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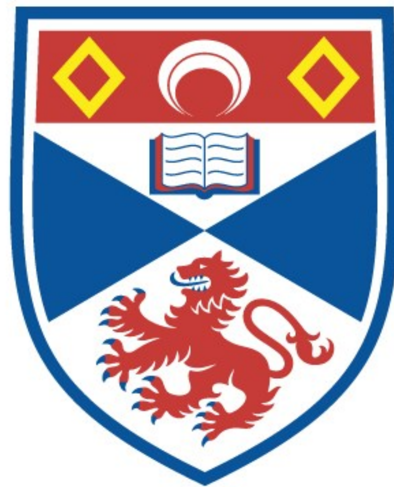

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THE EFFECT OF PREGNANCY ON THE METABOLISM OF RAT LIVER RNA

By

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Degree of Master of Science
of the University of St. Andrews.
May, 1973.

Declaration

I hereby declare that the following thesis is a record of research work carried out by me, that the thesis is my own composition, and that it has not been presented in application for a higher degree previously.

The research was conducted in the Department of Biochemisrtry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Professor G.R. Tristram and Dr. G.A.J. Goodlad.

CERTIFICATE

I hereby certify that Gary Yat-Wah Ma has spent seven terms engaged in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 51 and that he is qualified to submit the accompanying thesis for the degree of Master of Science.

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To

My Parents

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Introduction

1.1 General Aspects of Protein Metabolism during Pregnancy.

During gestation in mammals, there is a dramatic alteration in the processes of protein metabolism due to increased requirements of the tissues of the maternal organism, for the formation of new tissues such as the foetuses, placenta and also for the maturation of the embryo. Early observations by Poo, Lew and Addis (1939) on the well-nourished pregnant rat revealed that protein anabolism in this species was almost doubled towards the end of pregnancy and that there was a 28% increase in the amount of protein deposited in the liver. Some of the increased nitrogen retained during pregnancy of the rat was found to be deposited in the reproductive tissues and the foetuses (Boyne, Chalmers & Cuthbertson 1953, Jakobsen 1957). Blaxter (1964) found that tissue protein deposition started mainly during the second half of pregnancy in the rat.

The extent to which the nitrogen content of the extra-uterine tissues alters during pregnancy depends upon dietary state (Boyne et al. 1953, Spray 1950, Bourdel & Jacquot 1960, Morrison 1956). Diet also affects the number and condition of foetuses in the rat. If pregnant animals are fed a diet which has less than 6% protein, pregnancy cannot be maintained

until term, moreover, resorption of the foetuses becomes more frequent. This effect of protein deficiency can, however, be overcome by the administration of oestrogen and progesterone to pregnant rats (Hazelwood & Nelson 1965). In this case it appeared that the skeletal muscle acted as the main source for the mobilised protein precursor. Campbell, Innes & Kosterlitz (1953 a) found that if dietary deprivation of pregnant rats is restricted to the last week of gestation, there was no deleterious effect on the size of the foetuses. This led them to suggest that the maternal reserves built up during early pregnancy of the rat, at a time when demands by the foetuses are still small, are available for the dramatic growth of the foetuses during the final week of gestation.

The difference between the concentration of various nitrogenous compounds in the arterial supply and venous drainage from the uterus provides a means of estimating the metabolites utilised in the synthesis of proteins in the foetus and placenta and for the associated hypertrophy of the uterine wall. In pregnant rats, it was found that amino nitrogen was higher in the arterial than in the venous blood supply from the pregnant uterus, suggesting that plasma amino acids were used as building blocks for uterine, placental and foetal protein (Blaxter 1964). Jakobsen (1957) observed differences in the rate of uptake of individual amino acids. Setrikar & Barron (1958) determined amino - N, urea and the plasma protein

contents of the arterial and uterine venous blood of pregnant and non-pregnant goats and of the foetal blood in the umbilical artery and vein respectively. They demonstrated that during pregnancy amino nitrogen is lost by the maternal blood and gained by the foetal blood in the uterus and placenta respectively. However, urea is lost by the foetal blood in the placenta in foetuses 90 days of gestation age and older, but a regular rise in its concentration in the maternal blood perfusing the uterus was not observed. Setrikar and Barron deduced from their results that plasma proteins do not act as a source of nitrogen for uterine, placenta or foetal proteins. Moreover, the arterio-venous differences in amino acids were not found to correlate significantly with different stages of pregnancy (Setrikar & Barron 1958). There is an increase in both cell size and cell number in the uterine wall during pregnancy (Needham & Cawkwell 1957) while the total nitrogen per cell increases fourfold (Wakid & Needham 1960). Barcroft (1948) found evidence for an increasing blood supply to the uterus in sheep as pregnancy proceeded which would suggest that the increased demands of protein synthesis in this and associated tissues (placenta, foetuses) are being provided for by specific alterations in the circulatory system.

Not all tissues of the pregnant rat show an increased nitrogen content. Of the tissues which increase in size and

nitrogen content the most conspicuous is probably the mammary gland (Griffiths & Turner 1961). Increases have also been noted in the liver (Campbell & Kosterlitz, 1949; Clark, Steele & Goodlad 1971), kidney, gut (Boyne et al. 1953) and adrenal glands (Clark et al. 1971). In the rat, the serum albumin β - globulin and γ - globulin concentrations were lower in the serum of pregnant than of non-pregnant animals on the 21st day of pregnancy (Morgan, 1964). Moreover 6 and 20 hours after the intravenous injection of ^{131}I - labelled proteins into rats on the 20th day of pregnancy, relatively high concentrations of all three labelled proteins were found in foetal serum. The foetal concentrations of labelled albumin and transferrin were approximately equal and about $\frac{1}{3}$ those of γ - globulin. Morgan (1964) therefore concluded tentatively that in the rat near the end of pregnancy the three proteins were transmitted to the foetus relatively easily, albumin and transferrin to a very similar degree and both less than γ - globulin. Decreased serum protein concentrations have been reported in pregnant rabbits (Langman, van Druen & Bouman, 1959) and in pregnant cows (Prostyakov, Fortushnyl & Kovyndikov 1961). The decrease in plasma protein concentrations might reflect an increase in the plasma volume of the maternal organism. There is no evidence, however, from experimental animals to support this idea, although Malagoli & Donatelli (1962) reported an increase from 44 to 48 ml/Kg body weight in pregnant women.

1.2 Alterations in Liver Protein and Nucleic Acids of the Pregnant rat.

Before considering in detail the alterations in the liver which occur in pregnancy, it must be remembered that the liver contains a variety of different cell types. Although accounting for 90-95% of the liver mass, hepatocytes constitute only 60-65% of the total cell number (Daoust 1958). Cells of reticuloendothelial system, mainly Kupffer cells, constitute 35% of the cell population and there are also other types of cells such as those of the bile duct canaliculi and the blood vessels. Nicol and coworkers (1964) studied the phagocytic activity of the reticuloendothelial system of liver in the pregnant rat and observed that two peaks developed, one on day 12 and the other on day 19 of pregnancy. These increases may be related to the small increases in liver DNA synthesis and DNA content found in the pregnant rat between the 12th and 14th and 18th and 20th day of gestation (Campbell & Kosterlitz 1949). After the fourteenth day of pregnancy, Campbell et al. (1949) observed the RNA content of liver to increase, reaching a peak value on the 21st day. Histological examination of the liver of the pregnant rat (Campbell & Kosterlitz 1949) showed an increased basophilia distributed diffusely throughout the cytoplasm of hepatocytes. More recently, this extra RNA was found to be largely associated with the ribosomal fraction of the liver cell by differential centrifugation studies (Goodlad & Lumsden

unpublished observations) and by electron micropcopic examination (Brunt 1966). Thus it would appear that up to the 14th day of gestation in the rat an increase in the number of reticuloendothelial cells may account for the main increase in liver weight and nucleic acid content but thereafter proliferation of the hepatocytes occurs. Since from the 14th day of pregnancy onwards there is a disproportionate increase in RNA relative to DNA (Campbell & Kosterlitz 1949), it may be inferred that cell hypertrophy rather than increase in cell number is the main reason for the increase in liver size at this stage. In other instances such as regenerating liver, a similar pattern of changes in RNA and DNA has been observed in which increases in cell RNA content are more rapid and of greater magnitude than that of DNA (Brues & Marble 1937, Harkness 1957, Glinos 1958, Hymer & Kuff 1964). As a result of further studies, Campbell & Kosterlitz (1953) concluded that two independent factors were responsible for the increase in RNA in liver of pregnant rats, one being the dietary energy intake and the other a placental factor. Dietary protein intake did not affect the excess RNA produced during pregnancy whereas the magnitude of the increase was related linearly with dietary energy intake and placental weight. Surgical removal of the foetuses with retention of the placentae had no effect on liver RNA whereas removal of both placentae and foetuses caused a rapid return of liver RNA to its level in

the liver of the non-pregnant rat (Campbell et al. 1953). An alkaline extract of placentae when injected into non-pregnant rats was found to cause an increase in the RNA but not DNA content of liver. (Campbell & Kosterlitz 1953).

Relatively few studies have been made of protein synthesis in livers of pregnant rats. Little & Lincoln (1964) found that the incorporation of L-1-^{14}C-valine into protein by slices prepared from the liver of pregnant rats was increased during the second week of gestation. The increased demand of the necessary proteins during pregnancy may reflect the possibility of an increase in polyribosome populations which have generally been accepted as the prime cellular machinery for protein biosynthesis. From a study of the sedimentation profiles of ribosomal aggregates of livers of 18-day pregnant and non-pregnant rats in sucrose density gradients, it was found that in the former there was an elevation in the polysome population associated with a decrease in monomers and dimers (Lumsden & Goodlad personal communication). That there is not a general increase in protein synthesis in livers of pregnant rats is clear from the work of Jordan & Morgan (1969) who found a marked increase in the incorporation of ^{14}C-leucine into albumin by liver slices of 17 day pregnant rats, whereas the incorporation into the transferrin was increased to a lesser extent when compared with controls. This difference in the effect of pregnancy on the synthesis of a protein

destined for export and one destined to be retained inside the cell is of interest in view of the finding of Brunt (1966) that the <u>in vitro</u> amino acid incorporating ability of membrane-bound ribosomes was increased on day 18 and 20 of pregnancy in the rat whereas the incorporating ability of non-membrane bound ribosomes was similar to that in the non-pregnant rat on the 18th day and was actually depressed on the 20th day of pregnancy. Membrane-bound polyribosomes in liver are believed to participate primarily in the synthesis of albumin (Takagi & Ogata 1968, Hicks, Drysdale & Munro 1969, Takagi, Tanaka & Ogata 1970), and other serum proteins (Redman 1969, Ganoza & Williams 1969), whereas free poly-ribosomes synthesize predominately intracellular proteins, such as ferritin (Hicks et al. 1969, Redman 1969) and several unidentified proteins in the supernatant fractions of rat liver cells (Ganoza & Williams 1969, Takagi et al. 1970).

There are several circumstances apart from pregnancy where a rapid growth in the liver of the rat is found. Examples of these are partial hepatectomy, foetal and post-natal growth and the systemic effects of a tumour. Liver growth in young animals such as the rat, is very much dependent upon specific hormones. Thus hypophysectomy and thyroidectomy caused a retardation or cessation of growth of the liver and some other tissues. Administration of growth hormone to the hypophysectomised rat (Simpson, Evans

& Li 1949) triggered liver growth and elevated both liver RNA and protein content (Geschwind, Li & Evans 1950). Treatment of thyroidectomised or hypophysectomised rats with thyroid hormone caused a similar picture of increasing size, RNA and protein content of the liver (Pitt-Rivers & Tata 1959, Tata 1964). Moreover, administration of testosterone to young castrated male rats also caused an acceleration of growth of liver and other tissues although the main action of this hormone is on the prostate and seminal vesicles.

The ratio of liver weight relative to body weight is greater in embryonic and early postnatal rats than in the adult animal (Jackson 1913, Williamson 1948). Histological and biochemical studies have shown that diploid cells are common in foetal liver whereas at the time of weaning, there is a tendency for an accumulation of tetraploid and polyploid cells. These later cell types assume a greater proportion in adult liver (Swartz 1956, Narora 1957, Novikoff & Essner 1960, Swartz, Sams & Barton 1960, Swartz & Ford 1960, Swartz & Sams 1961). This development of higher polyploid numbers has been reported to be directly related to the influence of anterior pituitary growth hormone (DiStefano & Diermeier 1956, Carriere 1962). There is a relative slowing down of the rate of liver growth with maturity and the ratio of liver weight to body weight remains constant throughout most of adult life.

Oestrogens and androgens have been shown to influence the formation of octaploid nuclei in the liver parenchymal cells of intact and castrated rats. Swartz has shown that in the absence of either the male or female sex hormones fewer octaploid cells occur (Swartz et al. 1960, Swartz & Sams 1961, Swartz 1962). This worker has shown that the age at which orchidectomy is carried out is significant with regard to alterations of growth and polyploidy. Castration of males at the neonatal stage having a greater effect in this respect than at weaning or later.

Studies have been made on alterations in nuclei acid metabolism in the change from foetal to postnatal growth. The DNA concentration on the basis of dry weight of the foetal liver was found to be relatively constant from the 16th to 19th pre-natal days and then to drop to a third by the 21st day and to decrease progressively after birth. This decrease is part of the maturation process since it continues at a slow rate after birth. The RNA concentration, on the other hand, showed a steady rise (Wirth & Schreier 1956). The concentrations of DNA and RNA in rat liver on a wet weight basis reached a maximum level at 5 and 15 days after birth respectively (Oliver, Bollard, Shield & Bentley 1962, Devi, Mukundan, Srivastava & Sarker 1963, Lafarge & Frayssinet 1964). Fukuda & Sibatani (1953) calculated that the mass of the rat liver cell increased from 1.79 ng at 10 days of

age to 6.7 ng at 6 months of age. Concomitantly, data presented by Oliver, Blumer & Witham (1963) showed that during late foetal and early postnatal growth, there was a high content of free ribosomes which declined to low concentration 10 days after birth. Elevation in plasma proteins were found to accompany the decrease in free ribosomes (Oliver et al. 1962). It was suggested that the free ribosomes become attached to the membranes of the endoplasmic reticulum at this stage. Hence an increase in plasma protein may be a reflection of a change in the protein synthetic mechanism of the liver.

