. This suggests that inactive amylase is present in the interior of the vesicles and when the vesicles disintegrate the pre-existing amylase is released. The increase in activity of prothrombin in the microsomal particles might also be the only release of the preformed protein, especially since the synthesis of prothrombin occurs without any addition of energy source or any exogenous amino-acids. To test this 2 ml. of the microsomal suspension adjusted to pH 6 was taken in a 5 ml. universal container and 1 ml. of fine glass beads (Ballotini No. 12, English Glass Co., Leicester) was added. The container was covered with ice and attached to a Nelco-micro blendor. The blendor was then run at full speed for 2 minutes, then allowed to stand to permit settling of the glass beads. The supernatant was taken and incubated under the same conditions laid before and prothrombin is measured. The control group was untreated. In another set of experiments a portion of the incubated sample (control group) was taken out and treated with the ballotini beads and prothrombin was then measured. The results have been tabulated in Table VIII and in Fig. VIII.

From the tabulated results and also from the figure it is clear that there is no difference in initial prothrombin activity between the control and the test groups. In the test group (suspension first treated with ballotini beads and incubated thereafter) the activity failed to rise significantly and there was a considerable difference from the control series from the second hour onwards, but in the other group where the suspension was treated with ballotini beads after incubation there was no

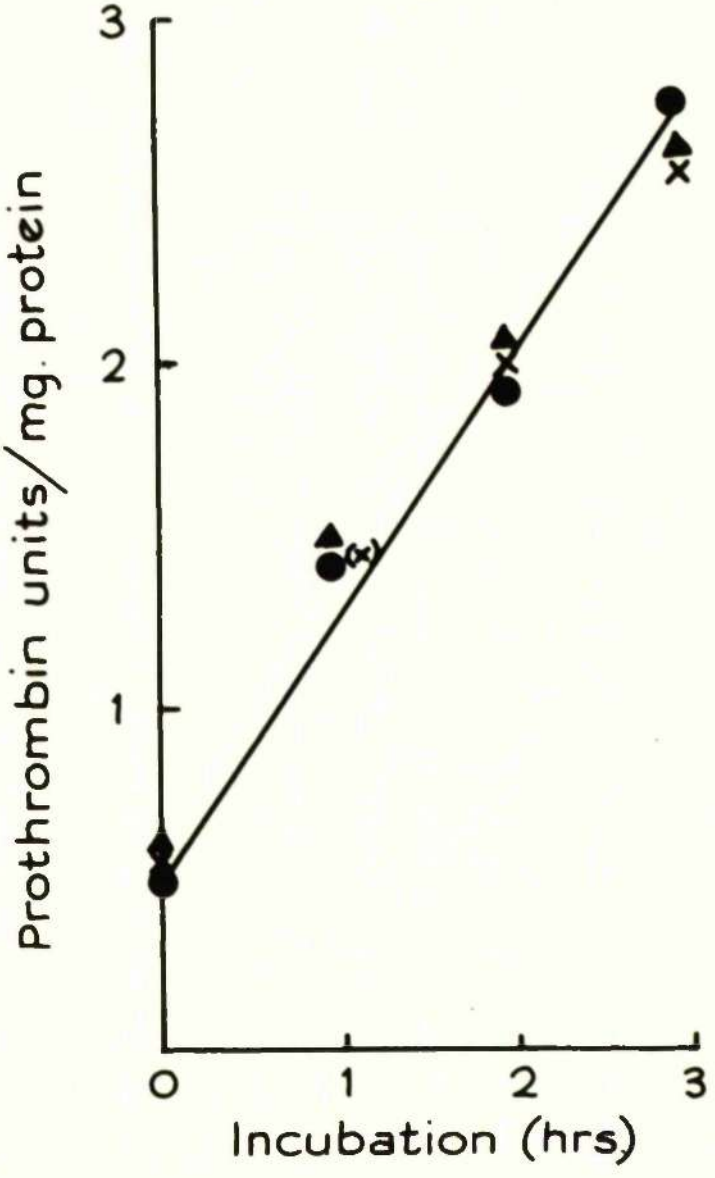


Fig. VIII

Effect of disintegration:- The heavy microsomes were incubated and then disintegrated with ballotini beads (▲), and by ultrasonic vibration (×), control (●).

such difference between the control and the test group (Fig. VIII). The lowering of activity in the first test group after 1 hour of incubation may be due to alteration of pH of the suspension; when disintegrated with ballotini beads, the suspension became slightly alkaline. Whatever might be the reason for the fall it is certain that there is no release of prothrombin bound to the microsomal vesicles, otherwise there would have been a higher value of prothrombin from the beginning.

For further confirmation the microsomal suspension in another series of experiments was disintegrated by ultrasonic vibrations and in that case also no difference of prothrombin activity has been noticed from the control group (Fig. VIII).

This experiment also suggests that the increased activity of prothrombin is not due to contact of the prothrombin with the glass surface of the incubating flasks as it has been suggested that contact with glass or a rough surface accelerates the conversion of prothrombin to thrombin (Waaler - 1958).

Effect of extra energy supply to the system: Although the use of nitrogen and of dinitrophenol did not show any immediate effect of deleting oxygen supply on prothrombin formation, we thought it advisable to determine whether addition of ATP and ADP to the medium might provoke an increased rate of synthesis. When the system is incubated with an added supply of ATP we did not notice any more synthesis of prothrombin than the control group which is incubated without any supply of ATP. (Table IX). Straub and others (1961) have demonstrated the synthesis of amylase with amounts

Table VIII

Effect of disintegration of the microsomal particle on the synthesis of prothrombin. The conditions for incubation and disintegration have been described in the text. The result is the average of three experiments - one rat in each.

Samples	Prothrombin Units/mgm of Protein Incubation Period in Hours			
	0	1	2	3
Control:				
Mean	3.03	5.67	6.40	7.10
S.D.	0.67	0.49	0.10	0.90
St. error	0.39	0.29	0.06	0.53
Test (suspension treated with ballotini and then incubated)				
Mean	3.27	4.50	3.57	2.53
S.D.	0.93	1.23	0.76	0.15
St. error	0.54	0.72	0.45	0.09

of ATP in the system as high as 50 μmole in the incubation medium. We therefore thought that the concentration of ATP as high as only 1.5 micromole per ml. might not be sufficient to stimulate an increased synthesis of prothrombin, and in another set of experiments where the system was incubated with ATP as high as 15 micromoles per ml., no extra synthesis of prothrombin was observed (Table X); rather, towards the later period of incubation the higher concentrations inhibited formation and there was a tendency for destruction of prothrombin to occur, as shown in Table X.

Clark, Goodlad, Chisholm and Munro (1960) have demonstrated that there is an increased content of ATP in rat liver after feeding with carbohydrate. Experiments were, therefore, carried out in which the rats were fed with 3 grams of glucose at 2 hours prior to killing. As shown in Table XI, there was an increased synthesis of prothrombin as a result of this pre-feeding treatment. The microsomal particles are known not to have the capacity to use carbohydrate in vitro and so we have not tried to see if there was any effect of addition of glucose to the system.

Oxidative phosphorylation and prothrombin synthesis: In the study of the incorporation of labelled amino-acids into the protein in mitochondrial systems it was observed that the addition of DPN (new nomenclature NAD) or DPN and ATP stimulated incorporation (Roodyn, Reis and Work -1960, 1961). Glock et al (1957) have determined the total

amount of DPN, DPNH (new nomenclature $NADH_2$), TPN and TPNH (new nomenclature NADP and $NADPH_2$ respectively) in the normal liver tissue, respectively 398 ± 10, 182 ± 8, 7 ± 1 and 217 ± 5 µgms per gram of liver tissue. The addition of equivalent amounts of these nucleotides to the incubating mixture used by us did not stimulate the formation of prothrombin activities as seen from the results presented in Table XII and Table XIII. Rather, it has been observed that in the groups where DPN or TPN has been added alone or along with ATP, the synthesis of prothrombin is much less than in the control flasks prepared from the same livers. DPNH was much less inhibitory (Table XII) and TPNH seemed not to affect formation at all (Table XIII).

Since some proteolytic enzymes (trypsin and papain) can convert prothrombin to thrombin (Alexander - 1958), the amount of protein in the microsomes was examined during incubation under our conditions. The extent of protein loss was small (Table XIV).

Table IX

Effect of addition of ATP and ADP to the system on prothrombin formation by heavy microsomes. (Varying amounts of ATP and ADP were dissolved in KRB solution and the pH was adjusted to 7.0 before adding to the system. The microsomal suspension in 0.25 M sucrose incubated in modified KRB solution at 37°C in an atmosphere of O_2:CO_2 (95:5) for 3 hrs. At the end of 1½ hrs. a fresh amount of ATP and ADP solution was added to each flask to compensate for loss of ATP and ADP during incubation).

Sample	Concentration in µmoles per ml. incubation medium	Prothrombin Units/mg. Protein			
		Incubation Period in Hours			
		0	1	2	3
Control	0	1.25	2.20	3.00	3.20
+ ATP	0.5	1.10	1.90	2.50	2.40
	1.0	1.15	1.90	2.50	1.90
	1.5	1.45	2.40	3.15	3.30
+ ADP	0.5	1.20	1.95	2.75	2.90
	1.0	1.30	2.15	2.40	2.55
	1.5	1.50	2.22	2.90	2.60

The results are the average data of two experiments.

Table X

Effect of higher concentration of ATP and ADP on prothrombin formation by heavy microsomes. (As before, the ATP and ADP were made in a solution of KRB and the pH was adjusted to 7.0. Varying concentrations of ATP and ADP were added to the system at the beginning of the experiment and at the middle of the 3-hr. incubation period. The system was incubated as before. The results were obtained in one experiment).

Sample	Concentration in µmoles per ml. incubation medium	Prothrombin Units/mg. Protein			
		Incubation Period in Hours			
		0	1	2	3
Control	0	0.70	1.60	2.30	2.50
+ ATP	2	0.60	1.20	1.90	2.50
	4	0.60	1.50	1.10	0.90
	8	0.70	0.60	0.60	0.60
	15	0.60	0.60	0.50	0.30

Table XI

Effect of feeding the rats with glucose before killing on prothrombin formation by heavy microsomes. (Rats fasted overnight were given 3 gm. glucose and killed 2 hrs. later; control animals were kept fasting. The conditions of incubation of the heavy microsomes fraction were as before. The results are the average data from three experiments).

Sample	Incubation Period in Hours			
	0	1	2	3
	Prothrombin Units/mg. Protein			
Control:				
Mean	0.63	1.50	1.93	2.53
S.D.	0.29	0.34	0.40	0.50
St. error	0.13	0.19	0.23	0.29
Test:				
Mean	0.90	2.00	3.27	4.20
S.D.	0.26	0.34	0.31	0.40
St. error	0.15	0.20	0.18	0.23

Table XII

Effect of DPN and DPNH to the incubating system on prothrombin formation by heavy microsomes. (DPN and DPNH in varying concentrations were dissolved in KRB solution and the pH was approximately adjusted with indication paper to 7.0 and then added to the incubating mixture. The system was incubated in KRB solution at 37° in an atmosphere of O_2:CO_2 (95:5) for 3 hrs. with constant shaking. The results are the average data of two experiments).

Sample	Concentration in micromoles per ml. of incubation mixture	Incubation Period in Hours			
		0	1	2	3
		Prothrombin Units/mg. Protein			
Control	0	1.60	3.05	4.20	4.55
+ DPN	0.5	1.45	2.15	2.30	2.20
	1.0	1.55	2.25	2.40	2.30
	1.5	1.60	2.30	2.35	2.15
+ DPNH	0.3	1.50	2.15	2.80	3.10
	0.5	1.45	2.15	2.85	3.20
	1.0	1.60	2.25	2.75	3.05

Table XIII

Effect of TPN and TPNH on prothrombin formation by heavy microsomes.

(TPN and TPNH in varying concentrations in KRB solution, adjusted to pH 7.0, were added to the incubation mixture. The system was incubated in KRB solution at 37° in an atmosphere of 0:CO (95:5) for 3 hrs. with constant shaking. The results are the average data of two experiments).

System	Concentration in micromoles per ml. of incubation mixture	Incubation Period in Hours			
		0	1	2	3
		Prothrombin Units/mg. Protein			
Control	none	1.05	1.90	2.70	3.10
+ TPN	0.001	0.95	2.00	2.70	2.65
+ TPN	0.005	1.05	2.05	2.70	2.45
+ TPN	0.010	1.10	2.15	2.85	2.75
+ TPNH	0.250	1.05	2.10	2.90	3.60
+ TPNH	0.500	0.95	1.85	2.30	3.05
Control*	none	1.20	1.70	2.70	3.20
+ TPN + ATP*	0.010 (TPN) + 1.50 (ATP)	1.20	1.80	2.20	2.30
+ TPNH + ATP*	0.250 (TPNH) + 1.50 (ATP)	1.00	2.00	2.20	1.90

* Result of one experiment only.

Table XIV

Protein content in µgm per 0.5 ml. of the heavy microsomal suspension at different incubation periods. 0.5 ml. of the suspension drawn from the incubating flask is diluted 1:40 and 1 ml. of this diluted solution is taken for the estimation of protein by Lowry's method. The result is the average of estimations on eight different samples.

	Protein in µgm per 0.5 ml. suspension			
	Incubation Period in Hours			
	0	1	2	3
Mean	1378.0	1318.0	1379.0	1323.0
S.D.	204.8	142.2	200.6	175.7
St. error	73.1	50.0	71.6	62.7

DISCUSSION

The results presented here definitely indicate that when the different fractions of liver cells are incubated in the conditions described in the experimental section, the capacity to form prothrombin is associated with the microsomes and notably with the heavy microsomes.

Pool and Robinson (1959) observed a net increase of Factor VII on incubation of liver slices; about 80% of this increase was in the tissues and 20% in the incubation medium. A much smaller quantity of prothrombin was formed in the tissues and none appeared in the medium. In contrast to Lasch and Koka (1955) they failed to obtain any evidence of synthesis of Factor VII and prothrombin by mitochondria, and hence suggested that intact cells were necessary for the synthesis of Factor VII. Their findings regarding the failure of synthesis of Factor VII and prothrombin by mitochondria corresponds to our findings but we do not believe that intact cells are necessary for prothrombin synthesis as we observed a considerable increase of prothrombin activity on incubation of the heavy microsomes under our conditions.

Pool and Robinson (1959) further noticed inhibition of synthesis by the liver slices on addition of sodium cyanide, dinitrophenol, chloroform, dicumarol, or tromexan to the medium, when kept in the cold, or when incubated in an atmosphere of nitrogen, and they suggested that the whole process is energy-dependent, requiring oxidative-phosphorylation, and probably vitamin K dependent. Our finding that the system is not affected by addition of metabolic inhibitor like dinitrophenol (3×10^{-4}M, pH 6.9)

to the incubation medium (Fig. 7) indicates that increased activity in our cell free system is not energy-dependent nor dependent on oxidative-phosphorylation. The results showing that there is no effect on formation of prothrombin by addition of NAD, $NAIH_2$, NADP, $NADIH_2$ to the incubation medium further supports the view that the process is not one of oxidative phosphorylation. Similar observations have been obtained by Nisman (1959) in the study of incorporation of activation of amino-acids by the disrupted protoplasts of E. coli where the addition of ATP and diphosphate nucleotide to the incubation flasks did not increase incorporation. In our study also the system has been incubated in the same conditions and our observation are also similar to his findings. We have further observed that addition of an energy source (ATP or ADP) to the incubation medium did not increase the activity of prothrombin though an energy-generating system is known to be essential for the synthesis of other proteins by liver microsomes from amino acids through carboxyl activation. With high concentration of ATP or ADP there was some inhibitory effect on the formation of prothrombin most probably due to adsorption of prothrombin by phosphate.

Unlike Lasch and Roka (1953) our system does not need any addition of serum to the incubation medium to obtain prothrombin activity. Slight prothrombin activity associated with the mitochondria and light microsome fractions in the initial stage of incubation is not significant and might be due to contamination with the heavy microsomes in the preparation of different fractions, as all the fractions used for the study are unwashed and rather crude preparations. Prothrombin activity associated with the

heavy microsomes was not increased after disruption of the particles with ballotini beads or with ultrasonic vibration, showing that increased prothrombin activity during incubation was not due to release of pre-existing prothrombin, in contrast to the suggestion of Pool and Robinson that increase of Factor VII in the tissues and in the medium on incubation might be a simple passive release process. Though they observed an inhibitory effect of synthesis of Factor VII and prothrombin by the liver slices in an atmosphere of nitrogen, Lasch and Roka (1953) did not observe a similar finding in their study of mitochondria, nor did we in our observations up to a two-hour period of incubation, after which the inhibitory action of nitrogen may have arisen from structural effects on microsomes. From the available data it is not possible to explain the mechanism of the increase in activity, nor are we in a position to say that this increased activity of prothrombin after incubation represents synthesis of prothrombin by the microsomal particles, for the whole process has occurred without any addition of an energy source or an energy-generating system, nor are free amino-acids added to the medium.

SUMMARY

1. The microsomal system of the liver is capable of showing an increase in prothrombin activity when this fraction is incubated in a modified Krebs-Ringers bicarbonate solution. The heavy microsomes (sediment obtained after spinning the mitochondrial supernatant at 18,000 g for 1 hour) are seen to be more active than the light microsomes in this respect.

2. Whether the particles are incubated in an atmosphere of O_2:CO_2 or N_2:CO_2, there is no effect on the increment in prothrombin up to 2 hours of incubation.

3. Pre-treatment of the rat with glucose 2 hours before killing resulted in a considerably increased rate of formation of prothrombin. This effect is apparent throughout the whole period of incubation.

4. The early stages of this formation are not dependent on oxidative phosphorylation, since incubation of the heavy microsomal particles with dinitrophenol, NAD, $NADH_2$, NADP, $NADPH_2$ or mixture of ATP and NADP, ATP and $NADPH_2$ does not inhibit nor increase the formation of prothrombin. After some 2 - 3 hours of incubation, there is an inhibitory effect of DPN or TPN which is not shared by DPNH and TPNH.

5. The increased activity is not due to release of preformed prothrombin bound to the microsomal vesicles as there is no increase in activity when the microsomes are disrupted with ballotini beads or with ultrasonic vibration.