Surgical ablation of a portion of the liver has been shown to induce very rapid growth in the parts remaining, which subsides only when the original organ mass is replaced. Harkness (1961) pointed out that this phenomenon is better termed liver restoration rather than regeneration because when entire lobes are removed they are not replaced. The original mass is restored by the enlargement of the remaining parts, a process which causes increases in mitotic rate in the liver after operation. Mitosis was shown to slow down as the normal liver weight was approached (Harkness 1957, Werinbren 1959). Leong, Pessotti and Brauer (1959) reported that the restoration process ceased before normal adult weight was reached. However, Bucher & Glinos (1950) have stressed that the age of the rat is a factor in determining how far the

process will proceed. Morphological as well as biochemical studies, revealed that there is disorganisation of the endoplasmic reticulum together with some loss of membranes and disaggregation of the ribosomes accompanying partial hepatectomy (Bernhard & Rouiller 1956, Bernhard 1958). The endoplasmic reticulum has been shown to begin to reform in close contact with groups of mitochondria (Bernhard et al, 1956) after 16 to 18 hours. These changes appeared first in cells of the centro-lobular area and progressed rapidly towards the periphery (Glinos 1958). By 36 to 48 hours after partial hepatectomy the normal structure was restored (Bernhard et al. 1956). An increased synthesis of DNA (Glinos 1958, Marsh, Drabkin, Braun & Parks 1966) became pronounced around 15 to 18 hours after partial hepatectomy. A sudden active incorporation of labelled precursor into DNA occurred with a peak at 22 to 36 hours, thereafter incorporation fell off somewhat but continued at an elevated level for some days. Laparotomy had no effect on the incorporation of thymidine into liver DNA whereas hepatectomy caused a striking increase in incorporation of ^{3}H-thymidine into liver DNA (Bollum & Potter 1959, Chandler & Neuhaus 1968). As might be expected, DNA content per nucleus is highest during the phase just preceding the onset of mitosis (Bucher 1963). In rat liver following partial hepatectomy, an early increase in the rate of incorporation of labelled precursor

into RNA occurred (Fujioka, Koga & Lieberman 1963, Bucher 1967, Bresnick, Williams & Mosse 1967), suggesting the synthesis of RNA reached a maximum at 6 hours after operation (Fujioka et al. 1963). Protein synthesis in regenerating rat liver was found to involve primarily an increase in cellular proteins as well as proteins for export (Marsh, Drabkin, Braun & Parks 1966, Marsh & Drabkin 1958, Majumdar, Tsukada & Lieberman 1967). However a net increase of protein synthesis was not easily detected until 12 hours after partial hepatectomy (Ferrari & Harkness 1954).

Evidence from the work of Von der Decken & Hultin (1958) showed that microsomes from regenerating liver were approximately twice as active as those from normal liver when incubated with the same soluble fraction. The RNA content of the microsomes was increased during regeneration, reflecting a high proportion of ribosomal relative to membraneous elements (McCorquodale, Veach & Mueller 1960). The greater activity of ^{14}C-leucine incorporation by regenerating liver microsomes appeared to be associated with the bound polysomes (Campbell, Lowe & Serck-Hanssen 1967) and their activity to be controlled by factors present in the membrane. Mention must also be made, however, of a report of an inhibitor of amino acid incorporation by ribosomes present in the cell sap, the concentration of which is much reduced following partial hepatectomy (Hoagland, Scornik and Pfefferkorn 1964).

From this survey it is apparent that the pattern of alterations in liver nucleic acids during pregnancy in the rat is in some respects similar while in other respects different from the patterns observed in other instances of intensive liver growth. In the pregnant rat there is no evidence for increased levels of oestrogens (Yoshinga, Hawkins and Stoker, 1969) or corticosteroids (Gala and Westphal, 1965) two hormones which could induce increases in liver RNA and protein.

1.3 Control of Liver Growth

Although the mechanism controlling the physiological changes of liver growth remains to be discovered, certain hypothesis have been put forward based mainly on studies with regenerating rat livers. Investigations using tissue cultures from parabiotic animals (Akamutsu 1953, Bucher, Scot & Aub 1951, Glino & Gey 1952) have suggested that factors in blood may be involved in the control mechanism. Glino (1958) showed that the mitotic rate in livers of rats after partial hepatectomy was suppressed when plasma volume was reduced by restriction of water intake. However, by varying the concentrations of sera, it was found that comparable out-growths of the liver in organ culture occurred with high concentrations of serum from partial hepatectomised rats and low concentrations of normal serum. Hence, Glinos suggested

the liver is the site of synthesis of inhibitory factors. Partial hepatectomy would stimulate mitotic activity of the hepatic cells owing to a low concentration of plasma proteins. When liver size advances, the accumulation of inhibitory factors would be increased and would suppress the rate of regeneration, leading to eventually cessation. Furthermore, by diluting normal serum by plasmapheresis mitotic activity was initiated in the intact liver and was increased with increasing duration of plasmapherais. Therefore, it was concluded that normal serum contains a growth-inhibitory substance whose concentration is reduced after hepatectomy (Glinos 1958, 1958a).

On the other hand, complete inhibition of mitosis was shown by exogenous administration of cortisone to hypophysectomised, partial hepatectomised animals (Hemingway 1960). Further work supporting a role for glucocorticoids as controlling factors was supplied by Guzek (1964) who studied the incorporation of tritrated thymidine into DNA of regenerating liver. He observed that all cell types were rapidly labelled in partially hepatectomised rats, if these animals were treated with cortisone there was a marked decrease in uptake of radioactivity into DNA. Since he found an increase in nucleotide precursor concentration, Guzek (1964) postulated that inhibition is at the level of thymidine triphosphate into DNA. With respect to the pregnant rat, Gala & Westphal (1965) showed that the level of

circulating glucocorticoid (corticosterone) is low until the day preceding parturition and Clark et al. (1971) showed that the livers of pregnant rats from the twelfth day onwards had an increased capacity to inactivate these hormones. Thus the increase in DNA content in the liver of the pregnant rat might be due to mitosis arising as a result of the release of glucocorticoid in relation. However, as discussed above, in the pregnant rat, cell proliferation may be restricted to the Kupffer cells only and not involve a general increase in liver cell population.

In tumour-bearing animals, too, the control of liver growth breaks down and the characteristics of liver growth under this pathological situation are an increase in relative weight accompanied by cell proliferation, an increased DNA turnover rate (Kelly & Jones 1950) and a disproportionate increase in RNA (Annau, Manganelli & Roth 1951). Hepatomegaly in tumour-bearing animals is accompanied by an increase in adrenal size and there is some evidence to suggest that the adrenals are hyper-functional with respect to both glucocorticoid and aldosterone production (Hilf, Burnett & Borman 1960, Rechcigl, Granthm & Greenfield 1961). It is difficult however to correlate a hypercortical condition as the above suggests with increase in liver growth in the light of Hemingway's work (1960) with cortisone.

1.4 Turnover studies on ribosomal RNA

The level of RNA present in the cytoplasm of liver depends upon an equilibrium between its rate of formation and its rate of degradation. Methods employed in the determination on rates of degradation of different species of RNA are based on the loss of specific radioactivity or of total radioactivity after a single adminstration of a labelled precursor. The decreases in these parameters are usually exponential with time and hence a decay constant may be evaluated which provides the information for calculation of the biological half-life of the molecular species being studied.

The rate of RNA turnover in rodent liver under various dietary conditions or hormonal treatments has been studied in the past few years (Loeb, Howell & Tomkins 1965, Hirsch & Hiatt 1966, Enwonwu & Munro 1970, Enwonwu, Stambaugh & Sreebny 1971). Most workers have reported that the half-life of the liver ribosomal RNA and ribosomal protein of normal rats is about 5 days (Loeb et al. 1965, Hirsch & Hiatt 1966, Wilson & Hoagland 1967, Blobel & Potter 1968), considerably less than the half-life of the average hepatic cell. This turnover provides a rationale for the relatively intense nucleolar activity and ribosomal RNA synthesis observed in cells of liver and other less rapidly proliferating tissue. At

present, there exists very little knowledge about the factors which control the rates of synthesis and degradation of ribosomal RNA. Dietary protein intake (Clark et al. 1957, Enwonwu et al. 1970) and food deprivation (Enwonwu et al. 1971) have proved to affect the rates of synthesis and degradation of ribosomal RNA in the rat liver. As a result of studies on rats undergoing prolonged starvation, Hirsch & Hiatt (1966) concluded that the loss of ribosomal RNA was due to a reduction in synthesis rather than an enhanced rate of degradation. Enwonwu and Munro (1970) suggested that RNA turnover in rat liver is regulated through changes in the population of free ribosomal subunits in the cytoplasm, the abundance of subunits being affected by amino acid supply. The increase in ribosomal RNA found in livers of adrenalectomised treated with cortisone has found to be associated both with a decrease in the rates of rRNA degradation and synthesis (Ottolenghi & Barnabei 1970).

1.5 Ribonucleases of rat liver

It is generally acknowledged that there are two main types of ribonuclease (RNase) present in mammalian tissue (Roth 1954, Shortman 1961), one showing optimal activity at pH 5.8 (heat labile and acid labile) while the other is optimally active at pH 7.8 (heat stable and acid stable).

The former is referred to as acid RNase, whereas the latter is termed alkaline RNase. However, Rahman (1966) showed the presence of another alkaline RNase with a pH optimum of 9.5 in rat liver.

It has been known for several years that ribosomes of E. coli (Tal & Elson 1960, Elson 1962, Szer 1969) and mammalian cells possess latent ribonuclease activity (Petermann & Hamilton 1957, Farkas & Marks 1966, Roth 1967) and also latent deoxyribonuclease activity (Tal & Elson 1961, Tashiro & Siekevitz 1965). These activities could only be demonstrated when the ribosomal structure was denatured. There is doubt as to whether ribonucleases and deoxyribonucleases are indeed structural proteins of the mammalian ribosomes or artifacts (Utsunomija & Roth 1966) arising during isolation. Roth (1967) presented data which he claimed, showed that the latent RNase is not an integral part of the ribosome but was due to contamination during homogenate preparation. However, in a recent report, Krechetova, Chudinova & Shapot (1972) showed that both RNase and deoxyribonuclease (DNase) activities were confined solely to the small ribosomal subunit. Latency could be ascribed only to the nuclease of intact ribosomes since the large subunit seems to protect the 18s RNA molecule to a certain extent from attack by the enzyme residing in the small subunit as long as the structure of the whole ribosomal particle is preserved.

Another ribonuclease, ribonuclease-H (RHase-H or hybridase) was detected by Stein & Hausen (1969) in thymus extracts. This enzyme digested the RNA moiety of DNA/RNA hybrids. The existence of the hybridase in certain other tissues, such as rat liver (Sekeris, Schmid & Roewekamp 1972), the integument of blowfly larvae (Doenecke, Marmaras & Sekeris 1972) as well as in bovine lymphocytes (Hausen, Stein & Peters 1969) has been shown. A biological implication of DNA/RNA hybrids has been put forward by Frenster (1965) and reviewed by Britten & Davidson (1969) that such hybrids have a stimulating effect on transcription. However, Sekeris and coworkers (1972) found, on the contrary, that hybrids restrict transcription and these workers suggested that the hybridase function is to eliminate the formed hybrids and thus stimulate transcription. Supporting these latter findings are data from Schmid et al. (1972) who showed that cortisol which stimulate RNA synthesis in livers of rats leads to a decrease of _in vivo_ formed DNA/RNA hybrids while causing an activation of the hybridase present. In keeping with these findings is the fact that administration of ecdysone, the insect steroid hormone, to the integument of blowfly larvae caused an increase of hybridase activity together with an activation of the transcription processes (Doenecke et al. 1972). Mölling and his colleagues (1971)

in studies on avian mycloblastasis virus (AMV) found that RNase-H was physically linked to a complex which has been shown to exhibit DNA polymerising activity on both DNA and RNA templates. The RNase-H was more stable on storage and handling than the DNA polymerase. These authors suggested tentatively that RNA was used by the DNA polymerase as a template to synthesise complementary DNA (reverse transcriptase) resulting in an DNA/RNA hybrid. Subsequently, RNase-H activity specifically digests the RNA in the hybrid, thereby releasing single-stranded DNA which can be replicated to yield the final product.

The distribution of RNase activity in the four major subcellular fractions prepared from rat liver homogenate has been studied extensively by Roth (1957, 1967) who has found in normal adult rat liver that 50-70% of the alkaline RNase at pH 7.8 was associated with mitochondria fraction (Roth 1957, de Lamirande, Allard da Costa & Cantero 1954, Schneider & Hogeboom 1952) whereas the microsome fraction accounted for 10-25% of the total activity and the nuclear and supernatant fraction about 10%. (Table 1)

Table I. Percentage distribution of RNase activity in rat liver (Roth 1967)

Fraction	Alkaline RNase[a] No treatment	Alkaline RNase[a] Treated with dilute sulphuric acid[b]	acid RNase[c]
Nuclei	8.2	18.6	14.8
Mitochondria	53.1	29.1	61.5
Microsome	25.8	18.6	10.1
Supernatant	12.9	33.7	13.4

a Assayed at pH 7.8 with Veronal-acetate buffer (Roth 1957)

b This releases latent RNase activity in the supernatant & other

c Assayed at pH 5.9 with Veronal-acetate buffer (Roth 1957)

As will be discussed later, the supernatant fraction of the cell, or cytosol contains a potent inhibitor of the alkaline RNase optimally active at pH 7.8. Roth (1967) therefore suggested that the 12.9% of the total cellular RNase assayable at pH 7.8 present in this fraction of the cell (Table I) was probably due to the enzyme optimally active at pH 9.5 or to acid RNase, the latter perhaps released from the lysosomes during homogenisation, neither of which enzyme are sensitive to the action of the inhibitor (Rahman 1966). Addition of

dilute sulphuric acid to the liver fractions overcomes the effect of the inhibitor and destroys the acid RNase (Rahman 1966, Roth 1967). As seen in Table I this treatment caused an increase in the amount of assayable RNase in the supernatant while the relative but not total amounts in the mitochondrial and microsomal declined. The increase in percentage of alkaline RNase at pH 7.8 in the nuclear fraction of sulphuric acid-treated homogenate as Roth suggested, is unexpected and difficult to interpret in light of present knowledge. However it is possible that under these conditions there is considerably more absorption of RNase by this fraction. There have been reports that some RNase-inhibitor is present in rat liver nuclei. Shapot and coworkers (1971) reported an inhibitor present in the nuclear sap of liver and hepatoma cells. Chakravorty and Busch (1967) showed the presence of an alkaline RNase and RNase-inhibitor in nucleolar preparations from normal and neoplastic tissues. It is possible that this RNase may be required for the post-transcriptional maturation hydrolysis of ribosomal RNA precursors. On the other hand, Roth & Juster (1972) presented data to show that no RNase-inhibitor, active against bovine pancreatic RNase existed in rat liver nuclei prepared by several methods which included those used by those workers who claimed the presence of such a factor. In this respect, Roth et al.

(1972) suggested that the positive report of a nuclear RNase-inhibitor was due either to contamination of the nuclear preparation and the passage of soluble cytoplasmic compounds into the nucleus through the pores of the nuclear membrane during extensive washings.

The 25.8% or less alkaline RNase at pH 7.8 present in the microsomal fraction may be due to mitochondrial contamination or adsorption by ribosomes of RNase released during cell homogenisation (Roth, Hilton & Morris 1964). The acid RNase at pH 5.8 present in various fractions of the rat liver is also recorded in Table 1. The mitochondrial fraction, including lysosomes, accounts for 61% of the acid RNase activity, whereas the small amounts of activity in the nuclear, microsome and supernatant fraction may be partly due to adsorption and partly to contamination by free acid RNase released during homogenisation.