SECTION II

Relationship of ribonucleic acid to prothrombin formation by microsomal particles

S E C T I O N I I

Introduction

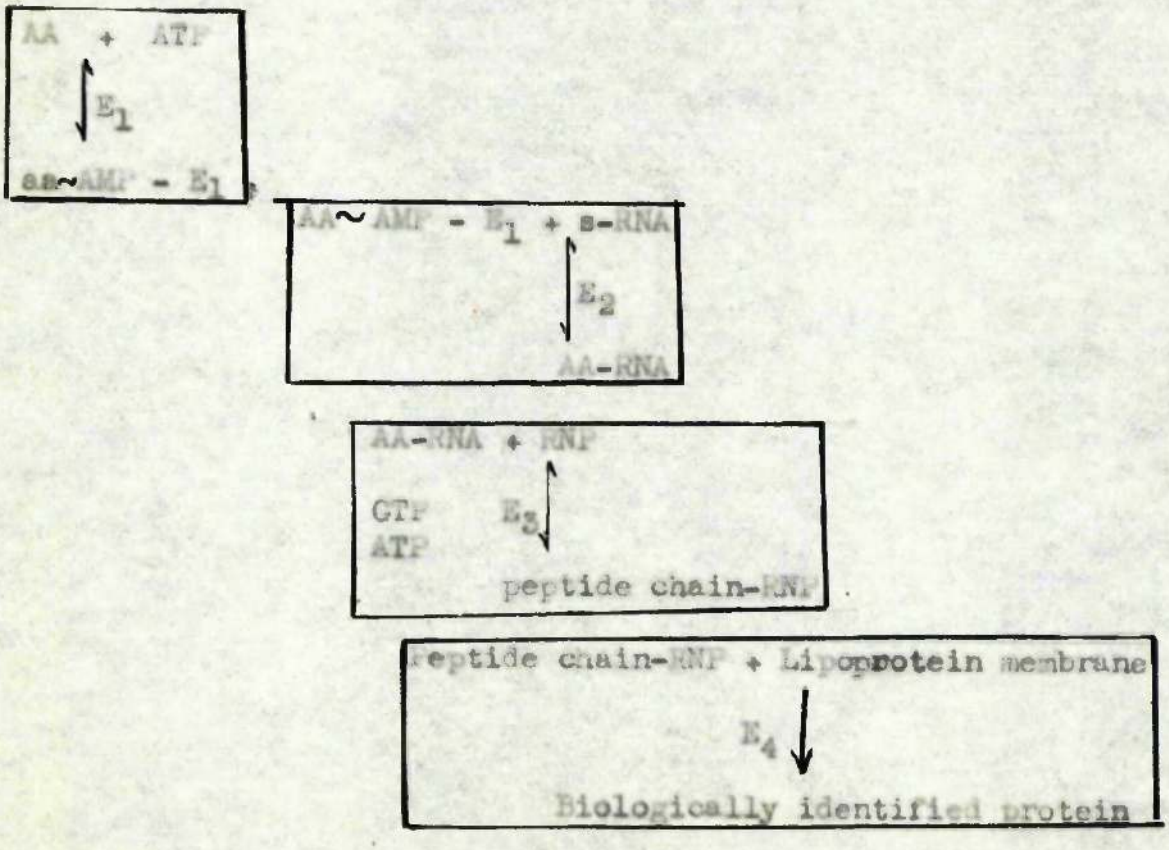
Observation of ribonucleic acid in abundance in the proliferating and in protein secreting tissues (Caspersson and Schultz - 1938), in the exocrine parts of the pancreas, in the pepsin-producing gastric mucosal cells, in the liver cells, in the nerve cells and in the young oocytes and embryoes undergoing differentiation, and a much less ribonucleic acid in the non-proliferating and non-secreting tissues like heart and kidney led to the hypothesis that ribonucleic acid of cells in some way or another is closely related to protein synthesis. Studies on microorganisms revealed a closer and quantitative relationship between the ribonucleic acid content of the cells and the rate of protein synthesis. It has been observed in some bacteria that the richer the RNA content, the higher is the rate of incorporation of labelled amino-acids and is another positive evidence for the relationship of RNA and protein synthesis (Keller et al - 1954, Littlefield et al - 1955, Allfrey, Daly and Mirsky - 1953, Borsook et al - 1950, Hultin - 1950, Siekevitz - 1952). It was demonstrated by groups of workers (Caldwell et al - 1950, Northrop - 1953, Wade - 1952, Price - 1952) in different laboratories that, during the logarithmic phase of growth of bacteria, the synthesis of ribonucleic acid runs parallel with the synthesis of protein. Gale and Folkes (1953) found that the capacity of disrupted staphylococci to synthesize protein is dependent upon their nucleic acid content. The capacity of synthesizing the enzymes after removal of nucleic acid could be restored by addition of RNA, in some cases the RNA of the same bacteria.

Chantrenne (1956) studied the problem from a different angle and showed that the synthesis of a specific enzyme protein is associated with synthesis of a new and possibly specific RNA; in recent years his conclusion has been subjected to criticism and it is believed to-day that simultaneous synthesis of RNA is not necessary for protein synthesis. However, the most recent evidence on messenger RNA suggests that it is used only once and this must be resynthesized.

Direct evidence of the relationship of RNA to protein synthesis has been demonstrated by the fact that ribonuclease strongly inhibits the incorporation of labelled amino-acids into protein in vitro experiments (Allfrey et al - 1953, Zamecnik and Keller - 1954, Straub, Ullmann & Acs - 1955), though it must be admitted that ribonuclease is also able to change the permeability of plant cells to ions (Lansing and Rosenthal - 1952), to evoke mitotic abnormalities (Kaufmann et al - 1954, 1957), to inhibit cell division, to liberate hydrolytic enzymes in homogenates and to destroy protoplasts. Ribonuclease does not inhibit respiration or phosphorylation in cells but it inhibits the incorporation of amino-acids into protein and thus can be regarded as having direct action on protein synthesis. The work of Ledoux (1954) on ascites tumour cells, showing the initial stimulation of RNA synthesis by ribonuclease, suggested that ribonuclease might inhibit protein synthesis by forming a protein complex rather than splitting the RNA. This view was later supported by Hultin et al (1957). Brachet (1954, 1955, 1956) observed that if the protein synthesizing system is exposed to ribonuclease for a period of 30 minutes there is complete blockage of protein synthesis, although during exposure to ribonuclease for

such a short period only a part of RNA is degraded. It has been therefore suggested that a partial degradation and/or with structural modification of RNA or destruction of certain type of RNA is sufficient to prevent the synthesis of protein (Jeener - 1959). Brachet and Six (1959) suggested that this certain specific type of RNA is soluble RNA.

In the cellular level the ribosomes contain about 80 of the total cellular RNA. Littlefield, Keller, Gross and Zamecnik (1955) have suggested that the cytoplasmic ribonucleoprotein particles are the site of initial incorporation of free amino-acids into protein. The free ribosomes are considered to be responsible for the synthesis of the cells own protein and the microsomal elements (endoplasmic reticulum) may provide for the synthesis of exportable protein (Petermann et al - 1954). Zamecnik, Stephenson and Hecht (1958) have outlined a four-step series of reactions in synthesis of protein as follows:-



The first two steps in the incorporation reaction can be studied in the absence of microsomes. To-day it is accepted that the formation of the amino-acyl adenylate compound is an enzymic reaction and this reaction is independent of ribonucleic acid, though a small inhibition of activation has been observed by Van der Decken and Hultin (1959) if the pH 5 enzyme is preincubated with the ribonuclease. The activated amino-acid in absence of the acceptor dissociates to a very small extent. The transfer of activated amino-acyl adenylate intermediate to s-RNA is performed by the activating enzyme itself (Berg and Ofengard - 1958). That protein can be synthesized in absence of lipoprotein material has been shown by Littlefield and Keller (1957), and Simkin and Work (1957), though recently Hunter and Godson (1961) demonstrated that in bacteria the greater part of the labelled protein is found in the phospholipoprotein fraction. Pure ribosomes are formed almost wholly of RNA and protein, and the lipoprotein material of microsomes is chiefly associated with the membrane of the endoplasmic reticulum. Palade (1959) and Siekevitz and Palade (1958, 1959) have suggested that the membrane serves to concentrate and to store the protein product formed by the ribosomes.

In the present investigation it has been the intention to investigate the probable role of RNA in prothrombin synthesis by heavy microsomal particles.

Materials and Methods

Animals: Male albino white rats weighing between 175 - 210 gms. from the departmental stock have been used in the present study.

Preparation of Microsomes: The preparation of heavy microsomes has already been discussed in Section I and because of the high activity of prothrombin formation associated with this fraction, the present study has been limited only to that fraction. The heavy microsomal fraction was suspended in 0.25 M sucrose solution and incubated in the modified Krebs-Ringers Bicarbonate solution (pH 6.9) as described in Section I.

Preparation of Membrane: For the preparation of the membrane from ribonucleoprotein particles the liver has been homogenised in 0.25 M sucrose solution in 9 times volume instead of the usual 2.5 times. The heavy microsomes were obtained by centrifuging at 18,000g for 1 hr., the supernatant after initial centrifugation of the liver homogenate for 10 minutes at 6,500g. The microsomal fraction was then suspended in 0.25 M sucrose solution (same volume as the original weight of the liver) and for uniform suspension it was homogenised at low speed, 3 - 5 up and down strokes. One ml. of suspension was taken in tubes for preparative ultracentrifuge (Spinco Model L) to each ml. of suspension, 10 ml. of sodium pyrophosphate (anhydrous, B.D.H.) solution in 0.25 M sucrose solution is added in such a way that the final concentration of sodium pyrophosphate solution in the tube becomes 0.1 M. The pH of sodium pyrophosphate in sucrose solution was adjusted with concentrated hydrochloric acid to 7.4. The suspension was then centrifuged in the preparative ultracentrifuge for 1 hr. at

105,000 x g, and the sediment thus obtained was the membranous part, the ribonucleoprotein particles becoming soluble in the pyrophosphate and appeared in the supernatant (Sachs, 1958). The membranous portion was then suspended in 0.25 M sucrose solution and incubated in the modified Krebs-Ringer Bicarbonate solution (pH 6.9) in the same way as the heavy microsomal fraction.

Estimation of prothrombin: Prothrombin has been assayed and has been expressed in the same way as described in the Section I.

Estimation of Ribonucleic Acid: Ribonucleic acid was estimated by the usual perchloric acid method by adding 5 ml. of 0.4N perchloric acid at 0°C to 1 ml. of the sample, centrifuged at 0°C for 10 minutes at 2,000 r.p.m. The precipitate is treated twice with 5 ml. of 0.4N perchloric acid at 70°C for 20 minutes with stirring every 5 minutes, the supernatants thus obtained were pooled, and the U.V. reading was taken at 260 and 290 mμ. The amount of RNA present was calculated from a standard yeast RNA. The difference of 260 and 290 mμ for the standard RNA was 0.165 for 10.11 micrograms of RNA.

RESULTS

Effect of Ribonuclease: Ogata (1960) observed the inhibition of incorporation of ^{14}C-leucine into microsomal protein when ribonuclease as low as 0.25 μgm per ml. added to his system. Ullmann and Straub (1957) noted the same effect with higher concentration of ribonuclease (20 μgm per ml.); and Koningsberger still increased the concentration of ribonuclease up to 100 mμ per ml. The ribonuclease concentration added to our system of

heavy microsomes in the study of prothrombin formation was higher than that used by these workers, but we also observed that, when the system was incubated with ribonuclease, there was no significant synthesis of prothrombin as seen in Table XV. After incubation of the system with the ribonuclease for 1 hour there is loss of RNA to a level 83% lower than that of the control group; even when the system is kept for 10 to 15 minutes in ice with ribonuclease there is a drop of RNA by 18%. Almost the same degree of loss of RNA in 5 to 15 minutes has been noticed by Champmen-Anresen and Prescott (1956) from whole amoeba.

Straub et al (1960) attempted to define the role of microsomal RNA in the synthesis of amylase. They incubated the system first with the addition of RNA alone and in another instance with "activated" RNA by incubating with ATP. In the former case they found no difference of synthesis of amylase but in the latter case there was synthesis of amylase. Similar results have been achieved by Koningsberger (1960). He noted that, when crude yeast protoplast system incubated in mannitol phosphate buffer, and glucose there was increased incorporation of ^{14}C valine. Like Straub and co-workers, we also did not find any alteration of prothrombin synthesis when microsomal RNA or pH 5 enzyme RNA were added to the incubating system. The result has been shown in Table XVI. The microsomal suspension prepared as usual was taken in three incubating flasks, one was taken as control, to another was added microsomal RNA and to the third pH 5 enzyme RNA without any energy source. All the flasks were then incubated as described under

Table XV

Effect of ribonuclease on the synthesis of prothrombin: Heavy microsomal fraction suspended in 0.25 M sucrose solution is incubated in modified Krebs-Ringers Bicarbonate solution (pH 6.9) at 37°C with constant shaking in an atmosphere of O_2:CO_2. Ribonuclease in Krebs-Ringers Bicarbonate solution (pH 7.0) is added to the incubating flask to make the final concentration 0.25 mgm/ml. The prothrombin and RNA was estimated at different intervals; protein was determined at the beginning and at the end of incubation. The results are the average of three experiments.

System	Prothrombin Unit/mgm of Protein				RNA in mgm/ml.			
	Incubation Period in Hours							
	0	1	2	3	0	1	2	3
Control								
Mean	1.63	2.60	3.27	3.77	19.97	18.00	17.20	17.00
S.D.	0.42	0.70	0.81	1.00	3.74	2.82	2.44	1.41
St. error	0.28	0.42	0.47	0.58	2.16	2.01	1.40	1.07
+ Ribonuclease								
Mean	1.30	1.83	1.77	1.83	16.30	2.60	2.10	1.70
S. .	0.40	0.49	0.45	0.49	4.10	0.71	0.79	0.16
St. error	0.23	0.28	0.26	0.28	2.37	.41	0.45	0.11

methods and prothrombin, protein, and RNA were measured at different time intervals. From Table XVI it is evident that synthesis of prothrombin does not differ in the other two systems from the control group.

Prothrombin activity associated with membrane of the microsomes: It has been observed that the pyrophosphate renders almost 80 - 85% of the microsomal RNA soluble and the resultant pellet is entirely composed of smooth membrane and about 15 - 20% RNA remains associated with it (Sachs - 1957). Spiegelman (1957) and Hunter et al (1957) suggested that the components in the initial stage of protein synthesis in bacteria are fairly closely related to the cytoplasmic membrane. Their view has been supported by Schachtschabel and colleagues (1959) and McCorquodale and Killig (1959). These workers working on E. coli have observed that a lipoprotein membrane containing some RNA is a much more active site of protein synthesis than free ribosomal particles obtained in the same experiment. Recently Butler, Godson and Hunter (1961) observed that the initial site of incorporation of amino-acids was mainly in the lipoprotein-rich membrane fraction and in the cytoplasm the amino-acids appeared in greater amount at a later stage. In our present investigation also the membrane portion seems to be able to form prothrombin as evidence from Table XVII. It is clear from the Table that the power of formation of prothrombin by the washed microsomes does not differ from the membranous portion of the microsomes fraction. It has been observed that 25 - 30% of RNA is associated with the membrane. In the membrane part at 0 hour incubation, the RNA is about 75% less than the washed microsomal RNA and

Table XVI

Effect of addition of microsomal RNA and pH 5 RNA on the synthesis of prothrombin: The microsomal RNA and pH 5 RNA prepared after Hoagland et al (1958) with phenol were added to the incubation system (without prior incubation with ATP). The system was incubated as before and prothrombin, protein and RNA determined at different time periods. The results are the data from a single experiment.

Sample	Prothrombin Unit/mgm Protein				RNA in µgm/ml. of Suspension			
	Incubation Period in Hours							
	0	1	2	3	0	1	2	3
Control	1.40	2.10	2.70	2.90	12.90	-	-	12.00
+ Microsomal RNA	1.00	2.00	2.30	3.20	17.00	-	-	14.80
+ pH 5 enzyme RNA	1.40	2.40	1.90	2.20	20.70	-	-	17.00

towards the end of the incubation period the RNA present in the membrane is about 20% of the total RNA associated with the washed microsomes. The total protein in the membrane at 0 and 3 hour incubation period respectively is 66 and 72% of the total protein in the microsomes. Similar loss of protein in the membrane of the microsomes after sucrose (0.25 M) pyrophosphate (0.1 M, pH 7.4) has also been observed previously in this laboratory (Logan - unpublished). Sachs using pyrophosphate of the same molar strength and the same pH for the fractionation of microsomes observed 39% loss of protein in the membrane and thus our findings are in close agreement to his.

The chemical analysis of the membrane fraction shows that it is rich in phospholipid. The works of O'Brien (1956), Sarkar and others (1956), suggested that conversion of prothrombin to thrombin is catalysed by an enzyme which needs phospholipid as well as calcium for complete activation (Sarkar - 1958). That the increased activity of prothrombin associated with the membrane is not due to its high content of phospholipid is proved in another set of experiments where ribonuclease was added to the incubating flasks containing the membrane suspension in 0.25 M sucrose - Krebs-Ringers bicarbonate. In this instance there was no increased activity of prothrombin (Table XVIII). In this experiment the heavy microsomal fraction taken as the control group were the unwashed fractions and as in the previous experiment the synthesis of prothrombin by the untreated membrane is slightly higher than in the control group. The RNA in this untreated fraction at 0 hour and 3 hours incubation period is 25

Table XVII

Synthesis of prothrombin by membrane of microsomal fraction: The heavy microsomal fraction was treated with sodium pyrophosphate (0.1 M, pH 7.4) to solubilise the ribonucleoprotein particles. The sediment obtained was suspended in 0.25 M sucrose solution and incubated at 37°C with constant shaking in an atmosphere of O_2:CO_2. Prothrombin, RNA and protein were estimated at different time intervals as described in the text. The results are the data from one experiment.

	Prothrombin Unit/mgm protein				RNA in µgm/ml. of suspension			
Sample	Incubation period in Hours							
	0	1	2	3	0	1	2	3
Control	1.60	3.70	6.30	9.20	25.40	25.60	24.40	22.60
Test	1.70	5.60	8.40	9.30	6.80	7.70	4.60	4.30

Table XVIII

Effect of ribonuclease added to the membrane fraction on the synthesis of prothrombin. One mgm of ribonuclease in Krebs-Ringers Bicarbonate solution was added to the incubating flasks with a final concentration of 1 mgm per ml. of suspension and incubated as before. The control group was the unwashed heavy microsomes, the test group was the suspension of the microsomal membrane obtained after treatment of the heavy microsomal fraction with pyrophosphate (0.1M, pH 7.4). The results are the average of two experiments.

Sample	Prothrombin Unit/mgm Protein — Incubation Period in Hours				RNA in µgm/ml. of Suspension — Incubation Period in Hours			
	0	1	2	3	0	1	2	3
Unwashed microsomes	1.10	2.50	3.70	4.70	23.50	21.90	21.00	20.20
Membrane	1.70	3.80	5.20	6.20	8.90	5.80	4.60	3.10
Membrane + ribonuclease	1.20	1.30	1.30	1.40	6.00	1.50	1.00	1.00

and 15.3% of total microsomal RNA respectively in contrast to the membrane treated with ribonuclease where at 0 hour and at 3 hours incubation the RNA is 25.5% and 4.9% respectively.

DISCUSSION

The results presented above indicate that RNA is some or other way related to the formation of prothrombin by the heavy microsomes when incubated under our conditions. The inhibitory effect of ribonuclease in protein synthesis is well established though the mechanism is not clearly understood. In our system also, the ribonuclease definitely inhibited prothrombin "synthesis" and this alone shows that the increased activity of prothrombin on incubation is not a mere autoactivation process. The inhibitory effect of ribonuclease in the synthesis of prothrombin by the membrane of the microsomes after pyrophosphate treatment also demonstrates that the increased activity is not due to the high phospholipid content of this fraction, for 20 - 25% RNA present in the original microsomes and not left in the membrane is biologically active and is capable of "synthesizing" prothrombin".

Jeener (1959) observed that the decrease of protein synthesis by ribonuclease was associated with an alteration of base ratio of the RNA, with an increase of 10% in uridylic acid. Ledoux and Vanderhaeghe (1957) noticed that in the cells of ascites tumour ribonuclease causes both in vivo and in vitro a rapid alteration in the metabolism of ribonucleic acid with more than 100% increased incorporation in uracil and cytosine but slightly or no liberation of guanine and adenine. They also noticed that during the first stage

of action the metabolism of protein is not altered. Though Kloet and others (1961) attributed the cause of inhibition of protein synthesis by ribonuclease to inhibition of respiration we are more inclined to believe that a specific base pattern is essential for protein synthesis. In our subsequent studies (to be discussed later) we observed that the base ratio of the ribonucleic acid associated with the membrane and with the ribonucleoprotein particles is quite different; similarity of the base ratio in the nuclei and the membrane RNA suggests that the base ratio of the ribonucleic acid associated with the membrane might be specific to prothrombin formation. To support this hypothesis further study is needed.

SUMMARY

1. Prothrombin formation by heavy microsomes of liver is dependent on ribonucleic acid, since addition of ribonuclease to the incubating system markedly inhibits formation of prothrombin. But the addition of samples of ribonucleic acid prepared from heavy microsomes or pH 5 enzyme does not alter the process of "synthesis".

2. The microsomal membrane prepared after pyrophosphate treatment is capable of synthesizing prothrombin. Here again the activity is not specifically associated with the high content of phospholipid but with ribonucleic acid in the membrane. The increased activity of prothrombin on incubating the membrane is completely inhibited by addition of ribonuclease to the system.

SECTION III

The nucleotide composition of ribonucleic acid in different fractions of rat liver.

SECTION III

Introduction

The endoplasmic reticulum, as explained previously consists of a membrane to which are attached the ribosomes, electron dense particles which are recognised to consist of about 40% RNA and 60% protein. Various chemical procedures have been used to separate the ribosomal component from the membranous portion of the endoplasmic reticulum. The RNA of the original endoplasmic reticulum (microsomes in the homogenised cell) does not, however, appear exclusively in the ribosomes separated by chemical treatment from the membranes. The question thus arises, is the RNA not appearing in the ribosomal component of the endoplasmic reticulum a different species? Two main lines of evidence can be summarised in support of the idea that a distinct and separate form of RNA is associated with the membrane. One comes from solubilization of the reticulum membrane with deoxycholate, leaving ribosomes as an intact sediment. The other approach is by using pyrophosphate or EDTA, which by cholating with Mg^{++} cause disruption of ribosomes and leave the membranes intact.

Using deoxycholate to solubilize the membrane the different workers have obtained different amounts of RNA in the ribosomes as shown below:

Deoxycholate soluble RNA in rat liver microsomes

	in Ribosomes	in Supernatant	Other Remarks
Palade & Siekevitz (1956)	80	-	-
Takanami (1959)	40	8	Suggested that DOC solubilized portion might contain RNA of high ^{32}P activity.
Moulé, Rouiller & Chauveau (1959)	40-64	45	-
Kuff & Zeigel (1960)	-	23	S_{20w} 5-65
Siekevitz & Palade (1960)	35-45	-	-

A difference of base composition of RNA among sub-fractions of the cytoplasm has been suggested by Reid and Barbara (1958) in rat liver and by Hays (1960) in roots. The base composition of s-RNA differs from the base composition of ribosomal-RNA (Dunn - 1959). The importance of base composition of RNA in protein synthesis has been suggested by Osawa and Otaka (1959), who showed that incorporation of ^{14}C-leucine was directly proportional to the content of pseudouridylic acid in RNA.

In the present investigation it has been one aim to study the base composition of different fractions of RNA from liver and some other properties of RNA associated with the membrane of the endoplasmic reticulum.

MATERIALS AND METHODS

Animals: Male white albino rats weighing between 175-215 gms. from departmental stock were used throughout the present investigation. Unless mentioned all rats were fed with the departmental stock diet and the rats were starved overnight before killing, to minimise the glycogen content in the phenol-extracted RNA (Kirby - 1956). In order to alter protein intake in the dietary experiments, each rat was caged individually and was trained to take the following diet at particular times:-

In the morning: 5 gms. of dextrose

+

1.0 gm. of V.M.R.

In the afternoon at about 4 p.m.:

4 gms. of high or low protein diet.

The diet was mixed with some water to prevent spilling. The type of diet that was given to the rats gives a total caloric intake of about 40 kcal. per day to each rat. In addition, one group of rats were fed with 2 gms. of casein and 0.5 gms. of sodium bicarbonate 1 hour before killing; thus this fed group of rats was in the absorptive state when killed. The others were in the post-absorptive state.

Isolation of nuclei: Most of the acid-soluble constituents and some of proteins are lost during isolation of nuclei in the usual citric method (Smellie et al - 1955). Nuclei isolated in the non-aqueous phase are metabolically inactive; whereas nuclei isolated with sucrose remain active metabolically and are considered to be "surviving nuclei" (Naora et al - 1961,

Composition of different Diet

High Protein Diet

42.0 gms. Margarine

67.0 gms. Potato starch

69.0 gms. Glucose

240.0 gms. Casein

Low Protein Diet

42.0 gms. Margarine

189.0 gms. Potato starch

189.0 gms. Glucose

V.M.R. Diet

32.5 gms. Sodium chloride

130.0 gms. "446"

250.0 gms. Vitamins in starch

62.5 gms. Agar powder

77.5 gms. Vitamin margarine

"446" Salt Mixture

NaCl	-	245.198 gms.
K citrate	-	533.000 gms.
K_2HPO_4	-	174.000 gms.
$CaH_2(PO_4)_2$	-	800.000 gms.
$CaCo_3$	-	368.000 gms.
Fe^{+++} citrate $3H_2O$	-	56.000 gms.
$Cu\ SO_4 5H_2O$	-	0.400 gms.
$Ca\ Cl_2 6H_2O$	-	0.200 gms.
$k_2Al_2(SO_4)_4 24H_2O$	-	0.200 gms.
Na F	-	0.002 gms.
$MgCo_3$	-	92.000 gms.

Vitamin for V.M.R.

Pyridoxine hydrochlor	25 mg.
Riboflavin	25 mg.
Aneurine hydrochlor	25 mg.
Nicotinic	100 mg.
Biotin	5 mg.
Menaphthene	5 mg.
Calcium pentothenote	0.2 gm.
Para-amino benzoic acid	0.5 gm.
Inositol	1.0 gm.
Choline chloride	10.0 gm.
Folic Acid	trace
Potato starch	500 gm.

but sucrose alone is not sufficient to maintain structural integrity. The medium requires calcium or magnesium to maintain the integrity of nuclei (Allfrey, Mirsky and Osawa - 1957). A very high amount of magnesium is observed in nuclei isolated in 2.2 M sucrose without calcium chloride (Naora et al - 1961). An optimum amount of magnesium in cells and nuclei is essential for its enzymic effect, specially the enzymes concerned with phosphorylation and also the synthesis of RNA (Kornberg - 1957, Grunberg-Manago, Ortiz and Ochoa - 1956) and proteins (Hoagland, Keller and Zamecnik - 1956) though it has been claimed that the presence of calcium or magnesium in traces precipitates the nucleoproteins (Miuskamp - 1901, Carter - 1940). Replacement of sucrose by 0.1 M Tris buffer, 0.25 M glycerol or 0.25 M ethylene glycol, all with calcium chloride, causes loss of all magnesium, or 48.5% loss of calcium when calcium chloride is replaced with magnesium in the above (Naora et al - 1961). Release of magnesium from nuclei is correlated with the loss of nucleotides, and hence, in the isolation of nuclei, sucrose with calcium chloride is used to prevent the loss of magnesium and thus to prevent the loss of nucleotides.

In the isolation of nuclei we have adopted the method of Wilczok and Charazy (1960) with slight modifications. Our procedure for isolation of nuclei is as follows: the rats were lightly anaesthetised with ether, the abdomen was opened immediately and the liver was perfused with ice-cold 0.25 M sucrose + 0.0018 M calcium chloride (Analar) through the portal vein. When the liver became pale, it was removed while the perfusion

needle was in situ. The liver was washed with ice-cold 0.25 M sucrose + 0.0018 M calcium chloride, dried in two layers of filter papers, weighed and homogenised with 10 vols. of 0.25 M sucrose + 0.0018 M calcium chloride. The homogenate was filtered through 4 layers of nylon gauze, and the filtered homogenate was then centrifuged over a density gradient of sucrose and calcium chloride, as shown in the flow sheet. All centrifugations were carried out at 0°C and all glass ware and tubes used had been cooled in ice previously. The last centrifugation was carried out in an M.S.E. superspeed head, when special care was taken to keep the diameter of the centrifuge tube less than 15 mm. and to adjust the height of the contents inside the tube. If tubes of proper diameter are not selected, or if the height of the contents inside the tube is more or less than 15 cm., instead of obtaining a light yellow precipitate of nuclei at the bottom of the tube, a red layer mixed with other cytoplasmic material is obtained at the surface of the 2.2 M sucrose solution. The specific gravity of 2.2 M sucrose is such that at 30,000 g only the nuclei can be precipitated. The photograph (Fig. 9) of the isolated nuclei by our method showed that the nuclei were very clear and free from other cytoplasmic material. Before proceeding to extract the RNA, the isolated nuclei in each batch were examined under the microscope to see if the nuclei were free enough from other contamination.

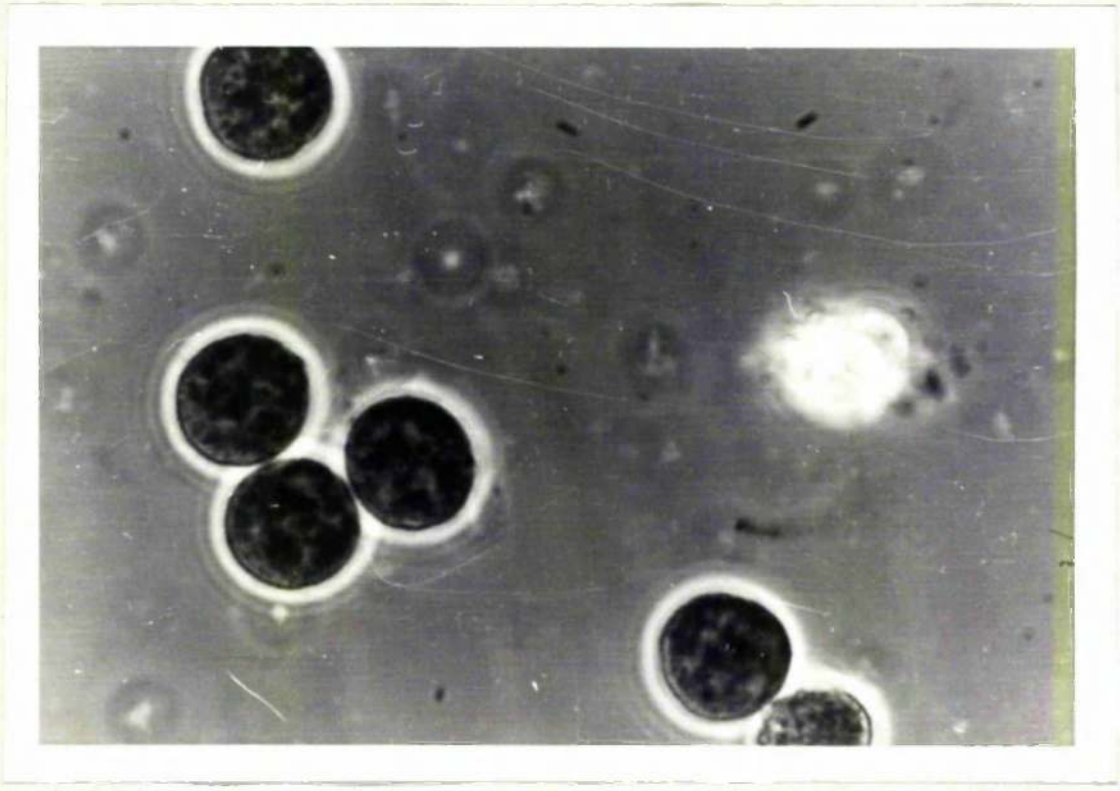
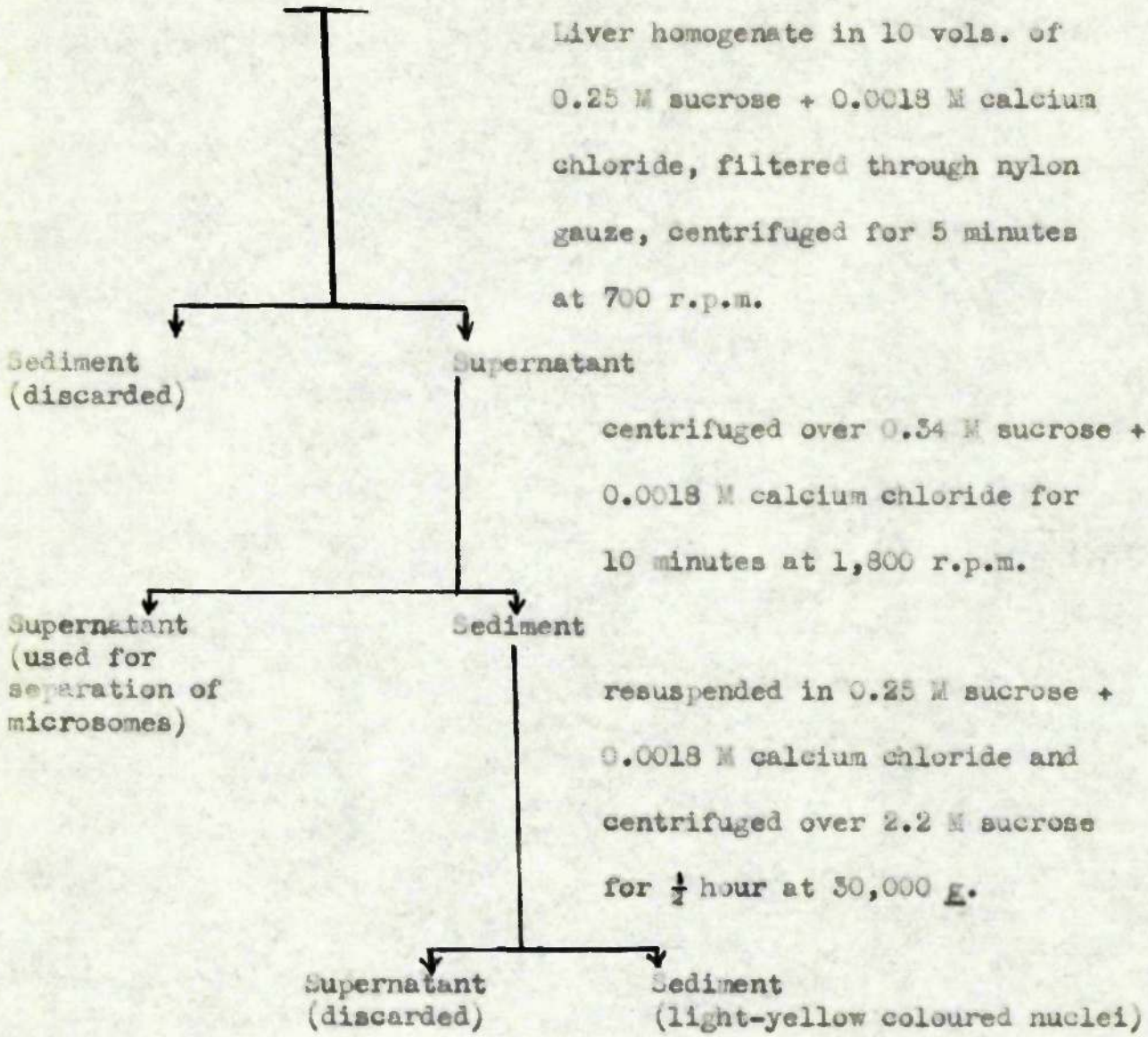


Fig. 9

Isolated nuclei photographed in phase-contrast microscope

Diagramatic Scheme for Isolation of Nuclei



Preparation of Microsomes: When the nuclei were isolated together with other fractions, the supernatant after centrifugation at 700 r.p.m. for 5 minutes was further spun for 5 minutes at 6,500 g and the spun supernatant was then centrifuged for 1 hour at 18,000 g to get the sediment of microsomes (heavy microsomes). At other times, the liver was homogenised in 9 vols. of 0.25 M sucrose solution, the homogenate was then centrifuged at 6,500 g for 10 minutes to sediment the unbroken cells, nuclei, and mitochondria and the supernatant thus obtained was further centrifuged for 1 hour at 18,000 g; the sediment from this was regarded as the microsomes ("heavy microsomes". A further microsome fraction ("light microsomes") was obtained by centrifuging the supernatant fluid from this fraction at 105,000 g for 1 hour in the Spinco Model L centrifuge.

Preparation of post-microsomal pellet: The supernatant after centrifuging down the microsomes was further spun for 3 hours at 105,000 g to obtain the "post-microsomal pellet".

Fractionation of microsomes: Sachs (1958) observed that, with increasing amounts of RNA and there is a parallel decrease in the incorporating ability of the microsomal pellets. In our preliminary studies we have observed that treatment of microsomes with 0.1 M sodium pyrophosphate in 0.25 M sucrose solution gave a separation of microsomal RNA similar to that observed with sodium deoxycholate, i.e. into membranous and ribosomal fractions. Further, it has been observed that, with this strength of sodium pyrophosphate all the lipoprotein is left in the pyrophosphate-insoluble pellets (Downie and Munro - unpublished). Hence, we have maintained the same strength

of pyrophosphate throughout our study. The microsomal particles were suspended in 0.25 M sucrose solution in such a way that 1 ml. of suspension contained the microsomes obtained from 1 gm. of liver. 1 ml. of this uniform suspension was taken in the centrifuge tube and to it 10 ml. of sodium pyrophosphate (adjusted to pH 7.4) was added to make the final concentration of sodium pyrophosphate 0.1 M. Thus each centrifuge tube contained liver microsomes obtained from 1 gm. of liver. Sachs (1958) observed that, to obtain a good separation of the membranous part of endoplasmic reticulum from the ribonucleoprotein particles, the aliquots of microsomes suspension should have a final concentration of about 3.5 - 4.0 mg. of protein per ml. In our investigations it has been observed that, if microsomal suspension obtained from 1 gm. of liver be treated with 10 ml. of sodium pyrophosphate (pH 7.4, 0.1 M), a good separation has been obtained. In our preliminary investigation, it has also been observed that, by varying the pH of sodium pyrophosphate or the period of contact of the microsomal suspension with sodium pyrophosphate at 0°C, there was a greater amount of RNA removed from the pellets at pH 9.9 than at pH 7.4, without any extraction of lipoprotein at these two pH range. The pH chosen in the present study was 7.4. The microsomal suspension with sodium pyrophosphate was then spun for 1 hour at 105,000 g, when the membrane sedimented at the bottom of the tube.