The specific activity of acid and alkaline RNase towards commercially prepared RNA has been studied by several authors (Zytko, de Lamirande, Allard & Cantero 1958, Reid & Node 1959) it was found that rat liver alkaline RNase degraded RNA in the same way as did bovine pancreatic RNase, i.e. attacking only phosphate bonds 3' with respect to pyrimidine nucleosides, whereas acid RNase attacked both cyclic nucleotides of the purine and pyrimidine bases.

Figure (I) shows the action of bovine pancreatic RNase towards an RNA chain. Aksenova & Nechaeva (1972) showed that brain tissue alkaline RNase at pH 7.8 split poly-U, had no effect on poly-A. A mixture of poly(A).poly(U) was not cleaved by this RNase.

The characteristics and distribution of the RNase-inhibitor present in the cytosol of several mammalian tissues have been studied by Roth (1958) and its purification has been attempted by Shortman (1961) and Roth (1958). These authors concluded tentatively that the RNase-inhibitor is a heat-labile, non-dialyzable factor, probably a glycoprotein (Roth 1956, Shortman 1962) which forms a complex with the enzyme. Bernherimer & Steele (1955) also reported a heat-stable dialyzable inhibiter in certain plant tissues, while Rost and his colleagues (1959) observed a heat-stable, non-dialyzable inhibitor in red blood cells. Nishimura in 1960, Smeaton and Elliott in 1967 reported evidence for a RNase-inhibitor in Bacillus subtilis. More recently an RNase-inhibitor from the cytoplasmic fraction of rat brain was isolated (Nechaeva 1972). This RNase-inhibitor formed inactive complexes with alkaline RNase of brain and pancreases but showed no effect on brain acid RNase.

There have been a number of attempts to assess the possible role of the RNase-inhibitor in regulating RNA metabolism, by estimating the alteration in the activities

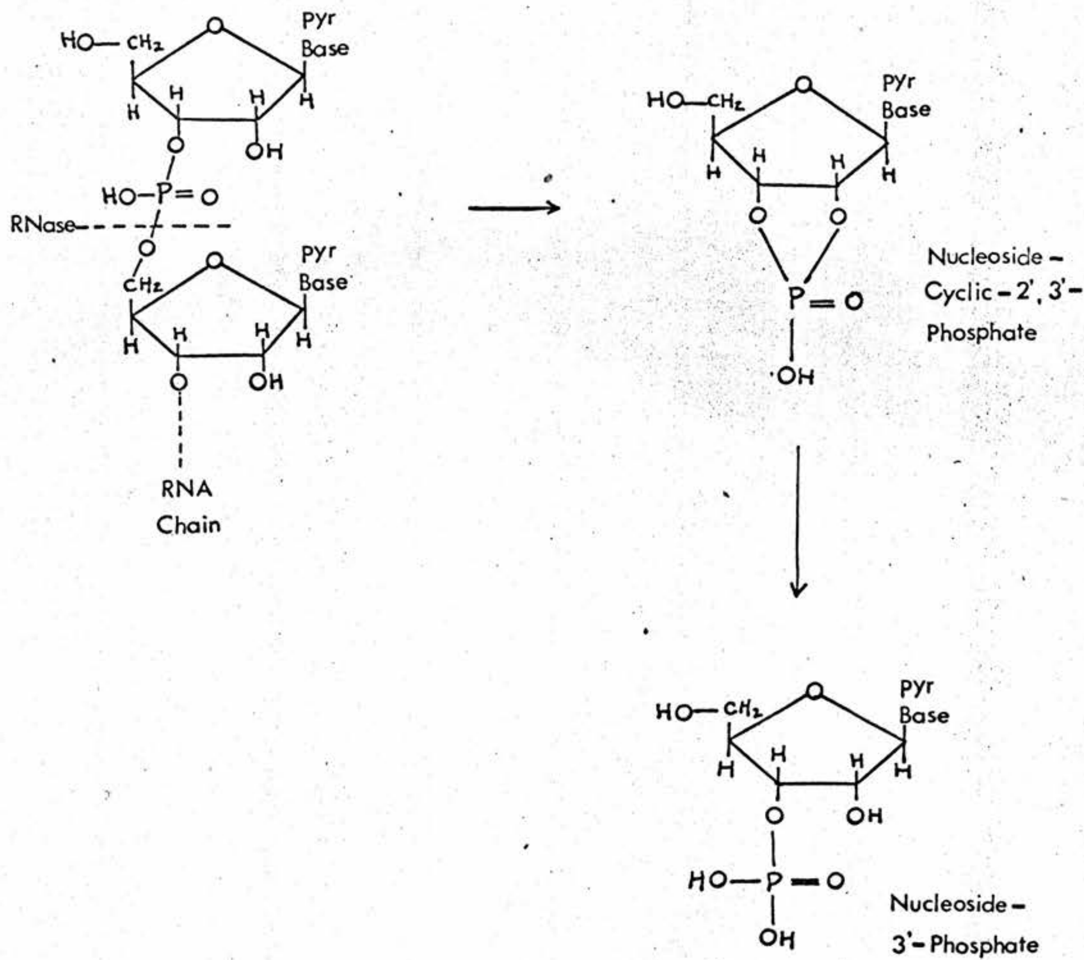


Figure 1 : Hypothetical model of Bovine Pancreatic RNase on Ribonucleic acid chain (Anfinsen & White 1961).

of RNase and RNase-inhibitor in various physiological conditions. Changes of RNase activity and the concentration of RNase-inhibitor have been studied extensively in rat livers and other mammalian tissues. During whole body X-irradiation of rats, Roth (1956) found in the livers of these animals, a significant decrease in the alkaline RNase of the separated mitochondrial fraction whereas the RNase-inhibitor activity in the supernatant fractions was depressed by 12 and 41% in two experiments after 1 day of irradiation and returned to the normal level on the second day. However, the RNase activity of whole liver homogenate, measured at pH 5.8, showed only a small change from control values in irradiated animals, while activity assayable at pH 7.8 in the whole homogenate and supernatant fraction normally very low from irradiation animals did not show significant changes from control. Studies of RNase and RNase-inhibitor have been made on liver and hepatoma sub-cellular fractions. Roth, Hilton & Morris (1964) have shown that acid RNase was markedly increased in the supernatant fraction of hepatomas with the exception of the Novikoff hepatoma in which there was only a slight increase. The RNase-inhibitor, on the other hand, tended to be greatly lower than in control. During different stages of anterior regeneration of Owenia fusiformis, both acid and alkaline RNase were found to increase in activity up to day 4 after amputation of the

anterior end. When differentiation became apparent, acid RNase decreased while alkaline RNase activity increased considerably (Fontes, Thouveny & Hebd 1971).

The level of RNase-inhibitor has been observed to increase moderately in regenerating rat liver (Shortman 1962), and substantially in the precancerous liver of rats fed 2-aminofluorene (Roth 1957). These observations suggest a direct relationship between RNase-inhibitor activity and rate of cell proliferation. In Novikoff hepatoma, an extremely rapidly proliferating tissue, RNase- inhibitor activity is considerably reduced compared to normal control animals (Roth et al. 1964). However some note should be taken of the fact that it is not definitely established that the Novikoff hepatoma is derived from liver parenchymal cells. Potter and his colleagues (1960) have suggested that the Novikoff hepatoma may have arisen from cells of the bilary duct.

In view of these facts, it is generally assumed that RNase inhibitor may have a direct relationship to the rate of turnover of RNA, not necessarily related to cell division or proliferation. Under normal conditions, one finds extensive RNA turnover in liver which keeps pace with a large scale synthesis of blood proteins (McFarlane 1964) and in brain cells it has been shown that neurones contain large quantities of RNA and are extremely active in the synthesis of RNA and proteins (Hyden 1960).

It has been suggested that the inhibitor may perhaps, prevent the degradation of RNA in certain specific cell structures (Roth 1967). The RNase-inhibitor has been suggested as preventing the degradation of m-RNA and thus maintaining polyribosomal integrity. In this respect, Blobel & Potter (1966) reported the presence of a potent RNase-inhibitor which prevents the breakdown of polysomes by either endogeneous RNase or by added pancreatic RNase. However the failure to demonstrate the presence of the inhibitor in liver of chicken and frogs (Roth 1962) would seem to cast some doubt on the substance playing a fundamental role in maintaining m-RNA stability. Furthermore, it must be remembered that liver and other tissues possess other enzyme activities e.g. phosphodiesterase I, phosphodiesterase II, which may also degrade cellular RNA.

As already discussed (p.17), the turnover of ribosomal RNA may vary under different conditions. In at least one instance, a decreased level of RNase-inhibitor has been found to accompany an increased turnover of ribosomal RNA (Enwonwu & Sreebny 1971). These authors observed in livers of malnourished rats that the RNase-inhibitor activity was reduced whereas free alkaline RNase activity, i.e. assayed in the absence of p-chloromercuribenzoate, was elevated (Enwonwu & Sreebny 1970). Moreover, in these rats the daily fractional rate of degradation of liver RNA was 25% (Enwonwu & Sreebny

1971), compared to a level of 17% in the well-fed animal.

1.6 Scope of present work.

It was felt that before further investigations could be made on the mechanism of and possible factors involved in the alteration in RNA in the liver of the pregnant rat there was a need to obtain more information on the nature of the changes occurring. Since previous work had suggested (Goodlad & Lumsden, unpublished results) that the increase in liver RNA during pregnancy was associated with the ribosomal fraction, a study was made of the turnover of ribosomal RNA during the stage of pregnancy when the increase in liver RNA occurs. The results obtained suggested that the change in RNA content was due to a decrease in its rate of catabolism and also an increase in its rate of synthesis. The possibility that the decreased rate of breakdown of ribosomal RNA might involve changes in liver ribonuclease activity was investigated by carrying out concurrent studies on the behaviour of liver acid and alkaline ribonuclease activities and ribonuclease inhibitor levels.

2. Experimental

2.1. Treatment of Animals

Wistar female albino rats weighing between 160-250 g were used throughout the experiments.

The oestrous cycle in rats is approximately 5 days. Anoestrous (dioestrous-prooestrous) lasts for 3 days while oestrous (oestrous-metoestrous) lasts for 2 days. The two phases can be differentiated by microscopic examination of vaginal smears. During anoestrous only small leucocyte cells can be seen, which are replaced by the larger cornified epithelial cells of oestrous. Large clumps of disintegrating epithelial cells are observed towards the end of oestrous, these in turn being replaced by leucocytes. The rat will conceive for about 12 hours following the appearance of the epithelial cells and at no other time.

Virgin females and males were caged together. Vaginal smears were examined daily as described by D'Amour & Blood (1948). A cotton wool bud, moistened with 0.9% saline was inserted into the vagina. The swab was then pressed on a glass slide moistened with 0.9% saline and the transferred cells examined under a light microscope. The morning on which sperm were found in the smear was designated Day zero of pregnancy. The pregnant rat and a non-pregnant female control of approximately the same weight were then put in

separate cages and weighed after an overnight fast. Both rats were fed 15g finely ground rat cake (North-Eastern Agricultural Cooperative) made into a paste with water at 4 p.m. daily. Groups of rats were killed on the 10th, 12th, 14th, 15th, 16th, 18th, 20th day of pregnancy, along with their respective controls, by stunning and exsanguination. The liver was quickly removed and placed in an ice-cold beaker and weighed. All weight measurements throughout the experiments refer to tissue wet weight.

2.2. Estimation of Total Liver RNA

The method adopted was that described by Fleck and Munro (1962, 1966). Acid-soluble nucleotides are extracted by treating liver homogenate or ribosome suspensions with 0.4 N perchloric acid at 0°. The residue, containing protein, RNA, and DNA, is subjected to hydrolysis with 0.3 N KOH for 1 hour at 37° when the RNA is degraded to acid-soluble nucleotides and the DNA and protein are unaffected. The absorption at 260nm of the acid-soluble fraction after hydrolysis is thus a measure of the RNA present.

1g of rat liver was homogenized with 10ml of ice-cold distilled water and the volume made up to 20ml with distilled water. 1ml 0.4N perchloric acid was added to 1ml of the final homogenate, and the mixture left in ice for 20 minutes before it was centrifuged at 0° for 15 min. in a MSE Mistral 6L centrifuge using the swing-out head at 2,000 r.p.m. The

precipitate obtained after centrifugation was washed twice with 5ml 0.2N perchloric acid and the supernatant and washings rejected.

1ml 0.3N KOH was added to the drained precipitate and digestion carried out at 37° for exactly 1 hour. The alkaline digest was then neutralised with 12N perchloric acid at 0° (external indicator). An equal volume of 1N perchloric acid was added to the neutralised alkaline digest and the precipitate was spun down at 2,000 r.p.m. for 15 min at 0°. The supernatant was collected and the precipitate washed twice with 1 ml 0.5N perchloric acid, the washings being added to the original supernatant. 0.5N perchloric acid was added to the combined supernatant and washings to bring the final volume to 5ml. The optical density of a suitable aliquot diluted to give a final perchloric acid concentration of 0.1N was read at 260nm on a Unicam SP502 spectrophotometer. For RNA, $E^{1\%}_{1cm}$ = 312 (Munro and Fleck, 1966).

2.3. <u>Studies on Ribosomal RNA Turnover in the Liver of the Pregnant Rat</u>

2.3.1 <u>Administration of ^{3}H-orotic acid and isolation of liver ribosomes</u>

The methods employed for the radioactive labelling of liver RNA and the isolation of liver ribosomes were essentially those described by Hirsch & Hiatt (1966). On the 13th day of

pregnancy, pregnant and control rats were weighed and given an intraperitoneal injection of 12.5uCi per 100g body weight 5-^{3}H-orotic acid (specific activity 21 curies per mmol, Radiochemical Centre, Amersham) in the form of a solution in 0.9% NaCl containing 25uCi per ml. The rats were then maintained in a 12 hour light-dark cycle commencing at 0700h and fed daily 15g of the diet described previously.

At 12.00 hr, groups of pregnant rats and their controls were killed by stunning and exsanguination 1, 2, 3, 5, and 7 days after they were given ^{3}H-orotic acid. The liver was quickly removed and chilled in several volumes of ice-cold Medium A (0.05M tris-HCl pH 7.5 at 0°, 1.0 mM $MgCl_2$, 0.025M KCl, 0.25M sucrose), blotted with filter paper and weighed. All subsequent operations were carried out at 0-4°. The liver was chopped into fine pieces with scissors and homogenized in 3 volumes (w/v) of Medium A, using a chilled Potter-Elvehjem (1936) homogeniser with a tight-fitting Teflon pestle. Cell debris, nuclei and mitochondria were centrifuged at 15,000 x g_{max} in a MSE Super 18 centrifuge for 15 min to yield a post-mitochondrial supernatant fraction. The supernatant thus obtained was removed and sufficient Medium A containing sodium deoxycholate (Mann-assayed) added to double the volume and give a final deoxycholate concentration of 1.3%. After this treatment the ribosomes released from the microsomes were sedimented

by centrifugation at 39,000 r.p.m. for 2 hr in a MSE Super 40 ultracentrifuge using an 8 x 25ml angle rotor at 0°.

The ribosomal pellet obtained after 2 hr centrifugation was rinsed gently with Medium B (0.05M tris-HCl pH 7.5 at 0°, 1mM $MgCl_2$, 0.025M KCl, distilled water), drained and 1 ml Medium B added to soften the pellets overnight at 0°. The ribosomal suspensions were centrifuged at 2,000 r.p.m. for 10 min at 0° in a MSE Mistral 6L centrifuge after gentle shaking to ensure complete dispersion of the ribosomal pellets. The Mg^{++} ion concentration ofthe resulting clear supernatant was brought to 0.05M by the addition of 0.1M $MgCl_2$ and left for 1 hour at 0° to permit aggregation ofribosomes which were later sedimented by centrifuging at $12{,}000g_{ave}$ for 10 min in a MSE Super 18 centrifuge at 0°. The supernatant was discarded and the cream coloured pellets were rinsed, drained and suspended in 2 ml 0.1M KOH. The ribosomal suspension was stored at 0° and its RNA and protein concentration and radio-activity estimated.

2.3.2 Estimation of Protein and RNA content of isolated ribosomes

Protein concentration was estimated according to the method of Lowry, Rosebrough, Farr and Randall (1951). A standard calibration curve was constructed by using bovine serum albumin. (Sigma Chem. Co. Ltd.)

A 0.5ml protein sample of approximately 10-200μg protein was pipetted into 5ml of reagent C [50ml reagent A (2% Na_2CO_3 in 0.1N NaOH) was added to 1ml reagent B (0.5g $CuSO_4.5H_2O$ dissolved in water and added to an aqueous solution of 1g sodium potassium tartrate. 10ml 1.0N NaOH were added and the volume of the solution made up to 100ml with distilled water)]. The solution was mixed thoroughly before 0.5ml reagent D (a mixture of equal volumes of Folin-Ciocaltean reagent (BDH) and distilled water) was introduced rapidly. The solution was carefully mixed and allowed to stand for 30 min at room temperature. The O.D. of the samples was read at a wavelength of 500nm.

A solution containing 100mg crystalline bovine serum albumin (Sigma Chemical Co. Ltd.) in 10ml 0.9% (W/V) NaCl was prepared and appropriate aliquots taken for a calibration curve. (Fig. 2)

The RNA content of the purified ribosomal suspension was estimated by the method of Fleck and Munro (1966). 1ml of the ribosomal suspension was introduced into a 10ml centrifuge tube together with 1ml ice-cold 0.4N perchloric acid. The mixture was left in ice for 20 mins before carrying out the subsequent procedures as described previously. (Experimental 2.2).

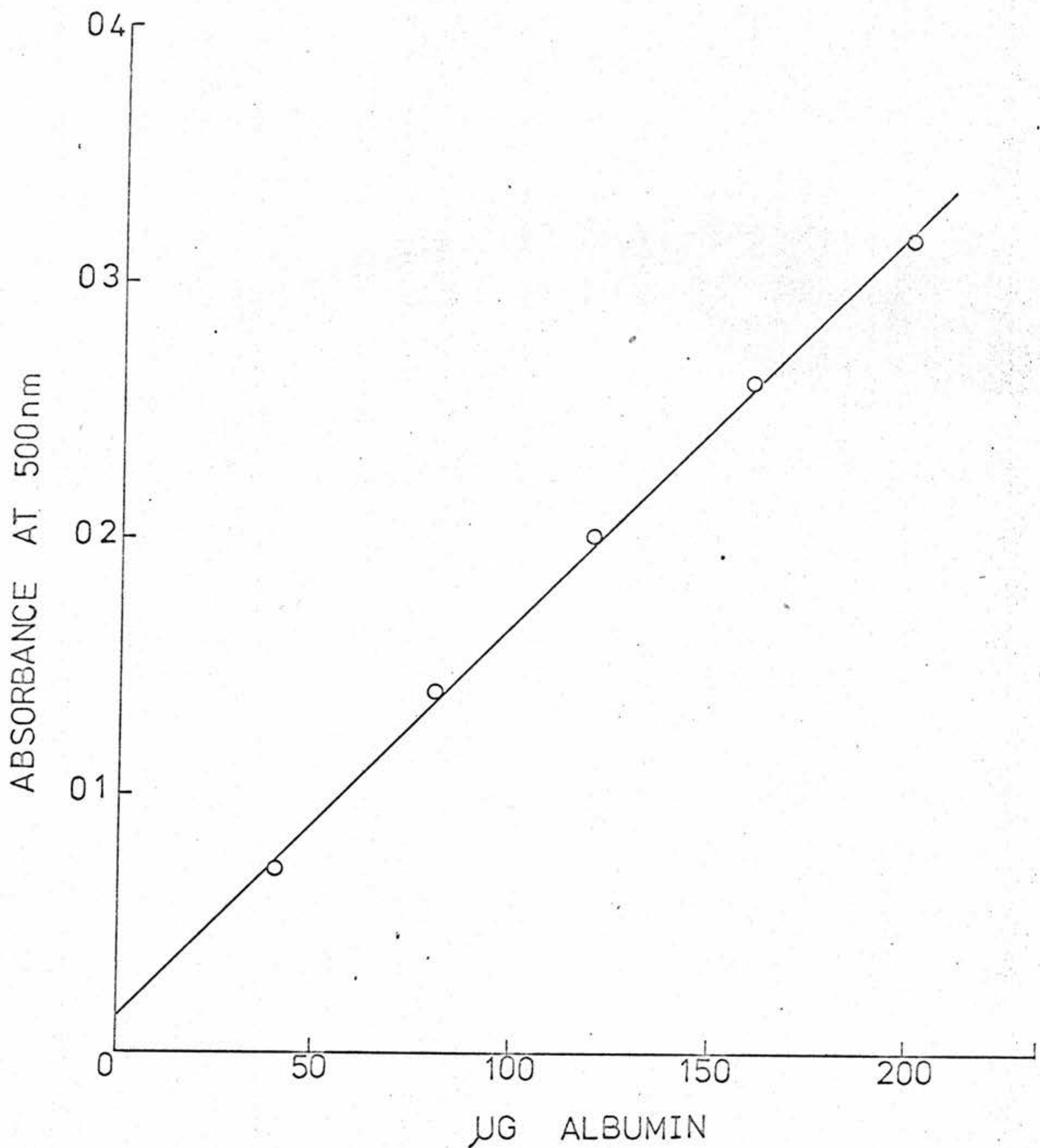


Figure 2: Calibration curve for Estimation of Ribosomal Protein. Absorption at 500 n.m. of increasing amounts of Bovine Serum Albumin using the procedure of Lowry et al.(1951).

2.3.3 Estimation of rates of synthesis and catabolism of liver ribosomal RNA

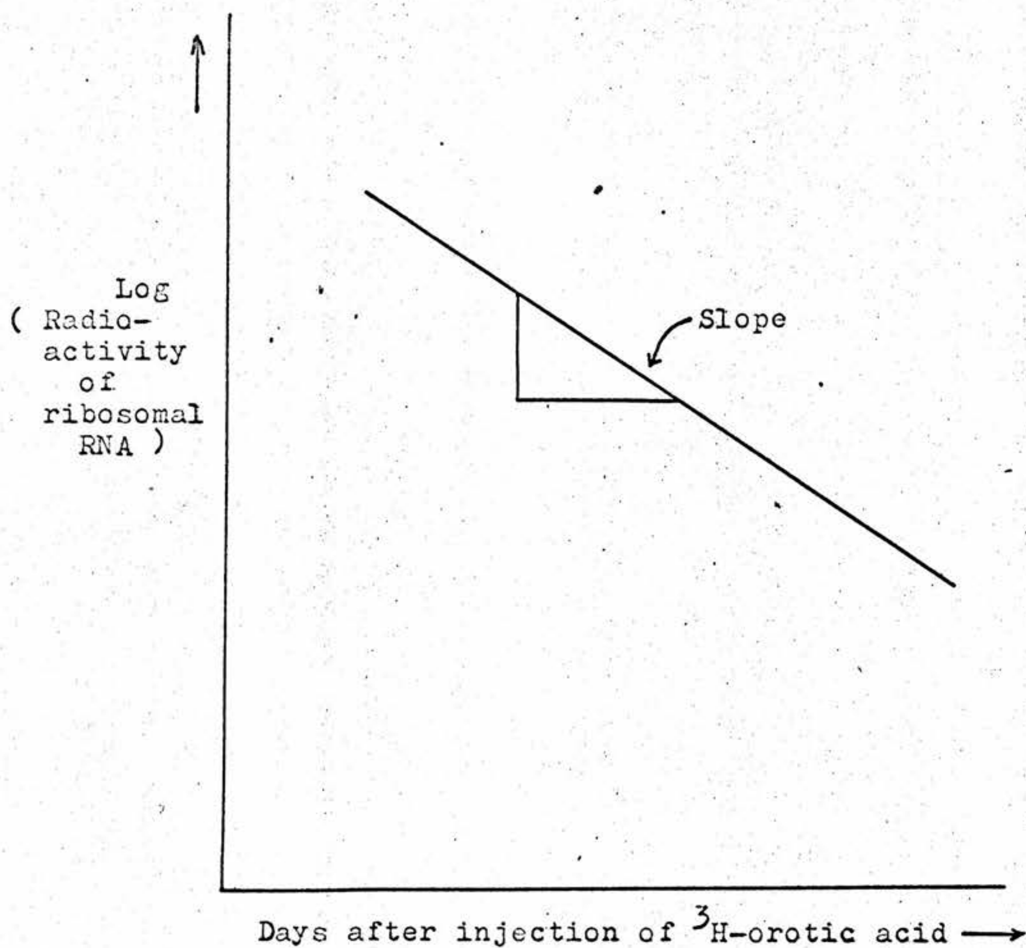
The rate of degradation of liver RNA in the normal rat follows first order kinetics (Ottolenglhi and Barnabeii, 1970, Enwonwu and Munro, 1970). The rate constant of the process can therefore be estimated by labelling the rRNA by injecting animals with a suitable radioactive precursor and plotting the logarithm of total rRNA radioactivity against a linear time scale. The slope of the resultant straight line equals $\frac{Kd}{2.303}$ where Kd is the degradation constant (fig. 3). There is much evidence to support the view that r-RNA constitutes 85-90% of the total cellular RNA in liver (Hirsch, 1967). In the present work, calculations were made taking the product of the specific activity of RNA in isolated ribosomes and total liver RNA content as an estimate of the radioactivity of total rRNA since complete recovery of the latter during cell fractionation procedures is not possible (Hirsch, 1967). A detailed justification of this procedure is presented in the discussion (p. 82)

The biological half-life, $t_{0.5}$, can be estimated using the relationship, $t_{0.5} = \frac{0.693}{Kd}$.

The daily fractional catabolic rate, (c) i.e. the percentage of the total rRNA catabolised per day is given by the relationship:

$$D_1 = CR_0 \quad \text{———————} \quad (1)$$

Figure 3 : Loss of Radioactivity from Ribosomal RNA with Time.



Kinetics of First Order Reaction:-

$$\frac{dx}{dt} = kd.x$$

Thus, $\log x = -\frac{kd}{2.303} t + \text{constant}$

Therefore,

$$\text{Slope} = \frac{kd}{2.303}$$

a toluene-based scintillator (NE 233, Nuclear Enterprises, G.B. Ltd.) were introduced. Radioactivity was recorded by assessing the time for 10^4 counts in an Intertechnique SL 30 liquid scintillation spectrometer at a temperature of 7°

The amount of RNA in the sample applied to the disc was estimated as described above and the radioactivity was expressed as counts per minute (c.p.m.) per ug RNA. In a preliminary series of experiments, the effect of varying the amount of a given ribosomal preparation plated on the disc on count rate was investigated. Figure 4 shows that the specific activity was independent of amount of RNA plated up to at least 300 ug.

The efficiency of this method of counting was determined in the following manner. A sample of RNA was counted in 5ml of NE 250 (Nuclear Enterprises, G.B. Ltd.), a dioxan-based scintillator. 0.05ml sample of ^{3}H-orotic acid, 10nCi per ml, 1,110 d.p.m. was counted in 5ml NE250 scintillator and found to give 600 c.p.m. A similar sample was then added to the vial containing the liver RNA which had been counted previously and the vial counted again. Another sample of ^{3}H-labelled liver RNA was then counted on a glass fibre disc. The results of these experiments are shown on Table 2, and it will be seen that the disc method of counting gave an efficiency of 41.8%

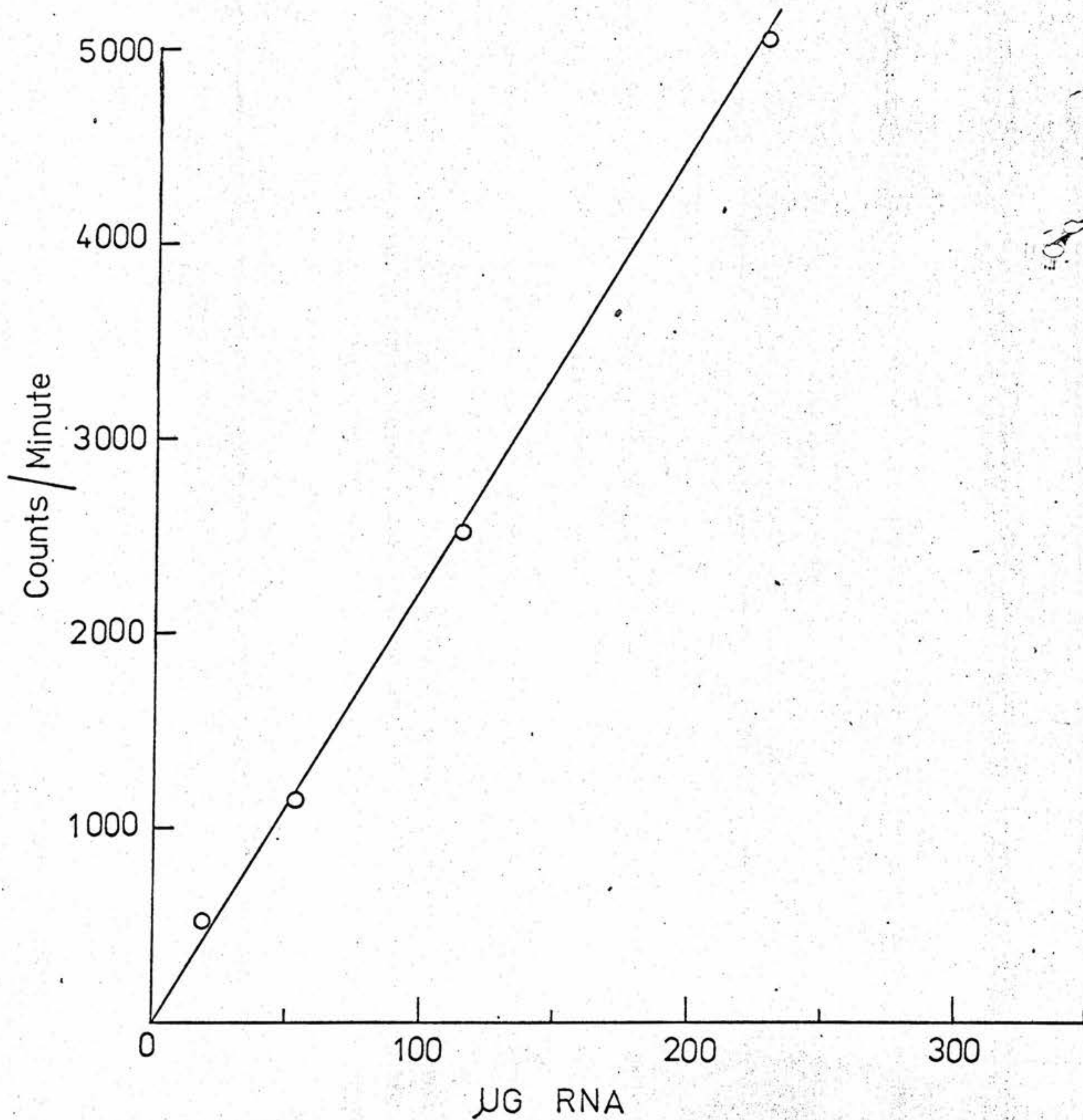


Figure 4 : Effect of amount of RNA plated on disc on count rate.