Extraction of RNA: The different fractions were suspended in and RNA was extracted with phenol (Hoagland et al - 1958) slightly modified. It has been observed (Littauer - 1961) that maximum yields of RNA were obtained

if the phenol treatment were carried out at 20°C; this preparation contained very little polysaccharide and the amount of protein is about 0.5%. RNA extracted with phenol at 0°C, on the other hand, contained about 2.0 to 4.0% protein and RNA yield was also somewhat lower (Littauer - 1961). On the other hand, stability of microsomes is dependent upon temperature and a marked change in microsomes is observed when the microsomes are kept at room temperature even for a short period (Hanson et al - 1960). For this reason we have decided to extract the RNA with phenol at 4°C. To the suspension of the membrane and the nuclei and to the supernatant an equal volume of freshly prepared 90% phenol was added with constant shaking for 1 hour at 4°C. The aqueous layer was separated by centrifugation at 2,000 r.p.m. for 1 hour at 0°C. The phenol layer was then mixed with distilled water and the aqueous layer was again separated after centrifugation at 2,000 r.p.m. for 1 hour, and this was repeated twice. The pooled aqueous layers were then treated with ether to remove phenol. RNA was precipitated as the potassium salt (by adding potassium acetate, 2%, pH 5.0) with ethanol. RNA thus obtained was then dialysed by first dissolving in water and dialysing against running distilled water. The sample of RNA prepared by us contained 0.1% protein when estimated by Lowry's method.

Hydrolysis of RNA: RNA thus prepared was hydrolysed in 0.3N KOH at 37°C for 18 hours (Crosbie et al - 1953). The use of higher concentration of KOH causes deamination of cytidylic acid (Davidson and Smellie - 1952), whereas use of 0.3N KOH does not cause any such deamination and at the same time gives a complete hydrolysis of RNA in 18 hours. After hydrolysis,

0.1 ml. of the sample was diluted to 4 ml. and the U.V. absorption at 260 mm. was read. The rest of the hydrolysed sample was neutralised with concentrated (10 N) perchloric acid in the cold to minimise the solubility of potassium percholate. The excess amount of potassium was removed as potassium perchlorate by centrifugation for 10 minutes at 2,000 r.p.m. at 0°C. The clear supernatant which contained the hydrolysed product was then concentrated by freeze-drying over H_2SO_4. The dried powder was then dissolved in an appropriate amount of distilled water (pH 7.0) so that 0.1 ml. of sample contained 0.20 - 0.24 mgm. nucleotides.

Chromatography: 0.1 ml. of this concentrated hydrolysed RNA was then applied to Whatman paper No. 1 and two-dimensional chromatography was carried out in (i) isopropanol-water (7:3, v/v) with ammonia in the vapour phase for 60 hours (Lipshitz and Chargaff - 1960), (ii) ammonium-isobutyric buffer pH 3.6 for 14 to 16 hours (Magasanik - 1950). With each batch, a blank sheet was treated in the same way and after the second dimension the papers were dried thoroughly for 6 to 8 hours.

Identification of nucleotides: When phenol-extracted RNA from yeast was hydrolysed as described above and separation was performed by two-dimensional chromatography, the separation of nucleotides occurred as shown in Fig. 10. Spots 1, 2, 3, and 4 were identified as guanylic, uridylic, cytidylic and adenylic acid by reading the eluate at pH 1.0 on a S.P. 500 spectrophotometer. For the identification of pseudouridylic acid, the chromatogram had to be exposed to the filtered U.V. lamp for a few minutes before any fluorescence could be seen; this then lay between guanylic and uridylic acids. The

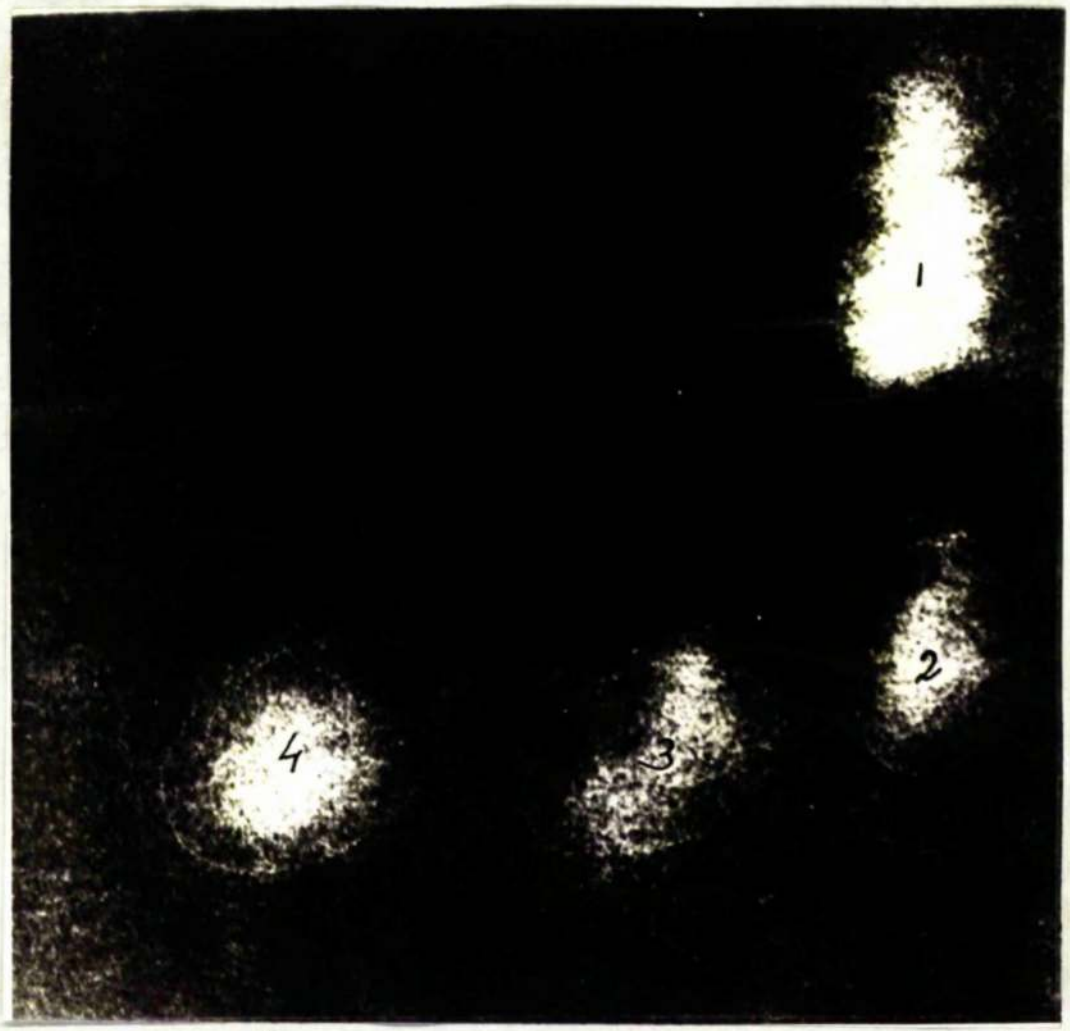


Fig.10

Separation and identification of yeast-RNA nucleotides by two-dimension chromatography

1 = guanylic acid

2 = uridylic acid

3 = cytidylic acid

4 = adenylic acid

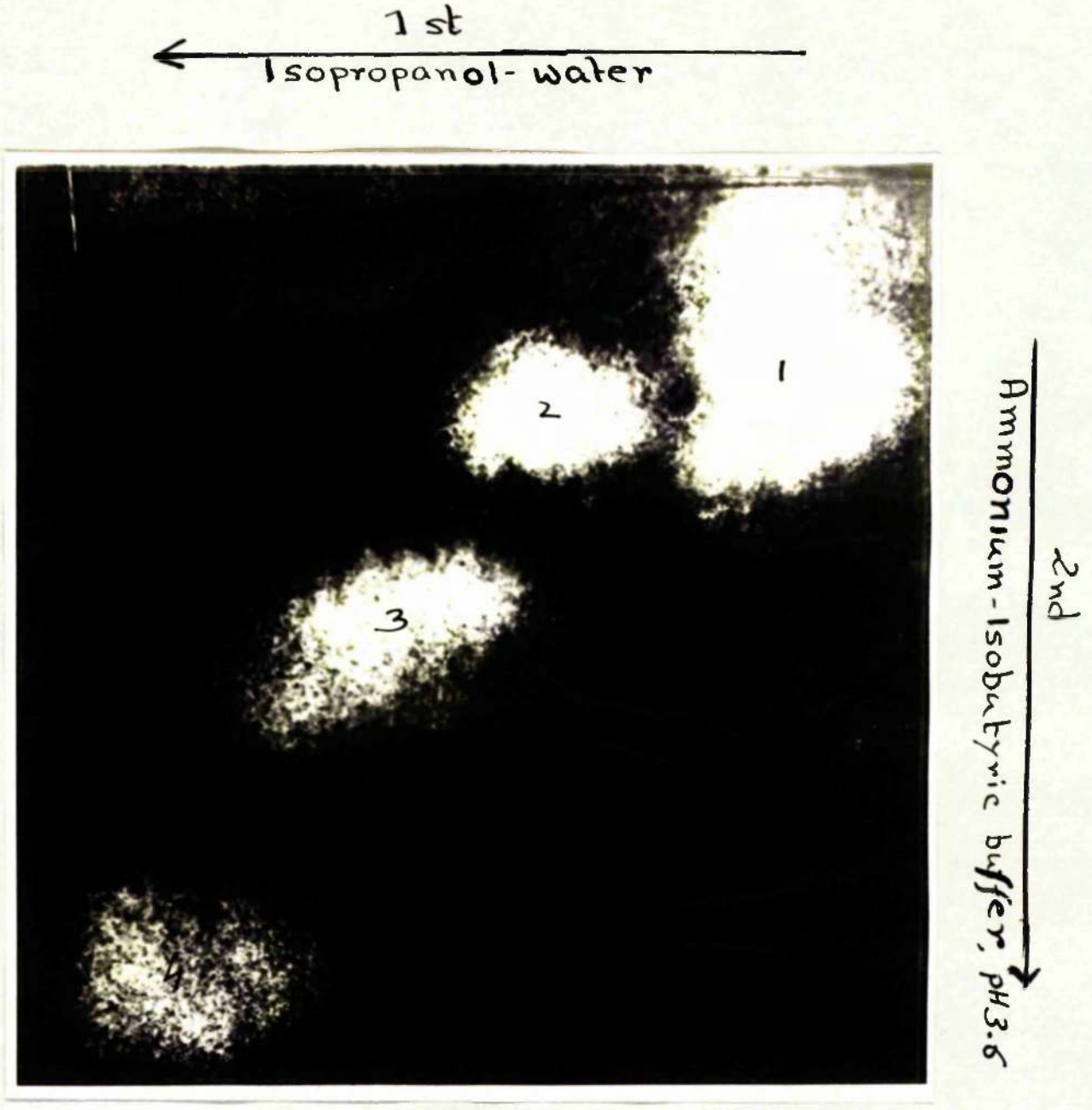


Fig. 10

Separation and identification of membrane-RNA nucleotides by two-dimension chromatography

1 = guanylic acid 2 = uridylic acid

3 = cytidylic acid 4 = adenylic acid

mobility of that area corresponds to the mobility data of pseudouridylic acid (Allen and Davis - 1957) and when the eluate from the spot was read in water at pH 1.0 and at pH 13.0, the maximum absorption was obtained at 264 mμ and 286 mμ respectively. The published maximum absorption for pseudouridylic acid at these two pH values is 263 mμ and 286 mμ respectively and thus the spot in between the guanylic and uridylic spots is confirmed as pseudouridylic acid.

Estimation of nucleotides: The nucleotides, after being identified on the paper, are marked very carefully so as not to overlap the area and each fraction was then eluted with water by capillary flow overnight, at room temperature. Corresponding areas from the blank sheet were also cut out and eluted similarly. Later (see below) blank areas on the same paper were used. The final volume for the different nucleotides in the U.V. 500 spectrophotometer were obtained (5 ml. for adenylic and uridylic, 8 ml. for cytidylic and guanylic and 4 ml. for pseudouridylic). An appropriate amount of normal hydrochloric acid was added to each fraction of the eluate to give the final strength 0.1 N acid and readings were usually taken at 260 and 280 mμ. The molar extinction coefficient used for adenylic, guanylic, cytidylic and uridylic were those of Wyatt (1955) and for pseudouridylic was that of Davis and Allen (1957). The 280:260 mμ absorption ratios were determined experimentally and coincided with those by Wyatt for all spots (1955).

RESULTS

Determination of paper Blank: In preliminary experiments in this laboratory, it has been observed that equal areas cut from the same sheet of filter paper do not give identical optical density figures, even the corresponding areas of different sheets run in the same tank do not give the same figure; however, the 230/260 ratio of the optical density of the blank corresponding to the different areas of nucleotides is found to be constant. The absorption spectra of pure nucleotides individually differs in all cases from that of the blank. Taking these two facts into consideration, it is theoretically possible to calculate the amount of the contribution of the paper blank to the absorption of the spot by measuring the optical density of two wave-lengths as follows:-

$$\frac{T_1 - C.\epsilon_1}{T_2 - C.\epsilon_2} = \frac{B1}{B2}$$

Where T_1 = optical density of nucleotide plus paper blank at λ_1.

T_2 = optical density of nucleotide plus paper blank at λ_2.

C = concentration of nucleotide.

ϵ_1 = extinction coefficient of nucleotide at λ_1.

ϵ_2 = extinction coefficient of nucleotide at λ_2.

B_1 = optical density of blank at λ_1.

B_2 = optical density of blank at λ_2.

$$\text{i.e. } T_1B_2 - C\epsilon_1B_2 = T_2B_1 - C\epsilon_2B_1$$

$$\text{or } C(\epsilon_1B_2 - \epsilon_2B_1) = T_1B_2 - T_2B_1$$

$$\text{Thus } C = \frac{T_1B_2 - T_2B_1}{\epsilon_1B_2 - \epsilon_2B_1}$$

$$= \frac{\frac{B_2}{B_1}T_1 - T_2}{\frac{B_2}{B_1}\epsilon_1 - \epsilon_2}$$

$$= \frac{\frac{B_2}{B_1}T_1 - T_2}{\left(\frac{B_2}{B_1} - \frac{\epsilon_2}{\epsilon_1}\right)\epsilon_1}$$

$$= \frac{\frac{B_2}{B_1}.T_1 - T_2}{-\epsilon_2\left(\frac{1 - B_2\epsilon_1}{B_1\epsilon_2}\right)}$$

It is seen from the above that the concentration of nucleotide can be found if the molar extinction coefficient of pure nucleotide and the ratio of pure nucleotide at the two wave-lengths ($\lambda_1 + \lambda_2$) are known, the other factors, i.e. B_1, B_2, T_1 and T_2 are obtained from the experimental data.

The two wave-lengths chosen in the present study were 260 and 280 because the 280:260 optical density ratio of the blank is quite constant, whether the blank area corresponds to adenylic, guanylic, cytidylic or

uridylic acid. Secondly, the maximum absorption of adenylic, guanylic, cytidylic and uridylic are respectively 257, 257, 278 and 262 mμ. Thus for adenylic, guanylic and uridylic acids, the absorption at 260 mμ is considerably higher than at 280 mμ, and for cytidylic acid, the absorption is higher at 280 mμ.

Chromatograms were run to compare the recovery of nucleotides by the conventional use of blank areas and by the new procedure using differential spectrophotometry. 0.01 ml. of chromatographically pure solutions of adenylic, guanylic, cytidylic and uridylic acids were applied to the Whatman paper No. 1, and chromatograms were run. The amount applied was estimated by measuring the optical density in 0.1N hydrochloric acid at 260 and 280 mμ. The percentage recoveries obtained by the two procedures has been shown in Table XIX.

From the Table, it is evident that the average percent recoveries in the new procedure are slightly better in some cases, though the difference over the conventional use of blank areas is not significant. However, the variability of recovery for individual nucleotides, as estimated by the new procedure varies between 2.5 to 6.9 , whereas the standard deviation by the conventional procedure varies between 7.0 and 21.5 . This reduction of variability of recovery shows the accuracy and is a justification of use of new procedures in the estimation of nucleotides.

Base composition of RNA isolated from different cell fractions of rat liver:

The base composition of phenol-extractable RNA isolated from nuclei, whole microsomes, membrane of microsomes and ribosomes has been studied and

Table XIX

Recoveries from the separation of known amounts of nucleotides by means of chromatography. Calculated by formula using appropriate blank and using 280:260 mμ readings. The results are from 6 chromatograms.

Chromatogram No.	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid
1.	100.3	93.2	97.2	105.9
2.	94.1	991.7	98.6	102.2
3.	94.3	97.5	91.0	91.7
4.	95.3	98.1	99.4	90.0
5.	96.9	99.5	97.6	93.8
6.	99.7	89.9	103.2	90.0
Mean	96.7	95.0	97.8	95.4
S.D.	2.5	3.9	4.0	6.9
St. error	1.0	1.6	1.6	2.8

Recoveries from the separation of known amounts of nucleotides by means of chromatography calculated by subtraction of an appropriate blank run on a separate sheet of chromatography paper.

1.	106.3	96.8	97.7	110.9
2.	93.2	64.9	105.0	101.1
3.	95.4	103.8	83.4	80.2
Mean	98.3	88.5	95.4	97.4
S.D.	7.0	21.5	11.0	15.9
St. error	4.2	12.1	6.3	9.2

the results have been tabulated in Table XX. From the Table it is seen that the amounts of guanylic acid and cytidylic acid in the membrane RNA are higher from the other fractions, though the difference of these two nucleotides of membrane-RNA over that of nuclear RNA is not significant. A significant difference in nucleotide composition is observed between the membrane and the ribosomes. The difference in adenylic acid concentrati in the membrane-RNA and in nuclear-RNA is within experimental error and hence not significant, but the amounts of adenylic acid and uridylic acid, specially the latter, in the membrane-RNA and ribosomal-RNA are highly significantly different. In the nuclei and in the membrane, except in two experiments, we did not notice any evidence of presence of pseudouridylic acid; the amount of pseudouridylic acid observed in the two experiments was also very low and because of our inability to detect any evidence of the presence of this nucleotide in RNA of membrane fraction, we thought it to be due to contamination from incomplete separation of ribosomes from the membrane of the microsomes. This might be due to uneven and non-uniform suspension of heavy microsomes in 0.25 M sucrose solution before treatment with pyrophosphate, and hence, in subsequent experiments, the microsomes were suspended in 0.25 M sucrose solution by homogenising the microsomes in low speed. In such preparations of microsomal suspension, no pseudouridylic acid in the membrane-RNA was detected. The pseudo-uridylic acid content of the whole microsomes and in the ribosomes is almost identical, the difference being again within experimental error.

It has been claimed by Monier et al (1960) that, when RNA is extracted

Table XX

Nucleotide composition of different fractions of liver: RNA from nuclei (isolated in 0.25 M sucrose + 0.0018 M calcium chloride) whole microsomes, microsomal membrane and ribosomes extracted with equal volumes of phenol, hydrolysed with 0.3N KOH at 37°C for 18 hours and the nucleotides separated by two-dimensional chromatography.