2.3.5 Estimation of Radioactivity of Acid-soluble Nucleotide Pool of Liver following ^{3}H-orotic Acid Administration

Since any variation in estimated turnover rate of r-RNA in livers of pregnant and control rats might be due to differences in the extent of reutilisation of ^{3}H-labelled nucleotide precursors arising from degradation of r-RNA, the amounts of radioactivity in the total acid-soluble pool and in the acid-soluble nucleotides of liver at 5 days after isotope administration were estimated.

2.3.5.1 Preparation of perchloric acid-soluble ($HClO_4$) fraction of rat liver

Groups of rats at specific days of pregnancy together with their non-pregnant controls were killed by stunning. The liver was immediately removed, weighed and homogenised in equal volumes of ice-cold distilled water in a Potter-Elvehjem homogeniser at a speed of 1,250 rev/min at 0°. 1ml of the aliquot was diluted with ice-cold distilled water to give a final dilution of 1 in 20. 1ml of the diluted homogenate was then pipetted in a pyrex tube followed by 1ml ice-cold 0.4N perchloric acid and the mixture left in ice for 20 mins before spinning down the precipitate in a MSE Mistral 6L centrifuge at 2,000 r.p.m. for 15 mins at 0°. The supernatant was collected after centrifugation and the precipitate was washed

twice with cold 0.2N perchloric acid. The supernatant and the washings were pooled together to form the perchloric acid-soluble fraction.

2.3.5.2 Separation of nucleotides by anion exchange chromatography from $HClO_4$ - soluble fraction of liver.

The method adopted was that described by Miller & Baggett (1972) with slight modifications. The $HClO_4$-soluble fraction was neutralised with 1N KOH at 0° using an external indicator. The mixture was left at 0° overnight and the precipitate of $KClO_4$ was spun down and discarded. The sample was passed through a 5cm x 6mm column of Amberlite Resin CG-400 Type II, 200 mesh (British Drug Houses Ltd.) The efficiency of the column was tested by passing 1ml of a solution containing 0.5mg purified cytidine-2'(3')-monophosphoric acid (British Drug Houses Ltd.) After the sample was adsorbed on the column, the column was washed with 20ml of water to wash down any unadsorbed material. The effluent was collected and its volume (25ml) measured and diluted to a final volume of 100ml. The absorbance of this fraction in the range 250-280 nm was negligible. The nucleotide fraction was then eluted from the column with 20ml 1 N HCl. The volume of the effluent (25ml) was made up to 100ml with distilled water and its absorbance

at 275 nm read on the Unicam SP 505 and compared with that of the solution before it was passed through the column. From these two readings it was calculated that the column had absorbed 95% of the sample originally applied.

1ml of the neutralised $HClO_4$ extract prepared from the liver was fractionated on the column. Nucleotide and other unabsorbed substances such as purine and pyrimidine bases were washed down from the column with 20ml distilled water, to give a nucleoside fraction. Nucleotides were then eluted from the column with 20ml 1N HCl. The HCl of the nucleotide fraction was subsequently removed by repeated vacuum distillation in a water bath at a temperature between 50° - 60°. The residue of nucleotides was then taken up in 2ml distilled water.

2.3.5.3 Determination of radioactivity of nucleotides of rat liver

A 0.2ml aliquot of the above nucleotide solution obtained after anion exchange chromatography was counted in 5ml NE 250 scintillator, with and without the added internal standard of 0.05ml ^{3}H-orotic acid (10nCi/ml, 1,100 dpm) for 100 min in an Intertechnique SL30 liquid scintillation spectrophotometer at a temperature of 7° and using a preset ^{3}H-counting channel. The neutralised perchloric acid extract was counted in the same conditions before separation of

nucleotides. The results were expressed as disintegrations per minute. The results of one such experiment are shown in Table (3).

The counting efficiency was also determined by the use of external standard counting. The principle of operation is that a γ -emitter source, ^{137}Cs, is placed close to the detection chamber, which generates Compton electrons in the liquid scintillator having an energy spectrum similar to that of a β -emitter. The spectrum will shift with changes in quenching. The shift may be observed in the form of a change in count rate of one channel set in the upper region of the Compton spectrum or by a change in ratio of two channels (see Fig 5). From this figure, one may observe that the external standard spectrum behaves in a similar way to that of ^{14}C in the presence of quenching except that there is less effect on the total count rate. It is evident that either the count rate in the upper external standard window or the ratio between the upper and lower windows may be used as a measure of spectral shift and subsequently related to the counting efficiency of the sample isotope. Hence the ratio ES_1/ES_2 of the count rates in channel A and B is a function of the sample quenching (see later). It is therefore possible to plot the curve representing the efficiency value according to the ratio ES_1/ES_2.

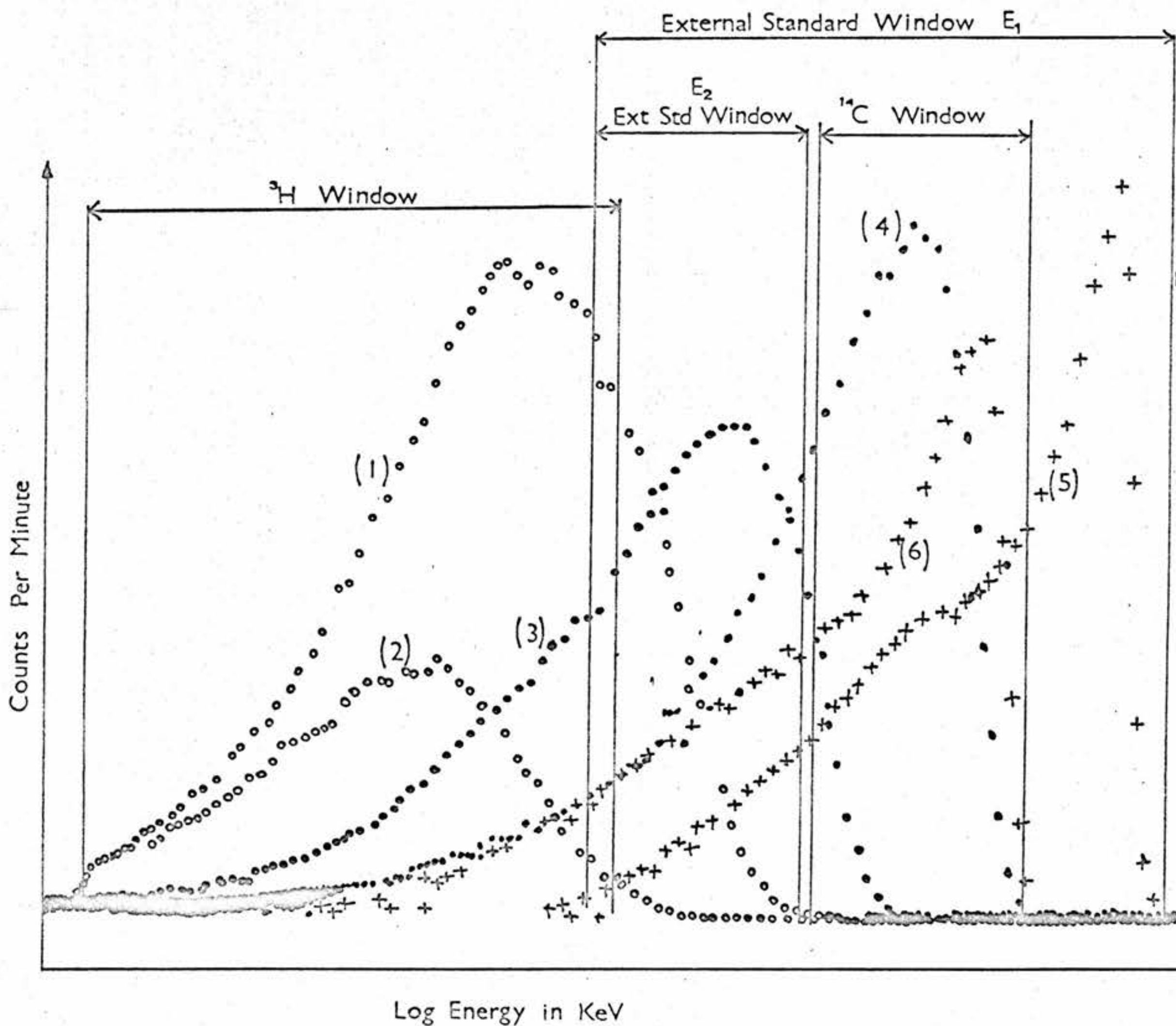


Figure 5 : Quenched and Unquenched Spectra of ^{3}H, ^{14}C and ^{137}Cs (External Standard). (1) ^{3}H-unquenched (2) ^{3}H-quenched (3) ^{14}C-quenched (4) ^{14}C-unquenched (5) ^{137}Cs-unquenched (6) ^{137}Cs-quenched.

The external standard curve is calibrated as follows:

A series of samples of different quench levels (methanol in this case) each containing identical known amount of standard (0.05ml ^{3}H-orotic acid, 10mCi/ml, 1,110 dpm) were prepared. A sample was counted in channel C preset for tritium of an Intertechnique SL30 liquid scintillation spectrophotometer for 100 mins at 7°. Sample number, counting time and content in channel C were printed out automatically. The external standard source was automatically positioned close to the detection chamber and channels A and B were switched to levels ES_1 abd ES_2 and counting was carried out for 30 sec. before contents in switched channel A and B (ET_{1a} for channel A and ET_{1b} for channel B) were printed out and the external standard removed. The sample alone was then counted automatically in switched channels A and B for 30 sec after which contents in switched channels A and B (ET_{2a} for channel A and ET_{2b} for channel B) were printed out. Calculation of the standard channel ratio ES_1/ES_2 was the difference between the count rate of the external standard in channel A, i.e. $ET_{1a} - ET_{2a}$, and channel B, i.e. $ET_{1b} - ET_{2b}$, such that:

$$\frac{ES_1}{ES_2} = \frac{ET_{1a} - ET_{2a}}{ET_{1b} - ET_{2b}}$$

By plotting the values of standard ratio ES_1/ES_2 on the X-axis

against the corresponding efficiency values in channel C, the efficiency curve was obtained (Fig. 6).

By using both methods to determine the counting efficiency of the sample in NE 250 scintillation, it was found that no significant difference in quench curve was observed, hence no correction was made for the data. The results of one such experiment are recorded in Table (4). It is seen that the efficiency of counting of nucleotide in NE 250 scintillator was 53.6%.

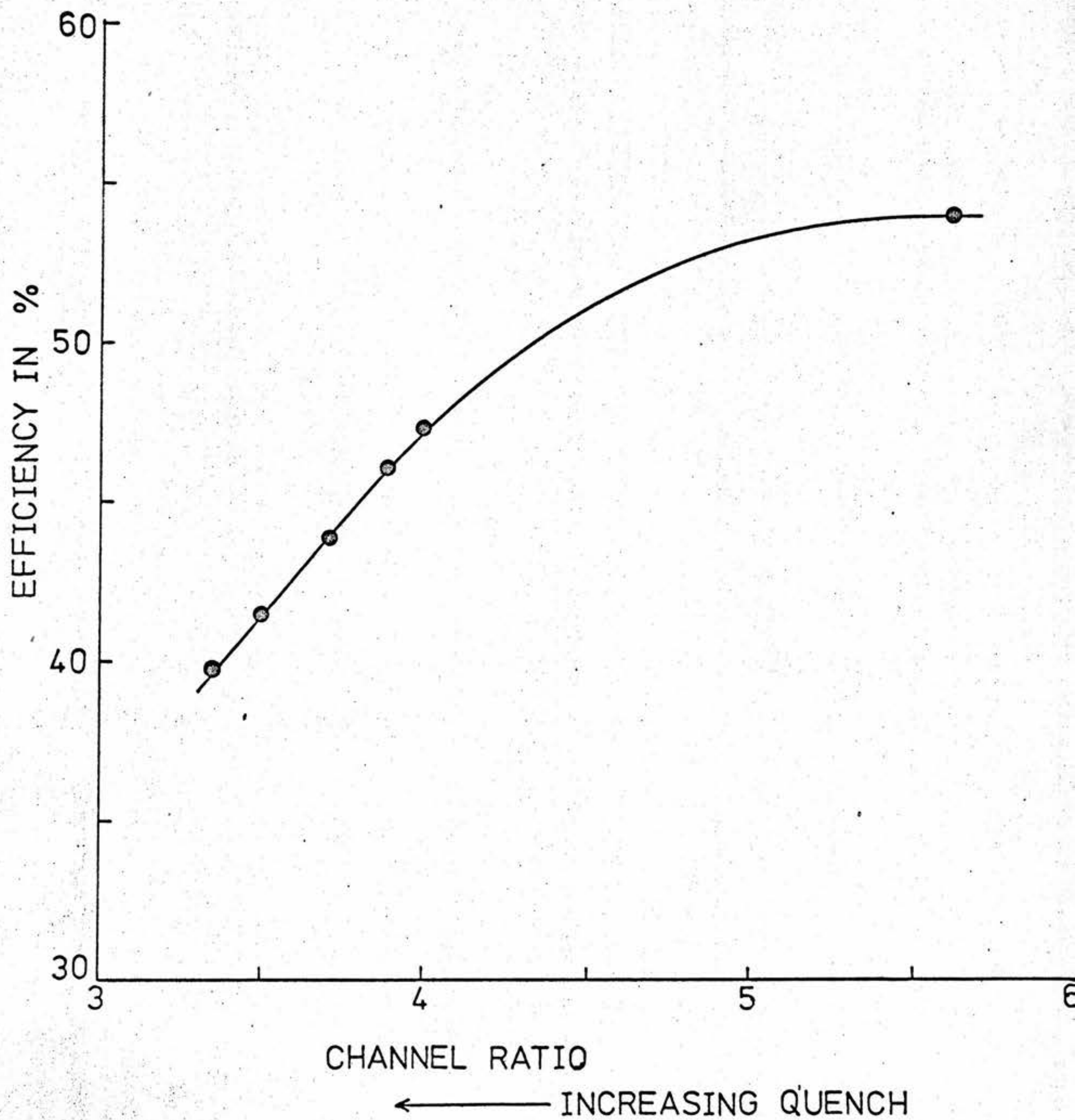


Figure 6: Quench curve obtained using External Standard Method on Intertechnique SL 30.