Sample	Average of	Adenylic Acid	Guanylic Acid	Cytidylic Acid	Uridylic Acid	Pseudo-uridylic Acid
Nuclei	3 expts. mean	18.7	31.8	31.3	18.4	0.0
	S.D.	2.4	1.8	1.3	2.5	0.0
	St. error	1.4	1.0	0.8	1.1	0.0
Whole microsomes	2 expts. mean	21.3	30.4	27.8	17.7	2.9
	S.D.	1.1	0.8	0.4	1.3	0.5
	St. error	0.8	0.6	0.3	1.0	0.4
Membrane	5 expts. mean	17.00	34.10	32.20	16.80	trace
	S.D.	1.70	1.50	3.60	1.86	
	St. error	0.76	0.68	1.62	0.81	
Ribosomes	5 expts. mean	20.90	27.00	27.10	23.00	2.20
	S.D.	2.27	3.10	3.50	4.60	2.30
	St. error	0.93	1.30	1.50	2.00	1.00

with phenol at room temperature, the cell wall is permeable to small molecules (e.g. sRNA) but does not allow the extraction of ribosomal RNA. This is especially true of nuclei, where phenol cannot extract the nucleolar RNA (Allfrey - 1961). So, in a series of experiments, the nuclei, membrane and ribosomes were treated with 30 cold trichloroacetic acid (TCA), allowed to stand in ice at least for half-an-hour, centrifuged at 2,000 r.p.m. for 10 minutes. The sediments were washed twice with 10 TCA. The washed sediments were then digested directly with 0.3N KOH for 1 hour at 37°C. It has been observed by Fleck and Munro (1962) that by this treatment the whole amount of RNA could be extracted with very little protein contaminati If digestion is prolonged, the amount of RNA extracted remains more or less the same but the amount of protein present is increased considerably. So, it can be accepted that after 1 hour digestion with 0.3N KOH at 37°C the whole amount of RNA has been extracted. After digestion, the sample was neutralised with cold perchloric acid (PCA 10N), washed twice with cold PCA (0.5N) and the pooled extract was further digested with KOH (final concentration - 0.3N) for 18 hours at 37°C, neutralised with cold PCA, freeze dried and applied on Whatman paper No. 1 as before for separation of nucleotides by two-dimensional chromatography. The results obtained from direct extracted RNA and phenol extractable RNA are shown in Table XXI. It is clear from the results presented in Table XXI that identical results for the different nucleotides of different fractions by these two procedures are obtained; the pseudouridylic acid concentration tends to be greater in RNA extracted with phenol from the ribosomes.

Table XXI

Comparison of base ratios of RNA extracted with phenol and with alkali

RNA from nuclei, membrane and ribosomes were extracted with equal volumes of 90% phenol and after Fleck and Munro's (1962) modification of the Schmidt-Thannhauser (1945) method (digestion 1 hour in 0.3N alkali). RNA thus obtained by the two different methods were hydrolysed with 0.3N KOH for 18 hours at 37°C. The nucleotides were separated by two-dimensional chromatography as described in the experimental section. The results are expressed in terms of molar ratios, with adenylic acid = 10.

Sample	Average of	Alkali RNA					Phenol - RNA				
		A	G	C	U	Ψ_s	A	G	C	U	Ψ_s
Nuclei	3 expts.	10.0	16.5	14.6	9.3	0.0	10.0	17.0	16.7	9.9	0.0
Membrane	2 expts.	10.0	21.4	18.3	10.6	0.0	10.0	20.1	18.9	9.9	0.0
Ribosomes	2 expts.	10.0	13.9	14.8	13.8	0.7	10.0	13.4	13.4	11.0	1.0

The results for the base composition of membrane and of ribosomes are from 5 experiments for phenol extractable RNA and 2 experiments for direct alkaline digestion.

A = Adenylic acid

G = Guanylic acid

C = Cytidylic acid

U = Uridylic acid

Ψ_s = Pseudouridylic acid

In the RNA directly extracted with alkali from nuclei and membrane, once more we did not detect any pseudouridylic acid, confirming our previous findings with the phenol-extractable RNA. In this procedure for RNA extraction also, the amounts of guanylic and cytidylic acids are again higher in the membrane fraction.

Finamore and Volkin (1961) studied two types of RNA in the amphibian egg, one of which was readily dissolved in the cold by 1.0N PCA. They observed more uridylic and adenylic acids in the acid-soluble portion of RNA over that found in the acid-insoluble portion of the RNA. There was an equivalent decrease in guanylic acid and cytidylic acid in the acid-soluble RNA, as compared with the acid-insoluble RNA. Consequently:

$$(G + U)_{soluble} = (G + U)_{insoluble}$$

and

$$(A + C)_{soluble} = (A + C)_{insoluble}$$

Similarly, we have also observed that:

$$(G + U)_{nuclei} = (G + U)_{membrane} = (G + U)_{ribosomes}$$

and

$$(A + C)_{nuclei} = (A + C)_{membrane} = (A + C)_{ribosomes}$$

Our results from the phenol extractable-RNA are as follows:

Fraction	G + U	A + C
Nuclei	(31.8 + 18.4) = 50.2	(18.7 + 31.3)= 50.0
Membrane	(34.1 + 16.8) = 50.9	(17.0 + 32.2)= 49.2
Ribosomes	(27.0 + 23.0) + 50.0	(20.9 + 27.1)= 48.0

This is also true for the RNA extracted by direct alkali digestion for 1 hour at 37°C.

Thus, it is suggested that the high guanylic acid content plus low uridylic acid content in the nuclear-RNA and in the membrane-RNA are equivalent to the low guanylic acid and high uridylic acids content of ribosomal-RNA; and high cytidylic acid content plus low adenylic acid content in the nuclear and in the membrane-RNA are equivalent to the low cytidylic acid plus high adenylic acid content of ribosomal-RNA.

Effect of nutritional status of rats on base composition of RNA of different fractions of rat liver: The effects of nutritional status on the morphological structure of the liver cell and on the biosynthesis of RNA have already been discussed in the general introduction section. Recently, it has been observed (Prosser, Wallinson and Munro - 1960) that the inclusion of protein in the diet increases the amount of RNA per 100 g body weight in the post-microsomal pellet but there is a decrease in the ability of this fraction to accept labelled amino-acids, suggesting addition of a new type of RNA, inactive in the uptake of labelled amino-acid in the post-microsomal pellets. Because of this, we have studied the base composition of RNA of different fractions of rat liver, including the post-microsomal

fraction. The rats were fed with different diets as described under the experimental section. The results obtained are tabulated in Table XXII.

From Table XXII, it is clear that feeding with 2 g. of casein + 0.5 g. of sodium bicarbonate before killing (fed-rats) causes a significant lowering of the amount of guanylic acid and cytidylic acid in the membrane-RNA. This occurs when protein was given to animals previously on low-protein diet (22.3% down to 17.3%) and to animals previously on adequate protein intake (26.5% to 17.0%). A similar significant lowering of guanylic acid was observed in the ribosomal-RNA in the fed groups from that of the fasted groups (18.9% to 15.8% and 16.8% to 11.4% respectively). The amount of uridylic acid of membrane-RNA in low protein group did not alter with prefeeding, but in the high protein group the amount of uridylic acid was lowered significantly when the rats were prefed with casein and sodium bicarbonate (12.0 to 9.8%). In the post-microsomal pellets in the high protein fed group there was a slight alteration of base composition but it is not significant, but the amount of pseudouridylic acid in the fed group was significantly raised. A significant increase of this nucleotide was also observed in the ribosomal-RNA in the two fed groups as compared with the two fasted groups.

It is of particular interest to note that a significant difference in base composition between the fasted and fed groups is mainly limited to guanylic acid which is reduced both in membrane and ribosomal-RNA in the fed groups, the effect being more marked in membrane-RNA and when the protein was fed to the group previously received high protein regime.

Table XXII

Effect of nutritional status of rats on base composition of RNA of different fractions of rat liver: RNA extracted either with phenol or with alkali and hydrolysed with 0.3N KOH for 18 hours at 37°C. Base composition studied as described under experimental section. Results are expressed as molar ratios with adenylic acid = 10.

Fraction	Nutritional tatus	No. of Expts.	A	G	C	U	Ps
Membrane	L.P. fast	3	10.0	22.3	25.5	9.5	0.0
	L.P. fed	1	10.0	17.5	16.5	8.0	0.0
	H.P. fast	2	10.0	26.5	25.5	12.0	0.0
	H.P. fed	2	10.0	17.0	19.8	9.8	0.0
Ribosomes	L.P. fast	3	10.0	18.9	15.9	8.4	0.3
	L.P. fed	1	10.0	13.8	16.7	10.3	0.5
	H.P. fast	2	10.0	16.8	16.1	9.2	1.1
	H.P. fed	2	10.0	11.4	13.8	9.3	2.2
Post-microsomal pellets	L.P. fast	1	10.0	16.9	15.8	9.7	0.9
	H.P. fast	1	10.0	15.9	15.9	9.2	0.8
	H.P. fed	1	10.0	17.1	16.4	10.7	1.1

L.P. = low protein
H.P. = high protein
A = adenylic
G = guanylic
C = cytidylic
U = uridylic
Ps = pseudouridylic

Ecteola column chromatography of membrane-RNA: Because of the difference of membrane RNA compared with the ribosomal-RNA, we decided to fractionate the phenol-extractable-RNA from the membrane in an Ecteola (ion-exchange adsorbent of cellulose treated with epichlorhydrin and triethanolamine) column as described by Goldthwait (1959). A column of 0.8 cm. in diameter and 3.5 cm. high was used. Ecteola was washed with 1N HCl, water followed by 1N NaOH and water until the pH of washing was neutral. Gradient system consisted of a constant volume mixing chamber containing 250 ml. of 0.01 M potassium-phosphate buffer, pH 6.85 into which flowed 200 ml. of 1N NaCl buffered with 0.01 M potassium phosphate, pH 6.85. The RNA solution was run through the column and 5 ml. collected in each tube and optical densities at 260 mμ were measured. The results are shown in Fig. XI. A small volume of ultraviolet-absorbing material with 1 M NaCl was obtained, but most of the RNA associated with the membrane was obtained by using an NH_4OH gradient elution. After the major portion of RNA associated with the membrane had thus been eluted, a small volume of RNA was eluted with 1N NaOH. This elution pattern may be compared with the pattern obtained by the same technique in this laboratory from other liver-cell fractions (Fig. XII). I am indebted to Mrs. Jean McLean for permission to reproduce this diagram. It will be noted that membrane-RNA (Fig. XI) resembles most closely post-microsomal RNA (Fig. XII) in pattern, having more UV absorbing material in NaCl gradient than found with whole microsomal RNA.

Ultracentrifugal study: A series of experiments were carried out to determine the sediment coefficient of membrane-RNA. The RNA was extracted

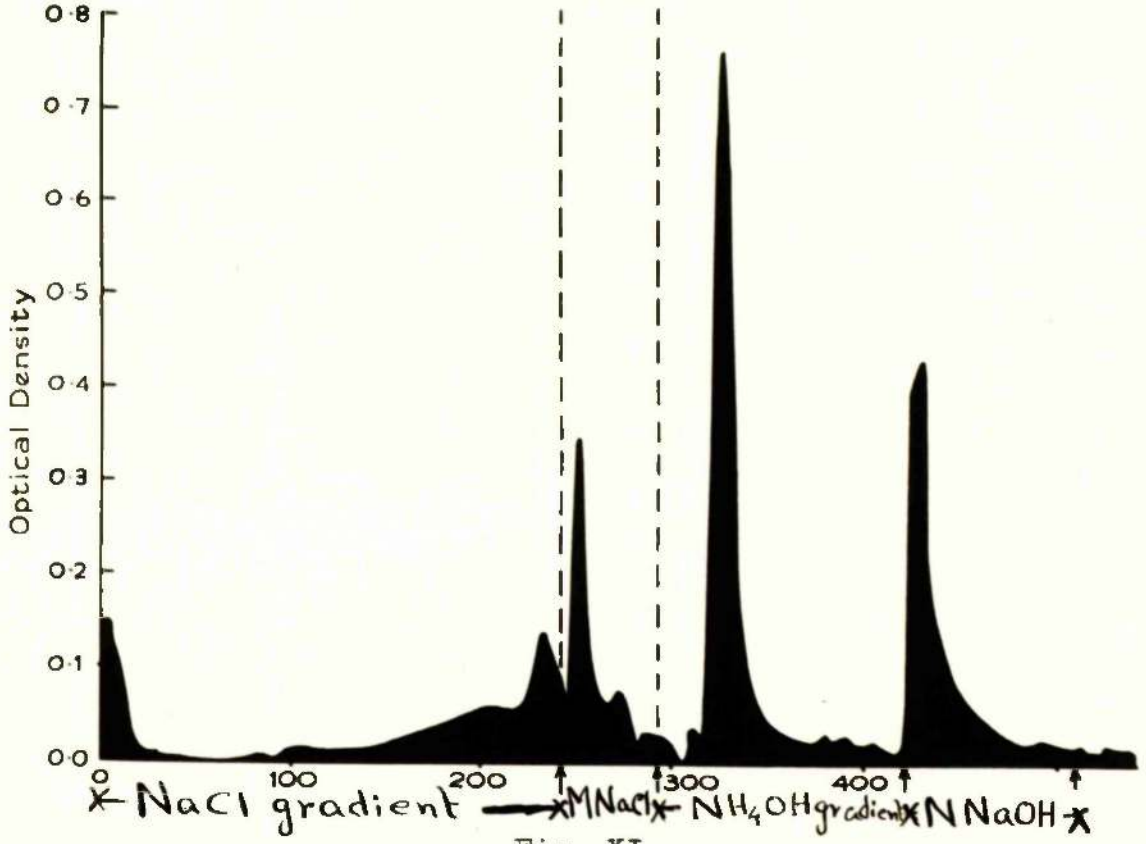


Fig. XI

ECTEOLA column chromatography of membrane-RNA

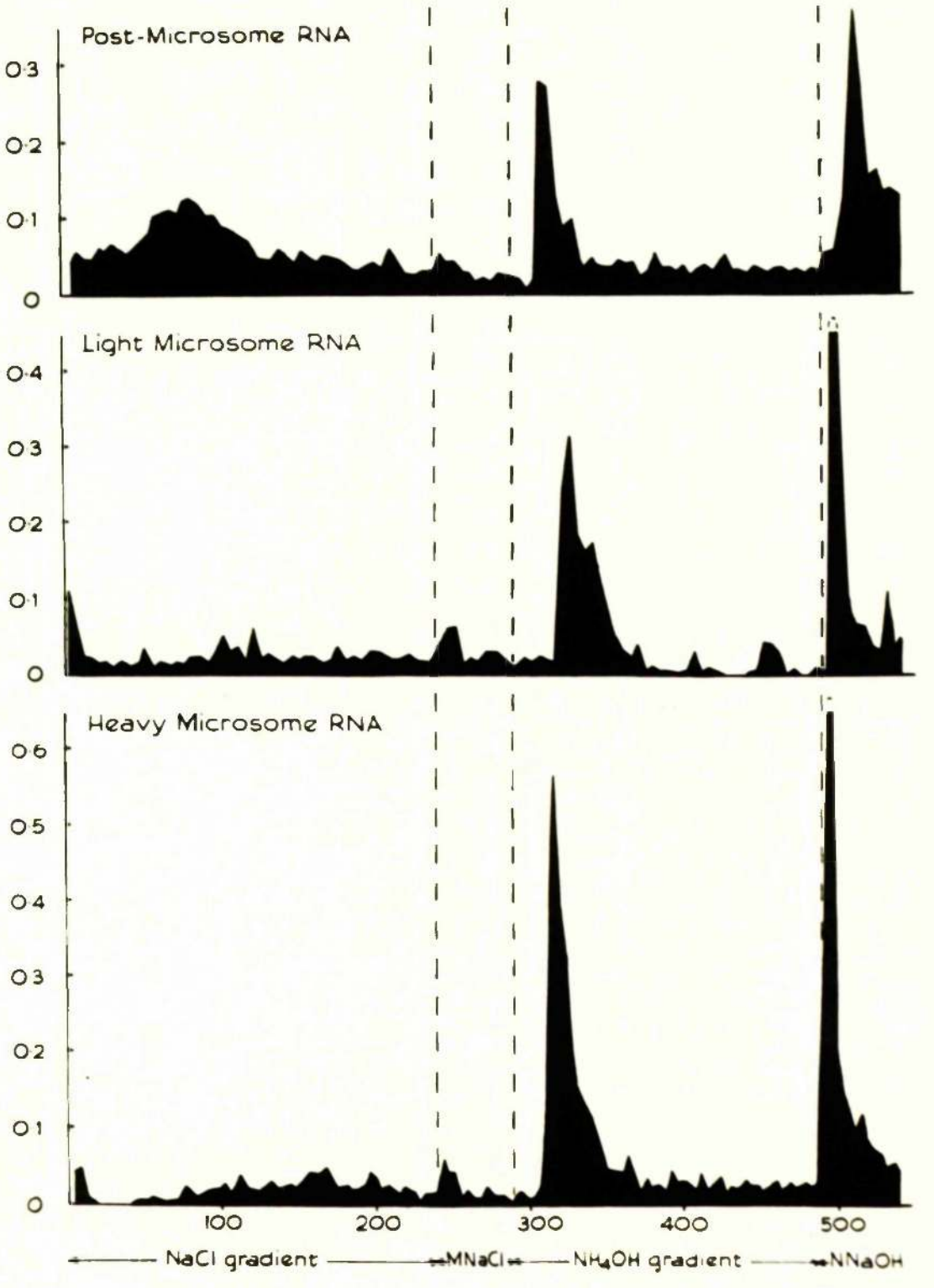


Fig. XII

ECTEOLA column chromatography of RNA extracted from microsomes and post-microsome pellets.

with phenol and RNA was dissolved with water so that the sample contained approximately 1% RNA. When the ultracentrifugal run was carried out at 59,780 r.p.m. at 17°C we observed a sedimentation coefficient (uncorrected) between 2 - 3S on two occasions.

Bosch and coworkers (1961) have observed that simple dialysis against double distilled water leads to degradation of some microsomal RNA fractions and suggested dissolving the RNA in 0.05 M phosphate-buffer with 0.2 M NaCl and dialysing against this solvent. Accordingly, we prepared, dissolved and dialysed our RNA in 0.2M NaCl containing 0.05 M potassium-phosphate buffer. The sample was run in the ultracentrifuge at 59,780 r.p.m and sedimentation coefficient at 17°C found to be 3S and 3.5S (uncorrected) on two occasions. This suggests that the RNA associated with the membrane is of low molecular weight and corresponds in size to s-RNA.

The ribosomal RNA removed from the microsomes by pyrophosphate treatment is not suitable for molecular determinations because of disintegration by this reagent, but we have compared membrane-RNA with whole microsomal RNA for molecular size. The phenol-extractable-RNA from the whole microsomes was dissolved in water and dialysed against running distilled water and ultracentrifugal run was carried at 44,770 r.p.m. The sedimentation coefficient (uncorrected) for the whole microsomal RNA was found to be about 30S and with a smaller peak.

DISCUSSION

The finding of an RNAse-sensitive system for prothrombin formation by heavy microsomes led us to examine the nature of the RNA associated with the membrane of these microsomes. These studies have provided us with data on (a) nucleotide composition, (b) molecular size and heterogeneity by Ecteola column chromatography and (c) molecular size by ultra-centrifugal studies. All these have emphasized that the RNA associated with the membran is a distinct species. We shall now consider how this species of RNA is related to the RNA patterns observed in different fractions of the liver cell by other workers.