Table 2 : Efficiency of counting ^{3}H-RNA in solution in NE 250 and on glass-fibre disc in NE 233

Conditions of counting	Sample counted	cpm	dpm	Efficiency (%)
In Solution in NE 250	Internal+ Standard (A)	600	1,110	54.05
	^{3}H-RNA* (B)	301	--	--
	^{3}H-RNA* & Internal+ Standard (C)	888	--	--
	(B - C)	587	1,110	52.9
	(B)	301	570 (calculated)	52.9
Glass-fibre disc in NE 233	^{3}H-RNA**	179	428 (calculated)	41.8

* Total volume = 0.2ml

+ 10nCi

** 75% of activity of sample B

2.4 Estimation of RNase Activity in Rat liver.

2.4.1 Preparation of liver extracts.

The liver was weighed, minced briefly with scissors in an ice-cold beaker, and homogenised in two volumes of cold 0.44 M sucrose solution, using a Silversen homogeniser. Additional 0.44 M sucrose was added to give a 1 in 4 homogenate with respect to the original weight of liver. Cell debris and nuclei were sedimented by centrifugation at 1,000 r.p.m. for 10 mins in the MSE Mistral 6L centrifuge at 0°. The 'supernatant' thus obtained was used for estimation of both acid RNase and alkaline RNase activity (see later).

The preparation of supernatant used for the assay of ribonuclease inhibitor was obtained by centrifuging the supernatant, obtained from the previous step, at 0° twice for 1 hourly periods at 81,000 x g_{ave} in the MSE Super 40 ultracentrifuge. The final supernatant was stored below -20°.

2.4.2 Assay of ribonuclease activity

The method used was essentially that described by Shortman (1961) which depends on the estimation of the absorption at 260nm of acid-soluble nucleotides produced by the action of liver enzymes on purified yeast RNA. The details of the method are as follows :

0.2ml buffer, 0.1ml of liver homogenate, 0.1ml water

(or 0.01 M p-chloromercuribenzonate (Sigma Chemical Co.,) adjusted to pH 8.5-9.0 with NaOH) were incubated with 0.2ml of 1% purified yeast RNA for 30 minutes at 37°. The yeast RNA used was supplied by Saravac Laboratories (Pty) Ltd. and was purified according to the scheme in figure 7. The reaction was stopped by cooling the mixture to 0° and adding 0.6ml 1 N HCl in 78% ethanol. This last mentioned solution was prepared by mixing 12ml A.R. concentrated HCl with 8ml distilled water and adjusting the final volume to 100ml with 96% ethanol. Undegraded RNA and protein were centrifuged down at 1,500 r.p.m. for 30 mins at 0° in the MSE Mistral 6L centrifuge. 0.5ml of the supernatant was diluted with 2.5ml distilled water and the absorbance measured using a Pye-Unicam SP 505 spectrophotometer. Sample and reagent blanks were run concurrently. 1 unit of ribonuclease activity is that amount of enzyme which under the conditions of assay caused an increase in absorbance of the acid-soluble fraction of 0.1. at 260 n

For the estimation of free alkaline RNase activity, the liver homogenate was diluted 1 in 5 with distilled water, the buffer was 0.03 M veronal-acetate, pH 7.8 (Roth 1952) and p-chloromercuribenzonate was omitted. Total alkaline RNase activity was estimated under similar conditions except that p-chloromercuribenzonate was present. In confirmation of a previous report of Shortman (1961), it was found that the

liver homogenate required to be frozen and thawed 8 times before assay to ensure maximal activity of both free and total RNases. This procedure was therefore carried out routinely.

Before it was assayed for acid RNase activity, the liver homogenate was diluted 5-fold, frozen in liquid nitrogen then rapidly thawed at 37°. The process of freezing and thawing was repeated 4 times. The buffer solution used was 0.03 M veronal-acetate, pH 5.8 (Roth 1952).

2.5 Assay of RNase-inhibitor in Rat Liver

The method described by Shortman (1961) was followed. A constant amount of purified pancreatic ribonuclease was incubated with purified yeast RNA at pH 7.8 in the presence of increasing amounts of liver supernatant (see above). The activity of the ribonuclease-inhibitor was assayed by estimating the amount of acid-soluble nucleotides produced from the increase in absorption at 260 nm. The incubation was carried out as follows:

0.2ml 0.03 M veronal-acetate buffer pH 7.8, 0.1ml of a 0.1% solution of gelatin, treated with EDTA to remove metal ions, containing 0.005ug crystalline pancreatic ribonuclease (Sigma Chemical Co. Ltd.), 0.1ml of water or a dilution of rat liver non-particulate fraction and 0.2ml

of 1% purified yeast RNA were incubated at 37° for 30 minutes. The reaction was stopped and the increase in acid-soluble nucleotide material formed during incubation estimated as described in the assay of ribonuclease activity. Sample and reagent blanks were run concurrently.

The activity observed when no liver supernatant fraction was present was taken as 100% and the activities observed in the presence of different amounts of liver supernatant were expressed as a percentage of this value. The percentage activity was plotted against equivalents of liver tissue present. Figure (8) shows the result of a typical experiment. One unit of ribonuclease inhibitor was defined as that amount which would cause a 50% inhibition of pancreatic ribonuclease activity under the above conditions of assay. Thus the amount of liver which produced a 50% inhibition was said to contain 1 unit of ribonuclease inhibitor and the amount of inhibitor in 1g liver was calculated.

Figure 7

Purification of Yeast RNA (after Kirby, 1956)

Stage I.

5% solution of RNA stirred
with 90% (w/v) phenol & centrifuged
at 1,750 r.p.m. for 1 hr. at 0°

Aqueous layer
collected

Residues washed 3 times with
water. The supernatant was
collected each time after
centrifugation at 1,750 r.p.m.
at 0° for 45 mins and added
back to original supernatant.

Aqueous extracts made 2% with
respect to potassium acetate.
RNA was precipitated by addition
of 2 vol. of ethanol, precip-
itate was spun down at 1,750
r.p.m. for 20 mins. at 0°

Supernatant
discarded

RNA pellet collected & washed
once with ethanol-water (3:1)
The washed RNA was dissolved
in 25-100ml water and the
solution was extracted 3 times
with an equal vol. of ether.

Volume of ether-free solution
of RNA was measured. Equal
vol. of 2.5M K_2HPO_4 solution
& 2-methoxyethanol (redistilled
b.pt. 122-124°) were added,
followed by 0.05 vol. of 33.3%
H_3PO_4. The cloudy mixture was
spun down at 1,750 r.p.m. at 0°
for 20 mins. The supernatant
was collected & dialysed over-
night against water, while the
residues were discarded. A few
drops of toluene were added to
the supernatant before dialysis
at 0°.

To stage II ←

Stage II

After dialysis overnight against distilled water, the solution was made to 2% with respect to potassium acetate. The precipitate brought down by the addition of 2 vol. of ethanol was centrifuged down at 2,000 r.p.m. for 20 mins. at 0°

↓

Supernatant discarded

The RNA pellet was collected & washed 3 times with ethanol-water (3:1). Precipitate was spun down each time at 2,000 r.p.m. for 15 mins at 0°. The RNA was dried over with $CaCl_2$ in a vacuum desiccator.

↓

A 3% solution of the dried RNA was dialysed against several changes of 0.01M EDTA, then against 0.15M NaCl overnight and later against water at 0°-5°

↓

The absorbance of the solution was determined at 260nm, the concentration was adjusted to 1% and the final solution was stored at -20°

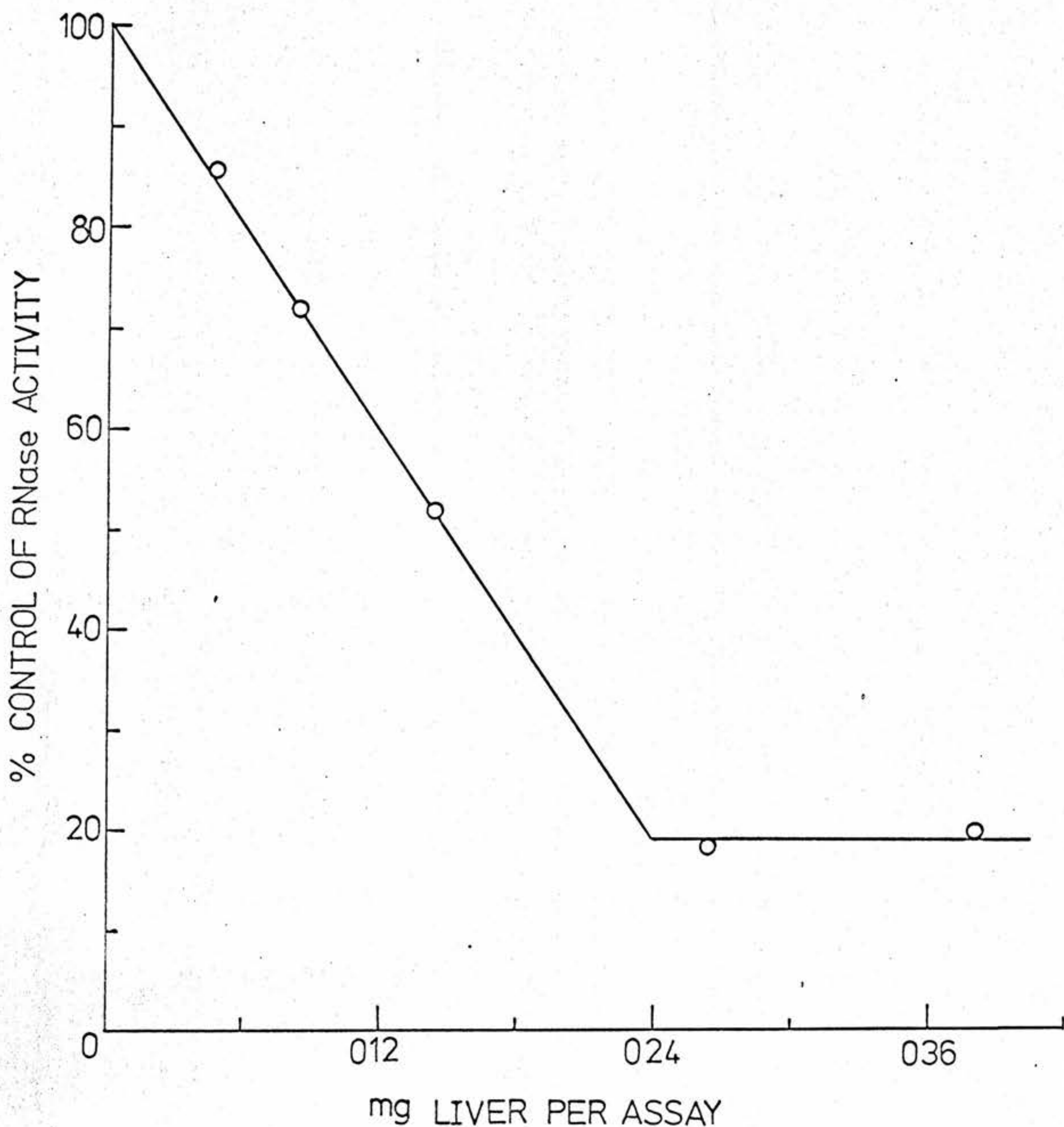
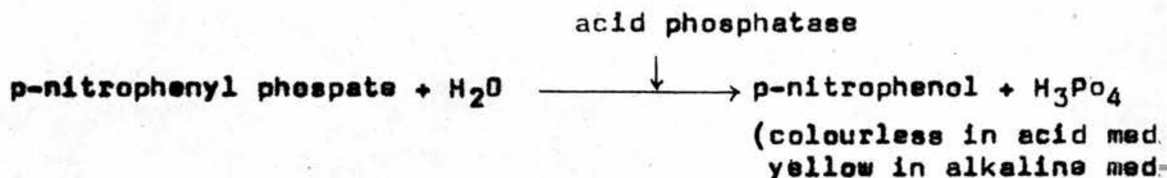


Figure 8 : Effect of increase in amount of Liver Supernatant on Activity of Pancreatic Ribonuclease. From the curve, 50 % inhibition would be produced by the equivalent of 0.14 mg tissue. Thus 1 g tissue contains 7692 Units RNase-inhibitor.

2.6 Assay of Liver Acid Phosphatase Activity

Groups of pregnant rats were killed between the 12th and 20th day of pregnancy along with non-pregnant control rats. The liver was immediately weighed, chopped finely with scissors and homogenised in 3 vol of ice-cold Medium A (0.05M tris-HCl, pH 7.5 at 0°, 1.0mM $MgCl_2$, 0.025M KCl, 0.25M sucrose) in a tight-fitting Potter homogeniser at 0°. Distilled water was added to give a final dilution of 1 in 400 to ensure disruption of lysosomal membranes so that full enzyme activity could be estimated (Beaufay & De Duve 1959).

The acid phosphatase activity of liver homogenates was assayed as described by Goodlad and Mills (1957). The yellow salt of the p-nitrophenol is liberated upon hydrolysis of the p-nitrophenyl phospate. The general reaction can be summarised by this equation :



Duplicate pyrex tubes were placed in a water bath at 37°, and 0.5ml 0.1M citrate buffer pH 4.8 introduced into the tubes followed by 0.1ml 0.025M p-nitrophenyl phosphate (Sigma Chem. Co. Ltd.) and 0.1ml distilled water. The reactants were preheated to 37° for 1 min then 0.3ml of a diluted liver homogenate

added to initiate the reaction. Incubation was carried out for 15 min, and 1ml 0.4N NaOH was added to each tube to stop the reaction. The volume was made up to 6ml with distilled water. Blanks were run in parallel where the p-nitrophenyl phosphate was added after the addition of 0.4N NaOH. Absorbance was determined at 400 nm in a SP505 spectrophotometer.

A standard calibration curve of p-nitrophenol (Fig 9) was prepared using recrystallised p-nitrophenol. A small quantity of crude p-nitrophenol was dissolved in 2% HCl (Vogel 1948) and the solution boiled for 10 min with added charcoal and then filtered through a hot Buchner funnel. The filtrate was left overnight when needle-shaped crystals settled. These were filtered and kept dried under vacuum (melting point 112°).

1 unit of acid phosphatase activity is that amount of enzyme which releases 1 u mole p-nitrophenol under the above conditions.

2.7 Statistical Analysis of Results.

Results are expressed as the mean of at least 3 replicates of each experiment ± standard error. Differences between data from pregnant rats and corresponding data from non-pregnant rats were tested for significance by a paired student's t-test. (Snedecor, 1956)

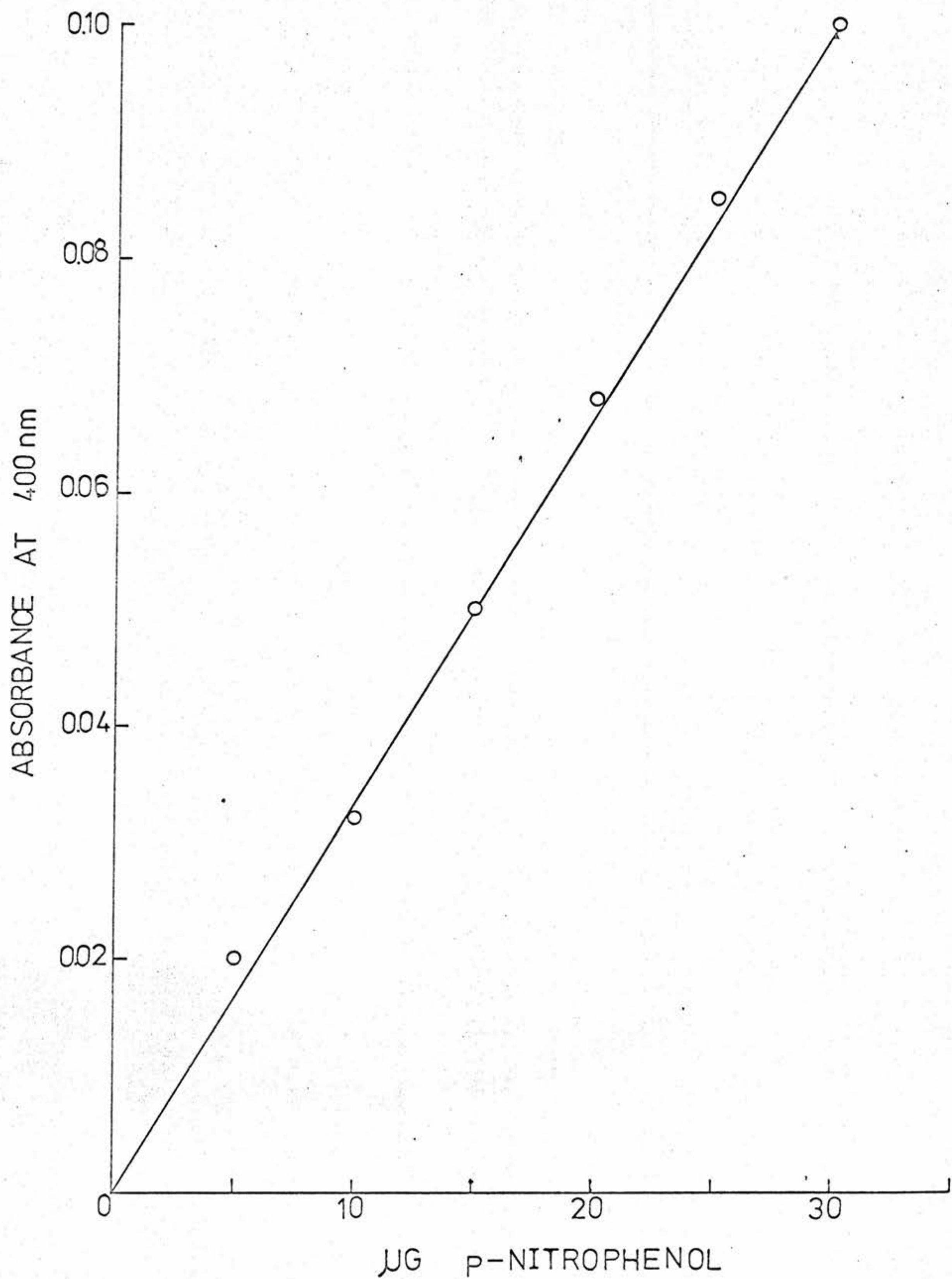


Figure 9: Calibration Curve for p-Nitrophenol.

3. Results.

3.1 Alteration in Body weight, Weight of Uterus plus Products of Gestation and Liver weight between the 10th and 20th day of Pregnancy.

Table 5 shows the alterations in body weight and in the weight of the uterus plus products of gestation (foetuses + placentae) during the latter half of pregnancy in the rat. The non-pregnant rats had a mean gain in body weight when they were fed 15g of ground rat cake daily from periods of from 10 to 20 days. There was a good deal of variability, however, in the extent of this gain between animals, the reason for this could be attributed to variations in the heating system in the animal house. The thermostats were set to control temperature at 70° but variations between 65° and 75° occured. Pregnant rats studied concurrently with the non-pregnant controls also gained weight on this dietary regimen. Part of this weight gain could be attributed to the growth of the uterus, placentae and foetuse. Nevertheless when the weight of the uterii and products of gestation was substracted from the final body weight, the weight of the mother still showed an increase over the initial body weight. The extent of this increase was generally greater than that observed in the non-pregnant controls (Table 5, Column 6). The maternal weight gain (column 6) decreased progressively as pregnancy progressed, presumably indicating the utilisation of maternal

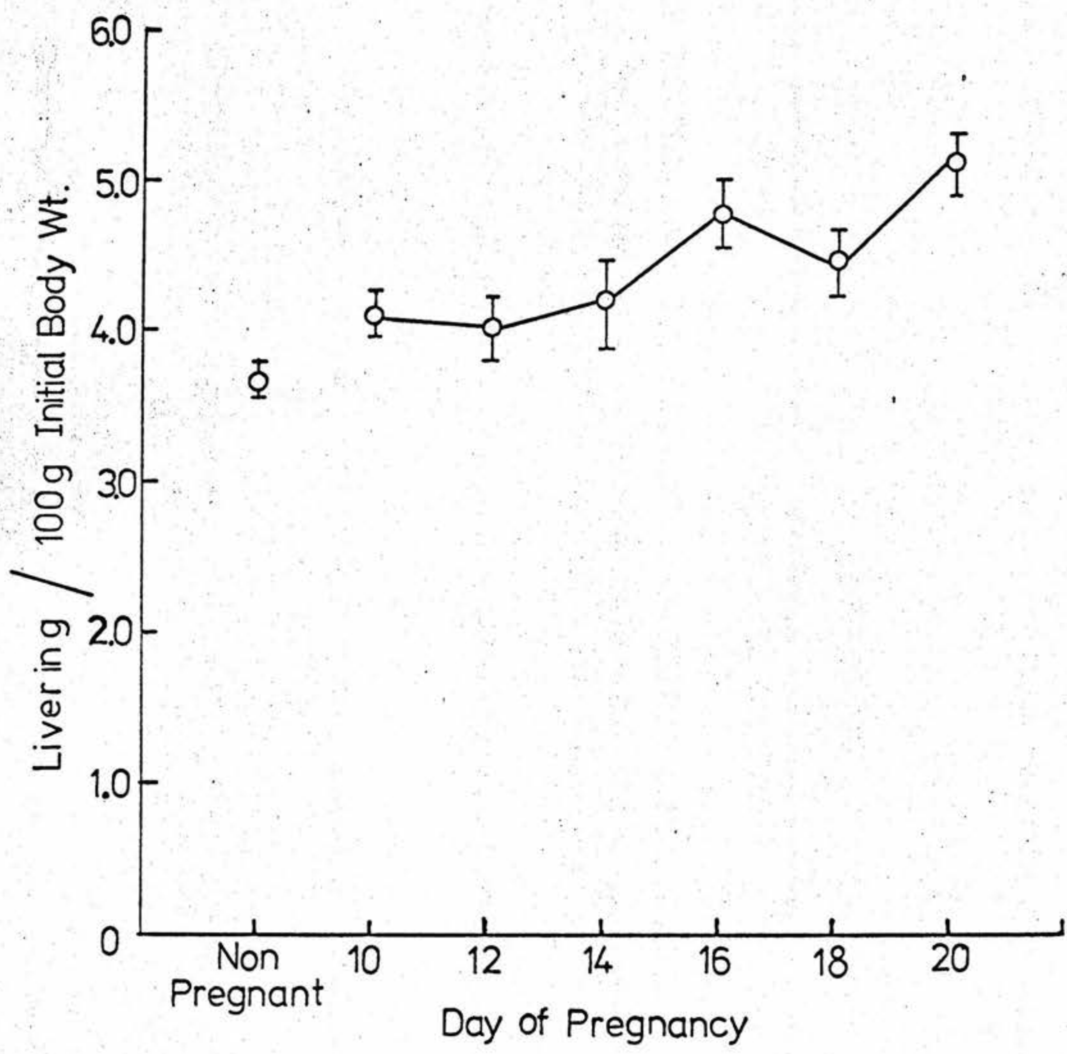


Figure 10: Alterations in Liver weight from 10th to 20th Days of Pregnancy in the Rat.

3.2 Effect of Pregnancy on Liver RNA Content

The changes occurring in the concentration and total amount of liver RNA from the 10th to 20th day of pregnancy are shown in Table 7 and Figure 11. The concentration of liver RNA was not significantly different from levels in corresponding control animals on the 10th, 12th and 14th day of pregnancy ($p > 0.1$). However, from the 16th day onwards there was a significant increase in the concentration of liver RNA in the pregnant rats. Total liver RNA was significantly increased above control levels in pregnant rats from the 16th day onwards, reaching approximately twice the control level on the 20th day of pregnancy.

3.3 Radioactivity of Liver RNA in Control and Pregnant Rats following a single injection of 5-^{3}H-orotic acid.

Pregnant rats along with non-pregnant control rats were injected with ^{3}H-orotic acid on the 13th day of pregnancy. Table 8 shows the specific activity of ribosomal RNA and the radioactivity in total liver RNA 1, 2, 3, 5, and 7days after the injection. The change in specific activity of ribosomal RNA with time is also recorded in Table 8. The ratio of protein to RNA in the final ribosome preparation was .95-.98 indicating there was little contamination from other cellular components.

The 13th day of pregnancy was selected as the day of

Table 7 : Alterations of liver RNA content between the 10th and 20th days of pregnancy in the rat.

Day of Pregnancy	No. of Animals	Liver RNA content	
		mg/gram Liver	mg/Liver/100gm initial body wt.
Non-pregnant	21	7.32 ± 0.14*	27.06 ± 0.40*
10	3	6.95 ± 0.28	28.38 ± 0.95
12	3	8.22 ± 0.19	32.90 ± 1.40
14	5	7.83 ± 0.17	32.70 ± 1.70
16	10	8.79 ± 0.21**	43.72 ± 1.81**
18	8	10.18 ± 0.30**	49.72 ± 2.25**
20	8	10.22 ± 0.22**	55.09 ± 1.78**

* Each value represents the mean ± S.E.M.

** Statistically different from mean value of corresponding control group of rats ($P < 0.01$)

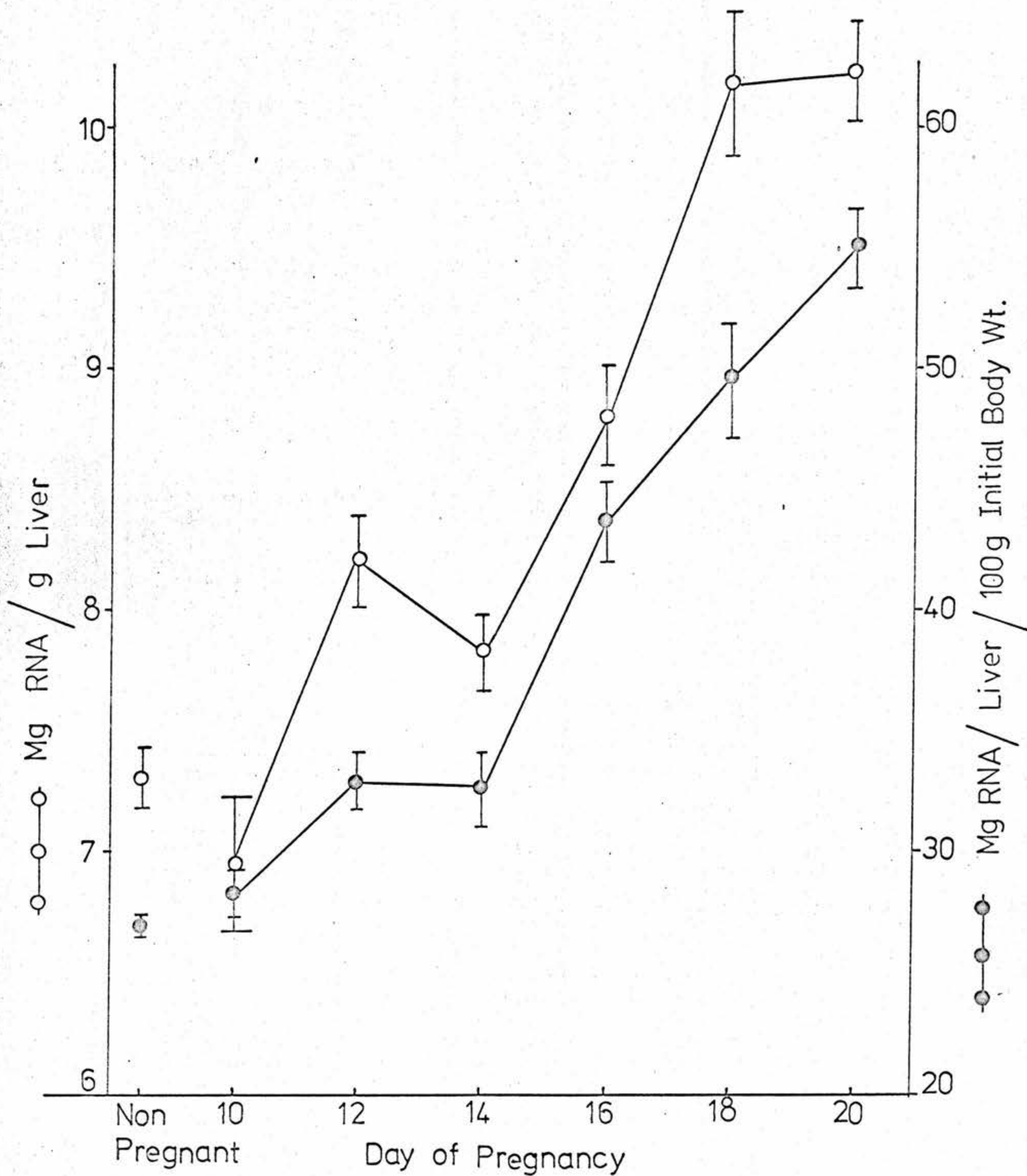


Figure 11: Alterations in Liver RNA from the 10th to 20th day of Pregnancy in the Rat. Both mg RNA per g liver and mg RNA per liver per 100 g Initial Body weight are shown.

administration of the labelled precursor because other workers had shown that in the normal rat labelling of ribosomal RNA is maximal at 48 hours after administration of ^{3}H-orotic acid (Hirsch & Hiatt 1966) and from table 7 and figure 11 it is apparent that liver RNA undergoes little alteration in pregnant rats between the 12th to 14th day of gestation. As seen in figure 13, the specific activity of ribosomal RNA in pregnant rats was higher one day after ^{3}H-orotic acid administrated (i.e. 14th day of pregnancy) than in control rats. The peak specific activity of the control rats occurred 2 days after ^{3}H-orotic acid was given. Specific activity of liver ribosomal RNA was higher in control than in pregnant rats on the 2nd and subsequent days after isotope was injected. In the absence of information on the size and radioactivity of the precursor nucleotide pools at the early stages following isotope administration, it is difficult to interpret the significance of these differences between control and pregnant rats.