The base composition of RNA from different sources has been studied by many investigators and their results along with our results have been tabulated in Table XXIII for comparison. Some have in fact, like ourselves, isolated the nucleotides which includes pseudouridylic acid; much of the published data, however, are for total base composition in which the pseudouridylic and uridylic acids both appear as uracil. From Table XXIII it is clear that our results for nucleotide composition of RNA from the different fractions of rat liver cells are in close approximation to the results of other workers. The slight difference in some cases is not significant, in some cases it is highly significant as for example Monier and coworkers' (1960) results for the base composition of ribosomal-RNA are highly different from our results, but they hydrolysed the RNA in 1N NaOH at 80°C for 1 hour and thus may well have degraded the RNA to a greater extent (Hutchison and Munro - 1961). So, the significant differences

Table XXIII

Comparison of base composition of RNA from different sources. The results are expressed as molar ratio with adenylic acid = 10. A = adenylic acid, G = guanylic acid, C = cytidylic acid, U = uridylic acid, Ps = pseudouridylic acid.

Cell-fraction	Origin	References		Cell-nucleotides A	G	C	U	Ps
	Rat-liver	(1) McIndoe & Davidson (1952)	(a)	10.0	14.8	14.3	12.9	-
			(b)	10.0	13.4	12.1	11.7	-
Nuclei		(2) Wilson & Chargaff (1952)	(a)	10.0	12.7	14.6	12.2	-
			(b)	10.0	13.0	14.9	11.5	-
		(3) Allfrey & Mirsky (1959)		10.0	16.2	14.2	8.6	0.0
		(4) Present investigation		10.0	16.8	16.7	9.0	0.0
Whole microsomes	Rat-liver	(1) Crosbie et al (1953)		10.0	16.9	14.7	10.3	-
		(2) Lipshitz & Chargaff (1960)		10.0	16.4	15.2	8.8	0.7
		(3) Present investigation		10.0	14.3	13.1	8.3	1.4
Membrane of microsomes	Rat-liver	(1) Chauveau et al (1962)		10.0	18.9	15.9	11.0	0.0
		(2) Present investigation		10.0	20.1	18.9	9.9	0.0

Cell-fraction	Origin	References	Cell-nucleotides A	G	C	U	Rs
Ribosomes	Rat-liver	(1) Magasanik (1955)	10.0	17.5	15.8	10.1	-
		(2) Chauveau et al (1962)	10.0	16.6	16.1	12.2	-
		(3) Present investigation	10.0	16.2	16.2	13.7	1.3
Ribosomes	Yeast	(4) Monier et al (1960)	10.0	10.7	7.7	10.1	0.3
	E. coli (70S values from 30S-50S)	(5) Spahr & Tissieres (1959)	10.0	12.5	8.7	8.7	-
	E. coli 30S (native)	(6) McQuillen (1961)	10.0	12.6	9.2	9.6	-
	50S (native)		10.0	14.1	8.1	7.7	-
	30S (derived by dissociation of native 70S)		10.0	13.1	9.8	8.5	-
	50S		10.0	14.4	8.5	7.6	-
S-RNA	E. coli	(1) Spahr & Tissieres (1959)	10.0	17.2	15.5	8.1	1.5
	E. coli	(2) McQuillen (1961)	10.0	17.3	14.7	8.7	-
	Yeast	(3) Monier et al (1960)	10.0	15.8	15.3	10.8	2.1
	Yeast	(4) Osawa (1959)	10.0	13.4	11.5	10.5	2.1
	Yeast	(5) Osawa (1959)	10.0	15.2	11.7	11.2	2.7
	Rat-liver	(6) Goldthwait (1959)	10.0	15.4	9.6	6.7	-
	Rat-liver	(7) Lipshitz & Chargaff (1960)	10.0	14.8	14.5	8.5	1.6
		(8) Chauveau et al (1962)	10.0	15.9	14.7	11.4	-
Post-microsomal RNA	Rat-liver	(1) Goldthwait (1959)	10.0	15.2	9.6	9.6	5.2

are more likely due to different procedures adopted for extraction of RNA, for hydrolysis of RNA and even for separation of the nucleotides. Allfrey and Mirsky (1959) using 0.5N KOH for hydrolysis of nuclear RNA at 38° for 24 hours obtained a low result for cytidylic acid and possibly again this may be due to deamination of cytidylic acid to some extent with that strength of alkali; but they did not observe any peak for pseudouridylic acid from the Dowex-1 column eluate, nor did we in our nuclear RNA extracted by phenol or by direct alkali digestion with 0.3N KOH for 1 hour at 37°C.

The base composition of membrane-RNA studied by us and by Chauveau et al (1962) shows a definite difference from that of ribosomal-RNA, not only by absence of pseudouridylic acid in the membrane-RNA, but also with a higher quantity of guanylic acid and cytidylic acid in this fraction than that in the ribosomal-RNA. These findings not only support the earlier concept of heterogeneity of microsomal-RNA, but also give some evidence of the presence of another species of RNA in the membrane fraction of the microsomes.

The base composition of membrane-RNA is more alike to the base composition of nuclear RNA, not only by absence of pseudouridylic acid in both instances, but also by a higher quantity of guanylic acid and cytidylic acid. Similarly a higher amount of these two nucleotides has been observed in s-RNA by other investigators. Edstrom et al (1961) have recently observed an identical base composition of RNA of whole cytoplasm and nucleolus from the starfish Oocytes and the composition is markedly and significantly different from the composition of RNA from the nucleoplasm. Though there

are other differences between the whole nuclear RNA and cytoplasmic RNA, even then it is suggested by them that at a given moment cytoplasmic RNA is completely derived from the nuclear-RNA. From the similarity of base composition of the nuclear, membrane-RNA and s-RNA except for pseudouridylic acid content, one can suggest that the same RNA entity is present in the three fractions if it is assumed that pseudouridylic acid combines with s-RNA at some later period. This hypothesis is further supported by the study of molecular sizes of RNA present in three fractions. We have already shown that membrane-RNA and s-RNA are small. Kendi and Warner (1960 have reported that thymus nuclei contain a low molecular weight RNA with a sedimentation coefficient of 4S. The sedimentation coefficient of s-RNA has been determined to be 3 - 4S. The sedimentation coefficient of membrane-RNA is also found to vary between 3 - 4S. Similar observation has been noted by Kuff et al (1960) who treated the ribonucleoprotein particl with 0.5% sodium deoxycholate and found approximately 23% of RNA and 42% of protein in the supernatant fluid. Ultracentrifugal analysis of this RNA by the UV absorption technique revealed a small boundary with $S_{20}w$ between 5 and 6S. The RNA of ribosomes consists mainly of two components with sedimentation coefficients of 15 - 18S and 23 - 28S (Timasheff, Brown, Colber and Davies - 1958; Littauer and Kisambern - 1959; Hall and Doty - 1959; Maeda - 1960) suggesting that membrane-RNA has quite a different S-value from ribosomal-RNA. It has also been noted by Hall, Storck and Spiegelman (1961), Otaka, Oota and Osawa (1961) and McCarthy and Aronson (1961) that RNA in the 70S ribosomes showed the existence of two metabolically

active types - one corresponds to the large RNA molecules (25S and 16S) and the other consists of smaller (4 - 10S RNA); though recently it has been claimed (Cox and Arnstein - 1952) that smaller molecular weight RNA from microsomes is the degraded product of the larger one formed as an artefact during isolation. The former groups of workers on the other hand, suggested that 4S-RNA is the basic unit of all RNA. If this hypothesis is taken to be a correct one, from our findings we can put forward that the membrane of the microsomes contains the basic unit of all RNA. Whether this basic unit has come from the nucleus or not we cannot say with the present data and further work will be necessary to support this hypothesis. But one can assume that most probably during the process of membrane development from the nuclear membrane, as suggested by Barer and coworkers (1960), the basic unit (4S-RNA) of the RNA is also carried from the nucleus into the cytoplasm. We shall defer to our general discussion the question of "messenger" RNA.

From the results obtained by us another obvious result is that the base composition of the RNA from the different fractions of rat liver under the influence of diet varies, most markedly and significantly in the membrane-RNA with a significant lowering of guanylic and cytidylic acid content when the rats were prefed with casein. The pattern of the membrane-RNA tends to be that of ribosomal-RNA. Whether this change of pattern of base composition of membrane-RNA is anything to do with the protein synthesis or not we do not know.

SUMMARY

1. A method for the estimation of nucleotides with less variation than the usual method has been developed and described.

2. The nucleotide composition has been determined for whole microsomal-RNA, nuclear RNA, membrane-RNA and ribosomal-RNA. The RNA associated with membrane prepared from the whole microsomes by treatment with pyrophosphate (0.1 M, pH 7.4) has been shown to have an unusual nucleotide composition, notably an absence of pseudouridylic acid and a high quantity of guanylic and cytidylic acids. Absence of pseudouridylic acid has also been observed in the nuclear-RNA.

3. The nucleotide composition of the RNA from the different fractions including the post-microsomes has been found to be dependent on diet. The amount of guanylic and cytidylic acids of membrane-RNA and ribosomal-RNA is significantly lowered in protein fed animals. This change of pattern is markedly significant in the membrane-RNA. The pseudouridylic acid content of ribosomal-RNA has been found to increase significantly with the inclusion of protein in the diet.

4. The molecular size of RNA associated with the membrane has been studied by Ecteola column chromatography and by ultracentrifugal study and found to be low in comparison to that of whole microsomal-RNA and ribosomal-RNA.

SECTION IV

Studies of incorporation of ^{32}P by the RNA of different fractions of rat liver

S E C T I O N I V

Introduction

It is accepted that there is specificity in protein synthesis, and this genetic information is derived from the DNA. Except for nuclear proteins, the actual synthesis of protein takes place in the cytoplasm, more specifically in the ribosomes. DNA itself is confined to the chromosomal elements of the cells, RNA on the other hand is present both in the nucleus and cytoplasm. Because of this widespread presence of RNA, the specificity of protein synthesis derived from the DNA is supposed to be carried by the ribonucleic acid; whether that RNA is a stable RNA of ribosomes or an unstable intermediate or "messenger" RNA is not yet definitely established.

The studies of RNA associated with the different components of ribosomes have suggested that the smaller molecular sized RNA is the precursor of the larger molecular sized RNA. It has been demonstrated by different investigators, including ourselves, that the membrane of the microsomes contains a small portion of the total cytoplasmic ribonucleic acid. Our data show that the nucleotide composition of membrane-RNA is different from that of ribosomal-RNA, suggesting that membrane-RNA is a distinct species from the ribosomal-RNA. We have also shown that the nucleotide composition of nuclear-RNA is more alike to that of membrane-RNA.

There is a great deal of evidence to suggest that nuclear-RNA is the precursor of the cytoplasmic-RNA. Can the membrane-RNA, which is similar

in many respects to the nuclear RNA, be also the precursor RNA of ribosomes? Can the RNA associated with membrane be the carrier of genetic information in the protein synthesis?

In the present investigation, we have attempted to answer these questions by incorporation study of ^{32}P into the nuclear-RNA, membrane-RNA and ribosomal-RNA.

Materials and Methods

Animals: Male white albino rats weighing between 175 - 205 gms. from departmental stock have been used. The rats were caged separately in individual cages and were fed with diets as described in Section III. Fifty µc of ^{32}P were injected into the gluteal regions of the rats which had been starved overnight. In the fed groups, the rats were fed with 2.0 gm. of casein + 0.5 gm. of sodium bicarbonate 1 hour prior to ^{32}P injection. The rats were sacrificed at different intervals after ^{32}P administration.

Preparation of different subcellular fractions: The rats were lightly anaesthetised with ether, the liver was perfused with ice-cold 0.25 M sucrose 0.0018 M calcium chloride. The liver was then removed, washed with the same solution, dried in between two layers of filter papers, weighed and homogenised with 10 vols. of ice-cold 0.25 M sucrose + 0.0018 M calcium chloride, filtered through the four layers of nylon gauze and a portion of filtrate was taken for isolation of nuclei; from the remaining half of filtrate, microsomes were separated. The microsomes pellets were then

suspended in 0.25 M sucrose + 0.0018 M calcium chloride, treated with 0.1 M sodium pyrophosphate solution in 0.25 M sucrose (pH 7.4) for the isolation of ribosomes and membranes. For the isolation of nuclei, microsomes, membrane and ribosomes the same procedure as described in Section III was adopted.

Extraction of RNA from the different fractions of liver cells: One half volume of 30% (w/v) A-R trichloroacetic acid (TCA) was added with stirring to the water suspended nuclei, and the membrane and to the solubilized ribosomes in pyrophosphate solution and allowed to stand for ½ hour in ice. The precipitated material was centrifuged down and washed twice with ice-cold 10% (w/v) TCA. The precipitate was extracted successively with acetone, ethanol, ethanol/chloroform (3:1), ethanol/ether (3:1) (twice), and ether. The residual material was dried at room temperature and the dried powder was then digested with 0.3N KOH for 1 hour at 37°C. After digestion the sample was cooled in ice, neutralised with cold concentrated 10N perchloric acid (PCA), and the pH of the sample was brought down to pH 1.0 with 2.1N PCA and then centrifuged at 2,000 r.p.m. for 10 minutes at 0°C. The precipitate (protein and DNA) was washed twice with 0.7N PCA; the three supernatant fractions thus obtained were pooled and digested with KOH (final concentration 0.3N) at 37°C was continued for 18 hours. This procedur minimizes release of peptides from protein. After the second digestion, the sample was cooled in ice and then neutralised with ice-cold concentrated (10N) PCA. Potassium-perchlorate from the solution was removed by centrifugation at 0°C for 10 minutes at 2,000 r.p.m. The neutral solution

was then made acidic (pH 4.0) with TCA before application of nucleotides to the paper for ionophoresis.

Separation of nucleotides: The hydrolysed RNA sample containing about 80 µg of phosphorus was applied in a narrow band on Whatman 3MM paper 72 x 7 cm. Ionophoresis was carried out in citric acid - trisodium citrate buffer (pH 3.5), at 720 volts for 18 hours as described by Davidson and Smellie (1952). After 18 hours, the ionophoretic strips were dried in an ultra-violet lamp and the nucleotide fractions were identified with a filtered U.V. lamp. The areas for the individual nucleotides was mapped carefully, cut and the nucleotides were eluted from the paper with water. The eluates of the different fractions were pooled and radioactivity was measured. In one or two experiments, the efficiency of separation was checked by using paper chromatography to separate the nucleotides (Lipshitz and Chargaff, 1960).

Estimation of radioactivity: 5 ml. of the pooled eluate was taken into the liquid counter and counted for 10 minutes in a liquid counter (Veall). The specific activities of different fractions have been expressed thus:

Sp. activities = counts/minute/100 µg phosphorus.

Estimation of phosphorus: 5 ml. of the eluate was taken and total phosphorus was determined by a micromodification of Allen's method (1940).

Estimation of inorganic phosphorus: The supernatant obtained after sedimenti the microsomes (18,000g for 1 hour) was treated with one-half volume of ice-cold 30% TCA (w/v), allowed to stand for ½ hour in ice, centrifuged at 2,000 r.p.m. for 10 minutes at 0°C, the precipitated material was washed

twice with ice-cold 10% TCA (w/v). The inorganic phosphorus present in the pooled supernatants was precipitated as the magnesium salt, $Mg(NH_4)PO_4$, by addition of Mathison's (1905) reagent (1 ml. to 10 ml. extract). The mixture was made alkaline with 10% NH_4OH and kept in the refrigerator overnight. After standing overnight, the sample was filtered through a Whatman No. 42 paper, washed repeatedly with 10% NH_4OH and the precipitate was then dissolved in 1N HCl. The amount of inorganic phosphorus was then determined by Allen's method (1940).

A difficulty arose in connection with the use of HCl to dissolve the $Mg(NH_4)PO_4$ for estimation, if the Allen estimation of inorganic phosphate is carried out without digestion. We have observed that if different volumes of 1N HCl are added to definite amounts of inorganic phosphorus, the readings of S.P. 600 at 640 mμ varied as shown below:

ml. of 1.0N HCl added	0	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
O.D. at 600 mμ	0.345	0.340	0.330	0.320	0.300	0.275	0.250	0.190	0.160	0.145

(The amount of molybdate and amidol reagents were added for "½"-Allen to each tube).

These varying readings could be corrected if the solution in 1N HCl was digested with 10N H_2SO_4 in a similar manner to total phosphorus. The effect of this is shown below. (The phosphate solution is not of the

same strength as above).

ml. of 1.0N HCl added	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
O.D. at 640 mμ	0.169	0.166	0.160	0.165	0.176	0.175	0.170	0.168	0.172

(The amount of molybdate and amidol reagents added for g -Allen). Consequently, the inorganic phosphorus in 1N HCl solution was digested with an appropriate amount of 10N H_2SO_4 to obtain a clear sample and the inorganic phosphorus was determined from this clear digested sample according to Allen's method (1940).

RESULTS

Separation of nucleotides: In the long ionophoretic run, Davidson and Smellie (1952) observed an adequate separation of nucleotides when the extraction and hydrolysis of RNA were carried out as described by Davidson et al (1951); but in such a separation, the uridylic acid from ^{32}P-labelled RNA was found to be accompanied by two components that appeared only in the autoradiograph and not in U.V. print, indicating traces of highly radioactive material. In our case also, the separation of nucleotides was adequate, though we have not examined the strip by autoradiography. Except cytidylic and adenylic acids, guanylic acid and uridylic acid were far away from each other, and the band for the inorganic phosphate (recognised by radioactivity) was always at the extreme end of the paper. We marked the areas under the U.V. lamp, and hence the contaminants that

might affect the uridylic acid were not likely to be included. However, occasionally we used to get a band between the cytidylic acid and the adenylic acid, especially if the initial digestion period for extraction of RNA was prolonged more than 1 hour. Whenever we have observed this band, it was marked and eluted separately. The optical densities of this eluate were examined on each occasion at pH 1.0 from 220 - 300 mμ and within this range of wave lengths we did not observe any peak for any nucleotides. The eluate was then examined for radioactivity and on each occasion we observed that this extra component did not contain any significant amount of radioactivity. Consequently, in our study of the radioactivity of the nucleotides of different fractions of liver cells we have not included this band.

For further confirmation that the eluted material did contain no materials other than the nucleotides, in a series of experiments the same hydrolysed material was subjected to ionophoretic and chromatographic separation and the nucleotides thus separated by the two procedures were compared. The results are shown in Table XXIV, from which it can be seen that the radioactivity of the individual nucleotides separated by the two procedures are identical, within the limits of error of the methods.

Specific activity of RNA of different fractions of liver cells: The incorporation of ^{32}P into the RNA of nuclei, membrane and ribosomes was studied at intervals of 1 hour, 3 hours and 8 hours after administration of ^{32}P and the results are tabulated in Table XXV. The rats were fed with low protein (fasted overnight), high protein (fasted overnight) and high

Table XXIV

Comparison of Sp. activities obtained by ionophoresis and chromatography separation of nucleotides: 50 μc of ^{32}P injected into the gluteal region of rat and RNA was extracted from the liver cells with direct alkali digestion (1 hour at 37°C with 0.3N KOH), the acidic solution was applied on Whatman 3MM paper for ionophoresis and neutral solution for chromatography on Whatman No. 1 paper. The results are the average of two experiments.

Sp. activities = counts/minute/100 μg phosphorus

Fractions		Ionophoresis	Chromatography
Nuclear (1 hour after ^{32}P administration)	Adenylic acid	3,440	3,740
	Guanylic acid	3,230	3,120
	Uridylic acid	3,160	3,530
	Cytidylic acid	3,060	3,620
Membrane (3 hours after ^{32}P administration)	Adenylic acid	3,662	4,000
	Guanylic acid	9.650	8,850
	Cytidylic acid	12,400	10,600
	Uridylic acid	9,950	6,240

Table XXV

Uptake of ^{32}P by RNA of different fraction of rat liver: 50 µc of ^{32}P was injected into the rats under different dietary regime namely - low protein fasted, high protein fasted, high protein fed. RNA was extracted by direct alkali digestion with 0.3N KOH for 1 hour at 37°C (Fleck and Munro - 1962) followed by 18 hours hydrolysis with 0.3N KOH at 37°C. Ionophoresis was carried out as described by Davidson and Smellie (1952).

Sp. activities = counts/minute/100 µg phosphorus

Fraction	Nutritional Status	Sp. activities after hour administration ^{32}P		
		1	3	8
Nuclei	L.P. fasted	432	2572	2556
	H.P. fasted	356	1812	1808
	H.P. fed	726	1898	1915
Membrane	L.P. fasted	85	460	1532
	H.P. fasted	44	306	707
	H.P. fed	102	512	878
Ribosomes	L.P. fasted	3	147	1062
	H.P. fasted	2	80	269
	H.P. fed	1	183	542
Sp. activities of inorganic phosphorus				
	L.P. fasted	27875	20785	14141
	H.P. fasted	13553	12358	9471
	H.P. fed	16085	12,551	8,526

(Results for L.P. fasted are from 2 experiments, and for H.P. fasted and H.P. fed are the average of 3 experiments).

protein (fed protein 1 hour before administration of the isotope).

It is obvious from the results that the highest incorporation of ^{32}P is in the nuclear RNA in between the 3 and 8 hours after injection of ^{32}P; at 8 hours the incorporation is more or less the same as at 3 hours, with a tendency towards decline. This finding is in accord with the earlier findings of others who have observed a very much more rapid uptake of ^{32}P by nuclear RNA than the cytoplasmic RNA. The activity of nuclear RNA rose rapidly and fell sharply after administration of the isotope (Smellie - 1955).

In our experiment after 1 hour of administration of ^{32}P the membrane-RNA showed some radioactivity but none whatsoever in the ribosomal RNA in that period. The uptake of isotope by membrane-RNA, though much less in comparison to that by nuclear-RNA at 1 hour, is gradually increasing whereas uptake by the nuclear-RNA is gradually decreasing. From 3 hours onwards there is incorporation of ^{32}P into ribosomal-RNA. The uptake of isotopes in the membrane-RNA even after 8 hours administration of isotope is still significantly higher than that of ribosomal-RNA.

Another interesting result presented in Table XXV is that with the variation of diet there is also variation of incorporation of ^{32}P. In the nuclei after 3 and 8 hours of administration of ^{32}P there is no difference between the H.P. fasted and H.P. fed though the difference is quite significant between the L.P. fasted and H.P. fasted or H.P. fed at these periods, whereas, in the membrane and ribosomes, the difference between the low protein fasted and high protein fed is not significant after 3 hours

of administration of isotope; but the difference between the L.P. fasted or H.P. fed and H.P. fasted is significant. At 8 hours the differences in uptake of ^{32}P by the ribosomal RNA of the three groups are significant. In general, prefeeding of rats with protein before injection restores the capacity of uptake of ^{32}P.

Though this observation was consistent, we performed another series of experiments with high-protein-fasted and high-protein-fed groups of rats. 250 μc of ^{32}P were injected into each rat in the gluteal region and RNA was extracted directly with alkaline digestion and hydrolysed as before. In this series of experiments instead of pooling the eluates of different nucleotides together, we measured the uptake of isotope by the individual nucleotides separately and the results have been tabulated in Table XXVI. From the results it is obvious that the Sp. activities (counts/minute/100 μg P between the individual nucleotides of nuclear-RNA and ribosomal-RNA are not very different, but it is markedly different in the case of membrane-RNA. Similar large discrepancies between the individual nucleotides of nuclear, deoxycholate-soluble and ribosomal-RNA have been observed by Shigeura and Chargaff (1958). The way they extracted and hydrolysed the RNA is slightly different from our method; moreover, the discrepancy between the individual nucleotides in our case is limited only to the membrane fraction, a fraction rich in phospholipid and phosphoprotein. High Sp. activities are seen in the two nucleotides (cytidylic and uridylic acids) which are at the two extreme ends of the ionophoretic strips. We, therefore, assumed that it might be due to contamination and so a pilot experiment was performed.

Table XXVI

Sp. activities of individual nucleotides of RNA of different fractions of rat liver: 250 μc ^{32}P was injected into each rat and RNA was extracted by direct alkali digestion followed by hydrolysis for 18 hours in 0.3N KOH at 37°C. The separation of nucleotides was carried out by ionophoresis (Davidson and Smellie - 1952).

Fraction	Nucleotides	Sp. activities		
		1 hour	3 hours	8 hours
Nuclei	Cytidylic acid	4,000	14,500	16,000
	Adenylic acid	2,440	14,650	18,500
	Guanylic acid	3,230	14,500	12,400
	Uridylic acid	4,160	15,200	13,900
Membrane	Cytidylic acid	2,982	12,400	27,900
	Adenylic acid	267	3,662	3,300
	Guanylic acid	213	9,650	6,400
	Uridylic acid	2,950	11,950	21,400
Ribosomes	Cytidylic acid	214	3,380	8,100
	Adenylic acid	154	2,330	8,500
	Guanylic acid	155	2,580	8,350
	Uridylic acid	174	2,400	8,150

One hundred μc of ^{32}P were injected into the rat and RNA from the three fractions was extracted with phenol under identical experimental conditions as described in Section III. The RNA was hydrolysed with 0.3N KOH at 37°C for 18 hours. The separation of nucleotides was carried out by chromatography and the nucleotides were eluted and radioactivity was measured in the individual nucleotides. From the results in Table XXVII, it is seen that, in the phenol extractable RNA of the membrane, the uptake of individual nucleotides does not vary so much as in the case of direct alkali extraction.

So, in a series of experiments, the high-protein-fasted and high-protein-fed rats were injected with 250 μc of ^{32}P and then RNA from the membrane and from the ribosomes was extracted by 90% phenol and hydrolysed with 0.3N KOH for 18 hours. The separation was again carried out by ionophoresis. The uptake of individual nucleotides was measured and the results have been tabulated in Table XXVIII. From this table, it is evident that one hour after isotope administration, the uptake by the ribosomal-RNA is much less than into membrane, and as in the case of direct alkali digestion the uptake by the ribosomal-RNA gradually increases.

It has been shown by us and also by others that the nuclear-RNA does not contain any pseudouridylic acid, and if the hypothesis that nuclear-RNA os the precursor of cytoplasmic RNA be accepted as a correct one, it is difficult to believe that the pseudouridylic acid is also derived from the nucleus. To study this we have performed a further experiment. As pseudo-uridylic acid cannot be separated by the ionophoresis, this nucleotide

Table XXVII

Sp. activities of individual nucleotides of membrane-RNA: 250 μc of ^{32}P were injected into the rats and killed, one after administration of isotopes. The RNA was extracted with phenol and the extracted RNA hydrolysed with 0.3N KOH for 18 hours at 37°C. The hydrolysed RNA was separated by chromatography.

Sp. activities = counts/minute/100 g of P

No. of Experiment	Nucleotides	Sp. activities
1	Adenylic acid	9,750
	Guanylic acid	7,780
	Cytidylic acid	7,350
	Uridylic acid	11,850
2	Adenylic acid	416
	Guanylic acid	598
	Cytidylic acid	448
	Uridylic acid	316
3	Adenylic acid	7,850
	Guanylic acid	7,650
	Cytidylic acid	7,450
	Uridylic acid	11,650

Table XXVIII

Sp. activities of RNA of different fractions of rat liver: 250 μc of ^{32}P were injected into H.P. fed and H.P. fasted rats and the RNA from the membrane and the ribosomes was extracted with phenol, hydrolysed with 0.3N KOH for 18 hours at 37°C. The nucleotides were separated by chromatography. The individual nucleotides are eluted, Sp. activities measured separately. The results presented here are:-

Sp. activities of nucleotides
(average of all nucleotides)

Fraction	Nutritional Status	Sp. activities at 1 hour	8 hours
Membrane	Fasted	239	2,020
	Fed	532	3,518
Ribosomes	Fasted	70	3,088
	Fed	63	2,445

was separated by chromatography as described in Section III. The nucleotides are eluted and uptake of ^{32}P measured and the results are tabulated in Table XXIX. From the presented results it is evident that, like other nucleotides of ribosomal-RNA, uptake of ^{32}P by pseudouridylic acid at the earlier periods is very low and gradually increases. It seems that uptake of ^{32}P by pseudouridylic acid tends to be lower than the other nucleotides.

In another experiment where the rats were injected with 100 μc of ^{32}P and killed 16 hours after injection, the uptake of the isotope by the ribosomal-RNA is now significantly above the uptake of ^{32}P by the nucleotides of membrane-RNA at that time period as seen from the results in Table XXX.

DISCUSSION

From the results presented above three important facts are obvious: (i) the uptake of ^{32}P by the RNA of different fractions of rat liver cells varies with the variation of diet, (ii) the uptake of ^{32}P in the ribosomal-RNA at one hour after administration is almost negligible, and (iii) the uptake of pseudouridylic acid is also very low and gradually increases with increase of time interval after injection of isotope.

Our finding of the highest uptake of ^{32}P by the liver-RNA in the low protein fasted rats are in concord with the earlier findings of Clark et al (1957) who observed a higher uptake of ^{32}P by liver RNA from the normal liver fasted overnight and lowest in case of rats fasted after a protein-containing diet. Prefeeding of rats with 2 gms. of casein and 0.15 gm.

Table XXIX

Sp. activities of pseudouridylic acid in comparison to other nucleotides of rat liver ribosomes: 250 μc of ^{32}P were injected into each rat, RNA extracted with phenol and hydrolysed with 0.3N KOH for 18 hours at 37°C. Separation of nucleotides was carried out by chromatography.

Sp. activities = counts/minute/100 g P

Nucleotides	Sp. activities		
	1 hour	3 hours	8 hours
Adenylic acid	0	958	9,900
Guanylic acid	8	519	4,860
Cytidylic acid	23	908	1,120
Uridylic acid	111	1,720	5,060
Pseudouridylic acid	0	395	895

Table XXX

Sp. activities of nucleotides of membrane and ribosomal-RNA: 100 μc of ^{32}P were injected into the gluteal region of the rats and after 16 hours RNA was extracted with phenol, hydrolysed in 0.3N KOH for 18 hours at 37°C and separated by chromatography.

Sp. activities = counts/minute 100 μg P

Fraction	Sp. activities of different nucleotides				
	Adenylic	Guanylic	Cytidylic	Uridylic	Pseudouridylic
Membrane	365	595	587	410	0
Ribosomes	1,640	2,100	2,080	1,545	480

of sodium bicarbonate prior to administration of isotope rapidly restored incorporation to a high level. Thus, our findings support the earlier hypothesis put forward by Clark et al (1957), namely that the rate of breakdown of RNA is influenced by the availability of amino-acids. Munro and Clark (1960) further suggested that the variation of RNA content of liver with the variation of diet seems to occur mainly in the endoplasmic reticulum. Though it is difficult to reach final conclusions at the present stage, it seems from the presented results that there is a tendency for the effect of diet on uptake of ^{32}P to be greater in the case of the membrane-RNA.

Our observation of high initial uptake of ^{32}P by the membrane-RNA corresponds to the findings of Shigeura and Chargaff (1958) though differs from the findings of Sachs (1958). The former group of workers suggested that inorganic phosphate as a precursor of RNA moves thus:

Cellsap RNA $\longrightarrow$ deoxycholate-soluble RNA (membrane) $\longrightarrow$ ribosome RNA

because the initial incorporation observed was greatest in the supernatant fraction (supernatant obtained after centrifuging at 105,000g for 1 hour) and next in the deoxycholate-solubilised fraction. We have not examined the supernatant fraction, but from our findings it seems that inorganic phosphorus as a precursor of RNA moves in the following manner:-

Nucleus $\longrightarrow$ Membrane of microsomes $\longrightarrow$ RNP particles

Our findings are in a way quite similar to the findings of other investigators who have observed by incorporation studies, including the autoradiographic technique, that when RNA precursors are presented to a cell they are first incorporated into the nuclear-RNA and later appear in the cytoplasm. These

findings support our hypothesis of movement of RNA in different cell fractions. Georgiev, Samarina and Zbarsky (1961) recently concluded that the macromolecule that participates the synthesis of ribosomes and "soluble-RNA", probably belonging to the nucleochromosomal apparatus, passes into the cytoplasm by way of the nuclear sap; this fraction was considered to be synthesized from free nucleotides and may serve as a precursor of cytoplasmic RNA. Perry and co-workers (1961), studying the kinetics of nucleoside incorporation into nuclear and cytoplasmic RNA in tissue culture cells, suggested that either all the RNA is made in the nucleus and transported to the cytoplasm as such, or some complex intermediate is made and then transported to the cytoplasm. Studies of molecular sizes of RNA of different fractions of liver cells and uptake of ^{32}P by the RNA supports the concept that a basic unit of RNA is synthesized in the nucleus and this basic unit is then transported to the cytoplasm. It this is so, pseudouridylic acid combines with the ribosomal-RNA at some stage of transportation.

SUMMARY

1. After injection of ^{32}P into rats, uptake of ^{32}P is highest from the beginning in the nuclear-RNA, the maximum being attained some time between 3 to 8 hours.

2. There is some incorporation of ^{32}P in the membrane-RN one hour after administration of ^{32}P, quite low activity at that period being observed in the ribosomal-RNA. The activity in both fractions gradually increases up to 8 hours after administration of isotope, the activity in the membrane-RNA

is higher than the ribosomal-RNA; at 16 hours after ^{32}P injection the activity in ribosomal-RNA is higher.

3. The activity in the pseudouridylic acid of ribosomal-RNA is initially low like other nucleotides. The uptake of ^{32}P by this nucleotide gradually increases with increasing time intervals after administration of ^{32}P, but may be less than that of other ribosomal-RNA nucleotides.

4. The uptake of isotopes by the RNA of different fractions varies with the nutritional status of the rat, the highest being in the low protein group fasted overnight, and lowest in the high protein group fasted overnight. The capacity to incorporate is markedly stimulated if the rats are prefed with protein just before injection of isotope. There is no difference of uptake of isotope by the nuclear RNA between the high protein fasted and high protein fed groups 3 and 8 hours after administration of ^{32}P.

GENERAL DISCUSSION

GENERAL DISCUSSION

The formation of prothrombin by microsomes:

The present-day accepted view on protein synthesis is that the amino-acid is first activated by its specific activating enzyme, forming an enzyme bound amino-acyl adenylate compound. This amino-acyl compound, by means of the same activating enzymes, reacts with an RNA molecule specific for the particular amino-acyl adenylate-enzyme complex, with the esterification of the amino-acid on the 2' or 3' hydroxyl of the terminal adenosine of RNA, the adenylate moiety of the activated compound being released. Many investigators, including Littlefield et al (1955) and Simkin and Work (1957), have shown that the ribosomes incorporate labelled amino-acids more rapidly than the membrane of microsomes. Since then, the ribosomes particles have been isolated from the nucleus (Frenster et al - 1960) and from mitochondria (Rendi - 1959) and it seems that no protein synthesis takes place except on ribosomes. On the other hand, initial high incorporation of labelled amino-acids into the protein of the lipid membrane of the microsomes as pointed out by Spiegelman (1959) and Campbell (1961) suggests that the membrane of the microsomes also plays a part in protein synthesis. The nature of this action is still in doubt.

In the present investigation, we have observed that only the heavy microsomes of the rat liver are capable of increasing prothrombin activity of the preparation on incubation. The other fractions, namely the mitochondria, the light microsomes and the cell sap do not show such an increase in activity of prothrombin. Before further discussion, it will be better to mention here that isolation of pure prothrombin is quite difficu

and hence incorporation experiments cannot be suitably extended to the study of the synthesis of prothrombin by the different fractions of the liver cells, as in the case of albumin synthesis. The method which has been applied to measure the prothrombin not only measures the prothrombin but also Factor VII. So, the increased activity of "prothrombin" by the heavy microsomes on incubation does not necessarily include prothrombin but also Factor VII which is, however, an inactive precursor of prothrombin, and hence, whether the observed increased activity is due to conversion of Factor VII from its inactive form to its active form prothrombin or simply a new formation of prothrombin by some other mechanism, the net product is increased activity of prothrombin. The heavy microsomes include ribosomes attached to the reticulum membrane, whereas the light microsomes mainly consist of the free detached ribosomes. The increased prothrombin formation by the heavy microsomes might be due to the attached ribosomes, thus supporting the earlier view (Cohn - 1959, Rendi and Hultin - 1960, Korner - 1960) that RNP particles prepared from liver are active sites of incorporation only when they are bound to the membrane of the microsomes; otherwise the membrane of the microsomes alone would be specific for the observed increased prothrombin formation. This view is more acceptable because of the distribution of enzymes in the two fractions. Fouts (1961) recently demonstrated that the rough-surfaced vesicles of microsomes are less active per mg. of protein for a number of enzymes, in comparison to the concentration of these enzymes associated with the smooth-surfaced microsomes.

Formation of prothrombin by the heavy microsomes on incubation cannot

be regarded as the synthesis of prothrombin in the truest sense, and definitely not through the carboxyl activation process, because, whether the membrane or the RNP particles be the sites of protein synthesis, both systems need a supply of energy and amino-acids. These were not supplied exogenously in our experiments. Spiegelman (1959) observed in the membrane fraction of bacteria that omission of ATP or the 5'-nucleotides results in a drastic loss of incorporation observed over 2.5 hours. Lipmann (1949) was the first to suggest that formation of peptide bond was by way of phosphorylated intermediates, and Zamecnik and Keller (1954) supported his view by demonstrating that incorporation of labelled amino-acids into protein could be obtained by activating the system by addition of ATP and of substrates like phosphocreatine for the continuous regeneration of ATP. The new formation of prothrombin by the heavy microsomes of the rat liver cannot therefore be a simple process of synthesis through the carboxyl activation mechanism as the increased activity has been observed without any addition of ATP or ADP and without any amino-acids. Addition of ATP or ADP alone also does not alter the picture up to a certain concentration of ATP or ADP. If a very high concentration of ATP or ADP (15 µmole/ml.) be added to the system, there is some inhibition of formation of prothrombin towards the end of incubation time; most probably the high concentration of phosphate adsorbs the prothrombin from the system. However, the increased prothrombin activity by the heavy microsomes on incubation on the other hand is not due to a simple release phenomenon of preformed prothrombin. Douglas and Munro (1959) observed an inactive form of amylase which could be released

as an active form by disintegrating pancreatic microsomes with ballotini beads and they concluded that the inactive amylase of fresh pancreatic microsomes is present in the interior of the vesicles. In our case, we disintegrated the microsomes with ballotini beads and with ultrasonic vibration; no release of preformed prothrombin was observed, suggesting that the increased activity of prothrombin by the heavy microsomes was not due to release of pre-existing prothrombin.

The mitochondrial system is capable of forming ATP and though it is possible that this ATP may be used for the synthesis of protein, it has been demonstrated by Roodyn and others (1961) that, when ATP was trapped with hexokinase, there was still incorporation of amino-acids into protein, suggesting that the synthesis of protein by the mitochondrial system might ge independent of availability of energy source. Though our preparation of different fractions of liver cells were not purified and there was every chance of contamination of one fraction with another fraction, it seems unlikely that there could be sufficient contamination of heavy microsomes with mitochondria to explain the formation of prothrombin by the heavy microsomes without any addition of energy source. In this connection, we would like to mention the very interesting result of increased prothrombin formation by the heavy microsomes of the liver cells when the rats were prefed with glucose two hours before killing. We cannot offer an explanation for this but at the same time we do not think that it is due to increase amount of ATP in the liver after feeding the rats with glucose, since adding ATP to the medium does not have this effect.

Work by Greenberg et al (1952), Winnick (1950), and Siekevitz (1952) have showed that conditions for obtaining the incorporation of labelled amino-acids are also the conditions for promoting oxidative-phosphorylation; addition of DNP inhibits the protein synthesis. The biosynthesis of protein studied in the cell-free system of bacteria is inhibited in an atmosphere of nitrogen (Beljanski et al - 1958). The incorporation capacity, once inhibited either by incubating in the atmosphere of nitrogen or by addition of dinitrophenol, cannot be restored by addition of ATP, which might be due to failure to reach the active site. The capacity of formation of prothrombi by the heavy microsomes is not altered if incubated in an atmosphere of nitrogen: carbon dioxide instead of oxygen: carbon dioxide; nor by addition of DNP to the system up to 2 hours. At the third hour period of incubation in nitrogen or in presence of DNP there is some inhibition of prothrombin formation, most probably due to disintegration of microsomal structures in the absence of oxidative phosphorylation. This experimental evidence suggests that formation of prothrombin by the heavy microsomes is not dependent on oxidative phosphorylation in the initial stage. This view is further supported by the fact that addition of NAD, $NADH_2$, NADP, or $NADPH_2$ to the system does not <u>increase</u> the formation of prothrombin by the heavy microsomes, but after 2 hours of incubation, there is an inhibitory effect of NAD and NADP, though not with $NADH_2$ or with $NADPH_2$. The absence of an inhibitory effect with $NADH_2$ and $NADPH_2$ could not be explained. It might be due to presence of $NADH_2$-cytochrome-c-reductase and $NADPH_2$-cytochrome reductase in the microsomes, but that cannot be ascertained. That it is

not due to simple oxidative phosphorylation is suggested by the fact that formation of proconvertin (Factor VII) by the liver cells is not influenced by dicumarol and vitamin K (Lasch, Pfisterer and Schimpf - 1957).

The occurrence of RNA in the microsome membrane and its relationship to prothrombin formation:

The increased activity of prothrombin by the heavy microsomes on incubation is not autoactivation. Whatever might be the mechanism, there is actual formation of new prothrombin which is evident from the relationship of ribonucleic acid to the prothrombin formation as well as from the experime mentioned above with ballotini beads. The ribonuclease destroys the integrity of RNA structure and is capable of inhibiting the formation of prothrombin by the heavy microsomes. This suggests that the process of formation of prothrombin is related to ribonucleic acid which might carry out the transformation of precursor protein to prothrombin. Straub et al (1960) observed that a "soluble system" of pigeon pancreas alone cannot form increased amount of pancreas amylase on incubation at 37°C. However, synthesis occurred when ATP, threonine and arginine were added to the system. A significant increase in synthesis was also observed when soluble system was incubated with "specific g RNA" (RNA extracted from the pancreas granules and this specific g RNA after incubation was able to replace the ATP, arginin and threonine. If RNA from the nonincubated sample be added to the soluble system there was no synthesis of amylase; this suggested that g-RNA in

presence of ATP, threonine and arginine, is converted to amylase-specific-g-RNA which in turn acts on the precursor protein to convert it into amylase. Whether the same principle is applicable or not to the formation of prothrombi we do not know, but addition of phenol-extracted RNA from the heavy microsomes or pH 5 enzymes does not alter the formation of prothrombin. The inhibitory effect of ribonuclease on prothrombin formation, not only observed in case of heavy microsomes alone, but on prothrombin formation by the isolated membrane. Though there are few instances in which it has been shown that the membrane of the microsomes might be more intimately concerned with protein synthesis, the function of the membrane has yet to be established definitely. The capacity of the membrane of microsomes to form prothrombin in our case might be another piece of evidence to support that hypothesis. We can, however, definitely conclude that the ribonucleic acid associated with the membrane has some biological activity, because addition of ribonuclease to the system inhibits the formation of prothrombin in parallel with loss of the RNA.

The one gene - one ribosome - one protein hypothesis has lost much of its ground and from experiments on virus infection of bacteria Brenner et al (1961) have suggested the presence of an unstable intermediate or "messenger" RNA carrying the information from genes to ribosomes for protein synthesis. This unstable intermediate or "messenger" RNA has the same base composition as the DNA of the virus. This messenger RNA is not only a minor fraction of total RNA (not more than 4%) but is also heterogeneous in size and is distributed unevenly in the different subunits of the bacterial

ribosomes. Nomura, Hall and Spiegelman (1960) studied the specific messenger RNA formed on phage infection and observed a lower sedimentation constant (8S) than the sedimentation constant of ribosomal-RNA (16S and 23S). Gros et al (1961) also obtained a similar sedimentation constant (8S) for the newly-synthesized messenger RNA which has a rapid turnover and which they found to be attached reversibly to ribosomes depending upon the magnesium concentration (messenger RNA). They did not observe any appreciable synthesi of typical 16S or 23S ribosomal-RNA during phage formation in the cell. The kinetic study noted by them revealed that 16S RNA molecules always become labelled before appreciable labelling of 23S RNA molecules and suggested that 23S RNA molecules form from two 16S chains. Similar observation has been noted by Aronson and McCarthy (1961). They also observed a species of RNA with sedimentation constant of 4-8S in all the components of ribosomes and this RNA becomes labelled very quickly and suggested that this lower molecular weight RNA is a precursor of the 28S and 18S RNA (ribosomal-RNA). From all these studies, it seems probable that a rapidly turning-over type of RNA, "messenger RNA", of molecular size (4S - 12S in various cases) is present in bacteria. Our own incorporation studies with ^{32}P into the different fractions of rat liver RNA it was found that, next to the nuclear-RNA, the membrane-RNA shows the greatest radioactivity at 1 hour after ^{32}P administration, whereas at that period the ribosomal RNA has no incorporation of ^{32}P. The ultracentrifugal analysis of the membrane-RNA has showed the sedimentation constant for this fraction of RNA is very low (3-4S) and is similar to the sedimentation constant of some of the nuclear RNA's (Rendi -1966

Hence it is suggested that the low molecular weight RNA in the membrane might be the precursor of the ribosomal-RNA, and be a form of mammalian messenger RNA.

Astrachan and Volkin (1958) first reported the existence of a species of RNA related to the composition of DNA. It has been suggested that the key to the genetic code lies in the base composition of the messenger or template RNA (Leslie - 1961) which, by all indications, is produced as single-stranded polynucleotides on the DNA "master genes". The symmetrical base ratios of DNA (A = T, G = C) is a recognised feature of the DNA duplex, the asymmetrical base ratios of RNA being a different molecular species of single-stranded template RNA (Leslie - 1961). In our study the base compositions of RNA of the different fractions of rat liver cell are "symmetrical" (A = U, G = C). We have not studied the base composition of RNA extracted with ice-cold dilute sulphuric acid to remove the histone protein and about 50% of the original RNA to obtain the asymmetrical base composition (Leslie - 1961). It was not our intention to test the genetic coding system: however, when we compare the base composition of our RNA from the different fractions of the rat liver cell with the base composition of duplex DNA of the liver cell (A = 29, T = 29, C = 21, G = 21, Leslie - 1961) we observed the reverse order between the base composition of nuclear (A = 18.7, U = 18.4, C = 31.3, G = 31.8) and membrane-RNA (A = 17.0, U = 16.8, C = 32.2, G = 34.1) with the base composition of DNA.

The origin of membrane-RNA from nuclear RNA is supported by other data, namely (i) the well established events of rapid labelling of nuclear

RNA followed by gradual levelling out of the difference between nuclear and ribosomal-RNA, (ii) the morphological origin of the membrane of the endoplasmic reticulum being in the nuclear membrane (Barer and co-workers - 1960). In concord with our findings, Schulman and Bonner (1962) recently observed in Neurospora Crassa, a deoxycholate (DOC)-solubilised RNA fraction with a high specific activity approximately eight times that of the particulate RNA fraction, and they suggest that this DOC-solubilised fraction contains the newly-synthesized RNA. They have noted that there is a naturally-occurring DNA-RNA hybrid in the DOC solubilised fraction of microsomes (membrane) which fulfils some of the requirements of an intermediate in the transfer of genetic information from DNA to RNA (Schulman and Bonner - 1962).

Munro and Clark (1959) have suggested that the breakdown of liver RNA when the diet fails to supply all the essential amino-acids is due to disintegration of the s-RNA. They have suggested the hypothesis that the supply of amino-acids for endoplasmic reticulum (microsome) formation determines the course of reactions:

acid soluble precursor compounds $\longleftrightarrow$ s-RNA $\longleftrightarrow$ microsomal-RNA

In our case, we have observed a significant alteration of nucleotide composition of RNA of rat liver with variation of the nutritional status, with a significant decrease of guanylic acid in the fed group. In the membrane RNA, a decrease was also noticed in the cytidylic acid content in the fed group. ^{32}P incorporation study also reveals a significantly high incorporation of the isotope in the membrane RNA in the prefed rats. On

the basis of these findings we can further modify the earlier hypothesis of Munro and Clark (1959) that changes of RNA content of liver cells with the protein level in the diet seems to occur in the RNA associated with the membrane of the endoplasmic reticulum of the liver cell.

SUMMARY

SUMMARY

The present investigation may be considered to consist of the two parts. The first part (Sections I and II) deals with the formation of prothrombin which showed that prothrombin formations occur in the liver microsomes and is dependent on the integrity of their RNA. In the second part (Sections III and IV) results of investigation on some of the physical and chemical properties, including the nucleotide composition and ^{32}P incorporatio of the different fractions of cell RNA, have been presented.

Section I - Prothrombin formation by the different fractions of rat liver:

1. The microsomal system of the liver is capable of showing an increase in prothrombin activity when it is incubated in a modified Krebs Ringer bicarbonate solution. The heavy microsomes (sediment obtained after spinning the mitochondrial supernatant at 18,000 g for 1 hour) are seen to be more active than the light microsomes in this respect.

2. Whether the particles are incubated in an atmosphere of O_2:CO_2 or N_2:CO_2, there is no effect on the increment in prothrombin up to 2 hours of incubation.

3. Pre-treatment of the rat with glucose 2 hours before killing results in a considerably increased rate of formation of prothrombin by incubated microsomes. This effect is apparent throughout the whole period of incubatio

4. The early stages of this formation are not dependent on oxidative phosphorylation, since incubation of the heavy microsomal particles with dinitrophenol, NAD, $NADH_2$, NADP, $NADPH_2$ or mixture of ATP and NAD, ATP and $NADH_2$ does not inhibit nor increase the formation of prothrombin. After

some 2 - 3 hours of incubation there is an inhibitory effect of NAD or NADP which is not shared by $NADH_2$ and $NADPH_2$. ATP in high concentration inhibits prothrombin formation towards the later part of incubation.

5. The increased activity is not due to release of preformed prothrombin bound to the microsomal vesicles, as there is no increase in activity when the microsomes are disrupted with ballotini beads or with ultrasonic vibration

Section II - Relationship of ribonucleic acid to prothrombin formation by microsomal particles:

1. Prothrombin formation by heavy microsomes of liver is dependent on ribonucleic acid, since addition of ribonuclease to the incubating system markedly inhibits formation of prothrombin. But addition of samples of ribonucleic acid prepared from heavy microsomes or pH 5 enzyme from cell sap (containing sRNA) does not alter the process of "synthesis".

2. The microsomal membrane, prepared after pyrophosphate treatment is still capable of synthesizing prothrombin. Here again, the activity is not specifically associated with the high content of phospholipid but with ribonucleic acid in the membrane; the increased activity of prothrombin on incubating the membrane is completely inhibited by addition of ribonuclease to the system.

Section III - The nucleotide composition of ribonucleic acid in different fractions of rat liver:

1. A method for the estimation of nucleotides by paper chromatography with less variation than the usual method has been developed.

2. The nucleotide composition has been determined for whole microsomal-RNA, nuclear-RNA, membrane-RNA and ribosomal-RNA. The RNA associated with membrane prepared from the whole microsomes by treatment with pyrophosphate (0.1 M, pH 7.4) has been shown to have an unusual nucleotide composition, notably an absence of pseudouridylic acid and a high quantity of guanylic and cytidylic acids, as compared with ribosomal-RNA. Absence of pseudouridylic acid has also been observed in the nuclear-RNA. The nucleotide composition of nuclear and membrane-RNA are very close to each other. It is thus possible that membrane-RNA arises from nuclear-RNA.

3. The nucleotide composition of the RNA from the different fractions, including the post-microsomes, has been found to be dependent on diet. The amount of guanylic and cytidylic acids of membrane-RNA and ribosomal-RNA is significantly lowered in protein-fed animals. This change of pattern is most marked in the membrane-RNA. The pseudouridylic acid content of ribosomal-RNA has been found to increase significantly with the inclusion of protein in the diet.

4. The molecular size of RNA associated with the membrane has been studied by ECTEOLA cellulose column chromatography and by the ultracentrifugal study and found to be low in comparison with that of whole microsomal-RNA and ribosomal-RNA.

Section IV - Studies of incorporation of ^{32}P by the RNA of different fractions of rat liver:

1. After injection of ^{32}P into rats, uptake of the isotope is highest from the beginning in the nuclear-RNA, the maximum being attained some time between

3 to 8 hours after administration.

2. There is some incorporation of ^{32}P in the membrane-RNA one hour after administration of the isotope, but very low activity at that period is observed in the ribosomal-RNA. The activity in both fractions gradually increases up to 8 hours after injection of ^{32}P, the activity in the membrane-RNA even at that period being higher than in the ribosomal-RNA; at 16 hours after ^{32}P injection the activity in ribosomal-RNA is higher than in the membrane-RNA. These findings are consistent with the formation of membrane-RNA first from nuclear-RNA.

3. The uptake of isotopes by the RNA of different fractions varies with the nutritional status of the rat, the highest being in the low protein group fasted overnight, and lowest in the high protein group fasted overnight. The capacity to incorporate is markedly stimulated if the rats are prefed with protein just before injection (1 hour) of isotope. There is no difference of uptake of isotope by the nuclear-RNA between the high protein-fasted and high protein-fed groups 3 and 8 hours after administration of ^{32}P.

From these findings, it has been concluded that the prothrombin molecule can be finalised by the microsome membrane, a type of RNA associated with the membrane being essential for the process. The membrane RNA appears to arise from the nucleus and to differ from ribosomal-RNA. It may represent a form of messenger RNA.

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Studies on the synthesis of liver-proteins with particular reference to Prothrombin

by

Pratul Goswami, M.B.B.S., D.Phil.(Med.)

The formation of prothrombin by liver cell particles has been studied under in vitro conditions. Mitochondria, a heavy microsome, and a light microsome fractions were isolated from rat liver and were incubated in a modified bicarbonate buffer solution at pH 6.9 in an atmosphere of oxygen. The heavy microsome fraction showed an increase in prothrombin activity.

This increased prothrombin activity by the heavy microsomes, lasting throughout a three-hour incubation period, was also seen if the system was incubated in an atmosphere of nitrogen up to a period of two hours. Feeding the rat with glucose before killing resulted in a considerably increased rate of formation of prothrombin by the incubated heavy microsomes. In spite of this observation, it was found that the early stages of prothrombin formation by the heavy microsome fraction was not dependent on oxidative phosphorylation, since incubation with dinitrophenol, nicotinamide-adenine dinucleotide (NAD), nicotinamide-adenine dinucleotide phosphate (NADP), and the corresponding reduced forms $NADH_2$ and $NADPH_2$ and adenosine-5'-triphosphate (ATP) neither inhibited nor increased the initial formation of prothrombin, though some effects after two hours of incubation were seen, but

were regarded as non-specific.

The increased activity of prothrombin during incubation was not due to release of preformed prothrombin bound to the microsomal vesicles, as there was no increase in activity when the microsomes were disrupted. Prothrombin formation by the heavy microsomes was, however, dependent on the ribonucleic acid of the membrane of the microsomes. Prothrombin formation was not seen if the system was incubated with ribonuclease, but this inhibitory effect of ribonuclease was not restored by addition of samples of ribonucleic acid prepared from microsomes or by pH 5 enzyme prepared from cell sap containing soluble ribonucleic acid (sRNA). Microsomal membrane, prepared by treatment of the microsomes with sodium pyrophosphate, showed an increase in prothrombin activity when incubated under the above conditions. This increment in activity was also inhibited with ribonuclease.

In view of the involvement of ribonucleic acid in the formation of prothrombin activity the nucleotide composition of ribonucleic acid in the whole microsomes, nuclei, microsomal membrane and ribosomes was studied. The ribonucleic acid from the membrane of the microsomes showed an unusual nucleotide composition, notably an absence of pseudouridylic acid and a high quantity of guanylic and cytidylic acids, as compared with that of the ribosomes. Absence of pseudouridylic acid was observed in the ribonucleic acid of the nuclei also. The amount

of guanylic and cytidylic acids of the membrane-ribonucleic acid and ribosomal-ribonucleic acid was significantly reduced in protein-fed animals, the change of pattern being most marked in the membrane-RNA. The pseudouridylic acid content of ribosomal-RNA was found to increase significantly with the inclusion of protein in the diet. The molecular size of ribonucleic acid associated with the membrane was found to be low in comparison with that of whole microsomal-RNA and ribosomal RNA when studied by ultracentrifugal analysis and by ECTEOLA column chromatography.

The uptake of ^{32}P was initially highest in the nuclear-RNA, the maximum specific activity being attained some time between 3 - 8 hours after injection. The membrane-RNA showed some incorporation of ^{32}P at one hour after administration, but little activity was found at that time in the ribosomal-RNA. The activity in both fractions gradually increased up to 8 hours after injection of ^{32}P, the activity in the membrane-RNA even at that period being higher than the ribosomal-RNA. At 16 hours after injection, the activity in ribosomal-RNA was now higher than in the membrane-RNA. The capacity to incorporate ^{32}P was markedly stimulated if the rats were prefed with protein one hour before injection of isotope.

From these findings it has been concluded that the prothrombin molecule can be finalised by the microsome membrane, a type of RNA associated with the membrane being essential for the process.

In view of the similarity between the nucleotide composition of the membrane-RNA and nuclear-RNA, and the labelling pattern of the membrane-RNA, it is suggested that membrane-RNA arises from the nucleus and may represent a form of messenger RNA. This concept would be consistent with electron micrograph evidence of the origin from nuclei of endoplasmic reticulum, from which the microsomes of disrupted cells are derived.