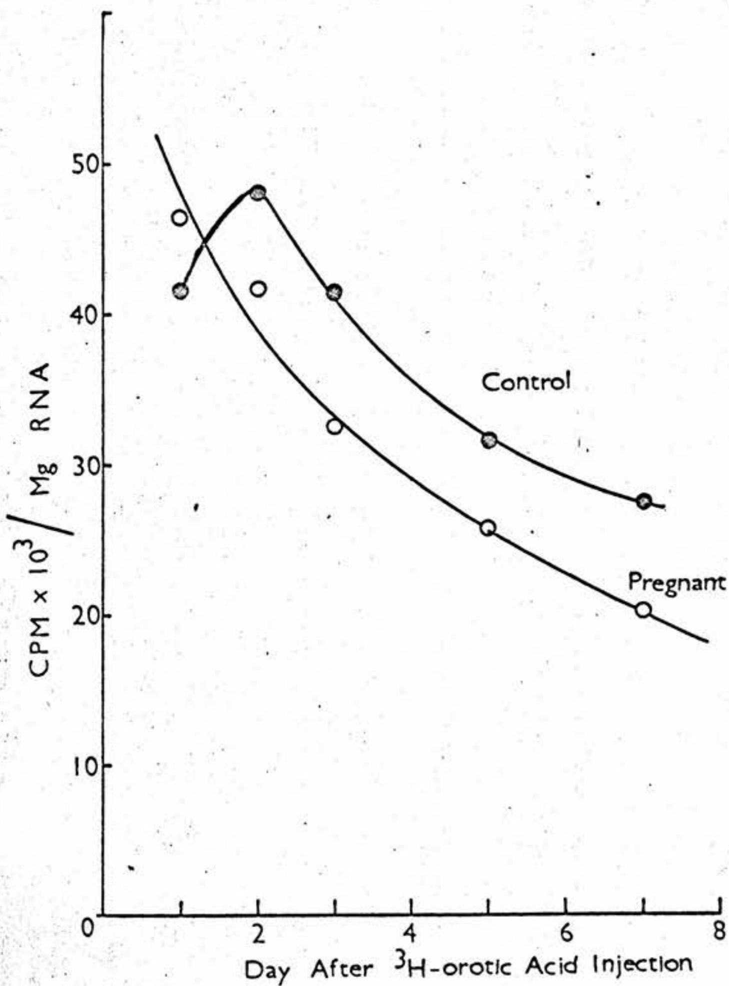
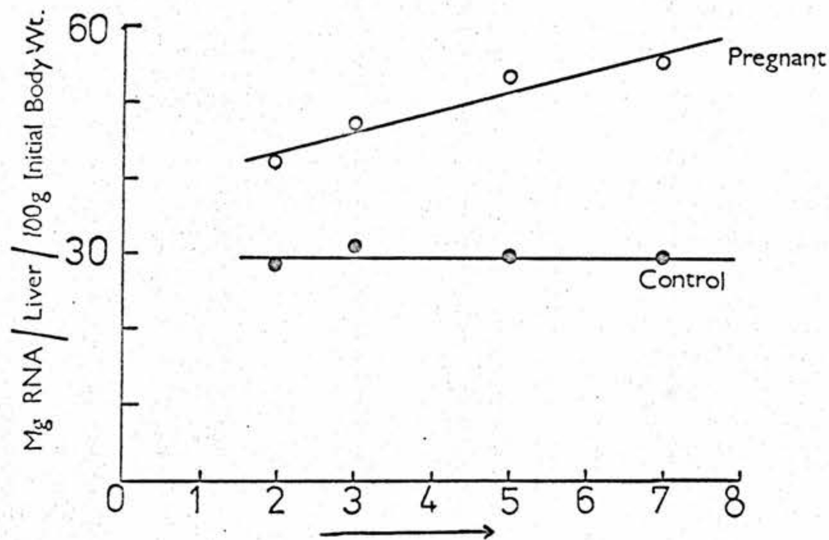


Figure 13: Specific Activity of Liver ribosomal RNA in pregnant and Non-pregnant Rats injected with ^{3}H-orotic acid.

3.4 Effect of Pregnancy on the Turnover of Liver Ribosomal RNA

When the total radioactivity of liver RNA was plotted on a logarithmic scale against a linear time scale a straight line relationship was obtained in the case of both pregnant and non-pregnant rats from 2 days after ^{3}H-orotic acid administration onwards (figure 12). In figure 12 the lines represent those calculated by the method of least squares from the data obtained experimentally. Data calculated from the slopes of these lines are given in Table 9. Biological half-lives for ribosomal RNA in liver of pregnant and non-pregant rats were 7.81 and 5.68 days respectively and the corresponding rate constants for degradation were 0.0887 and 0.1220 respectively. Calculated turnover times for degradation (i.e. time for entire liver ribosomal RNA pool to turnover) were 11.27 and 8.10 days respectively for pregnant and non-pregnant rats. The fractional rate of degradation of liver ribosomal RNA in pregnant and control rats was thus 8.87 and 12.30% per day respectively.

The amounts of RNA in the livers of the rats used in the labelling experiments are also shown in figure 12. It is seen that the level of RNA in the livers of the control rats remained constant over the period studied. On the other hand, there was a net increase in RNA content of 29.13% over the 5 days, equivalent to a daily increase of



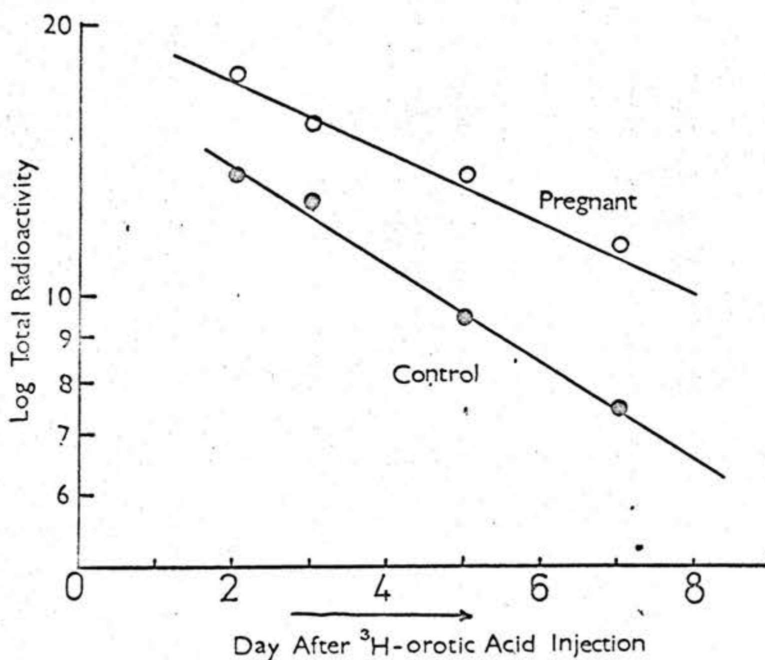


Figure 12: Total Radioactivity in Liver ribosomal RNA of Pregnant and Non-pregnant rats after ^{3}H-orotic acid administration (bottom graph). The changes of total liver RNA per liver per 100 g Initial Body Weight of both Pregnant and Non-pregnant rats are also recorded. (Top graph)

5.83%. Using equation (4) (Experimental, p. 37), it was calculated that the rates of synthesis of ribosomal RNA in livers of pregnant and non-pregnant rats were 6.21mg and 3.47mg per day respectively. Thus pregnancy has a dual effect on rat ribosomal RNA in that it causes its rate of catabolism to be retarded and, at the same time increases its rate of synthesis. An increased rate of synthesis of ribosomal RNA in the liver of the pregnant rat is also suggested by the fact that while the loss of radioactivity from RNA is less than in control rats, the specific activity of RNA is also less in livers of pregnant rats.

3.5 Radioactivity of total Acid-soluble Pool and Acid-soluble Nucleotides in livers of pregnant and Non-pregnant rats injected with ^{3}H-orotic acid.

The decreased loss of radioactivity from liver RNA in pregnant rats might be due to a decrease in labelling of RNA precursor pools relative to that in livers of control rats. To examine this possibility studies were made of the radioactivity remaining in these pools 5 days after rats were injected with ^{3}H-orotic acid (i.e. on the 18th day of pregnancy). Table 10 shows the radioactivity remaining in the total acid-soluble pool before it was submitted to ion-exchange chromatography. It is seen that there was no significant difference in the amount of radioactivity

Table 9 : Data on degradation and synthesis of ribosomal RNA in livers of pregnant and non-pregnant rats calculated from results reported in figure 12 (bottom graph).

	Condition of Rat	
	Pregnant	Control Non-pregnant
rate constant of degradation	0.0887	0.1220
biological half-life	7.81 days	5.68 days
turnover time of degradation	11.27 days	8.20 days
fractional daily rate of degradation	8.87%	12.20%
Mean daily change in total liver RNA	5.83%	0.00%
Daily synthesis of liver RNA	6.21mg	3.47mg

remaining in the preparation from non-pregnant rats and pregnant rats. These levels of radioactivity were only about 6% of that in livers of rats 3 hours after they were given ^{3}H-orotic acid.

When the radioactivity remaining in the acid-soluble nucleotide fraction was estimated, again no significant difference between the pregnant and non-pregnant rat was observed (Table 11). The radioactivity in both these fractions was only about 4% of that in the total acid-soluble pool.

It would thus appear that the difference in turnover time of liver RNA in pregnant and non-pregnant rats cannot be attributed to differences in precursor pool labelling.

3.6 Effect of pregnancy on Level of Alkaline RNase Activity of Rat Liver

The level of free alkaline RNase activity in livers of rats on the 10th to 20th day of pregnancy are shown in Table 12. In both pregnant and control rats the observed activities were very low and variable. Shortman (1962) also reported low and variable levels of free RNase activity in rat liver and concluded the variability was due to destruction of the inhibitor due to homogenisation procedures. Consequently it was decided to continue with studies on total RNase and RNase-inhibitor only.

Table 10 : Estimation of Radioactivity of Total Acid-soluble pool after administration of ^{3}H-orotic acid

	dpm x 10^{-3} per g tissue
5 days after injection	
Non-pregnant :	256 ± 33.5*
Pregnant :	230 ± 8.5*
3 hours after injection	
Non-pregnant :	4,566 +
Pregnant :	3,926+

* Results are expressed as the Mean ± S.E. of the mean of 4 animals

+ Result from one control and one pregnant rat

Table 11 : Nucleotide pool in Livers of Pregnant and Non-pregnant Rats after 5 days ^{3}H-orotic acid administration.

	dpm x 10^{-3} per g tissue	
Pregnant	10.77 ± 1.37*	p = N.S.**
Non-pregnant	10.18 ± 2.10*	

* Each value represents the Mean ± S.E. of the mean of 4 animals

** The difference between pregnant and control rat was found to be not significant (Student's t-test).

Total alkaline RNase activity per g liver was found to be lower than normal during pregnancy (Table 13). When levels in pregnant rats were expressed as a percentage of level in their respective non-pregnant controls, there was a significant decrease in concentration of the activity only on the 18th and 20th days of pregnancy. (Table 14). The total enzyme activity per liver was increased relative to control levels from the 10th day onwards (Table 14). The increase was significant on days 12, 14, 18 and 20 respectively. The increase in enzyme was less than the increase in total liver weight.

The level of RNase-inhibitor, on the other hand, tended to increase markedly towards the end of pregnancy (Table 15). The activity in the pregnant rats expressed as a percentage of that in their respective controls is shown in Table 16. The increase in pregnant rats was apparent both when the amount of inhibitor was expressed per g of liver and per total liver.

3.7 Effect of Pregnancy on Level of Acid RNase Activity in Rat Liver

Levels of acid RNase activity increased markedly towards the end of pregnancy (Table 17). When the levels in each individual pregnant rat were expressed as a percentage of that in their corresponding controls (Table 18) it was found that the concentration of the enzyme increased by 35-39% and the total amount by 80%

Table 12 : Effect of pregnancy on level of free alkaline ribonuclease activity in rat liver

Day of pregnancy	Group	Units per g tissue
10	Pregnant	144*, 181, 216
	Non-pregnant	317, 294, 168
12	Pregnant	381, 259, 180
	Non-pregnant	345, 432, 103
14	Pregnant	79, 129, 244, 259, 432
	Non-pregnant	69, 72, 244, 86, 495
16	Pregnant	216, 98, 288
	Non-pregnant	388, 86, 388
18	Pregnant	158, 248, 192, 122, 132
	Non-pregnant	403, 173, 78, 72, 98
20	Pregnant	72, 127, 230, 538, 144
	Non-pregnant	216, 236, 201, 345, 98

* Results are values obtained with individual rats

Table 13 : Effect of pregnancy on level of total alkaline ribonuclease activity of rat liver.

Day of Pregnancy	No. of Animal	Units per gm tissue
Non-pregnant	24	1615 ± 41.80*
10	3	1362 ± 53.29
12	3	1823 ± 58.23
14	5	1434 ± 89.93
16	3	1286 ± 74.92
18	5	1553 ± 108.10
20	5	1445 ± 50.39

* The results are expressed as the Mean ± S.E. of the mean of duplicate experiments for each animal.

Table 14 : Liver total alkaline RNase activity in pregnant rats expressed as a percentage of control level.

Day of Pregnancy	No. of animals	% of control of total alkaline RNase specific activity*	total activity*
10	3	98 ± 2.42**	120 ± 5.47
12	3	98 ± 3.22	123 ± 3.75+
14	5	96 ± 9.00	113 ± 7.52***
16	3	92 ± 4.16	116 ± 6.02
18	5	91 ± 3.27+	115 ± 5.03+
20	5	82 ± 3.21***	110 ± 4.52***

* Specific activity = Units per g liver per 30 min

Total activity = Units per whole liver per 30 min

** Each observation represents the mean ± S.E. of the mean as determined in duplicate sets of experiments for each experimental animal.

Statistical significance of the difference between pregnant and non-pregnant rats. (Student's t-test)

+ $p < 0.05$

*** $p < 0.01$

Table 15 : <u>Effect of pregnancy on ribonuclease inhibitor in rat liver.</u>

Day of Pregnancy	No. of Animals	Units* per g tissue
Non-pregnant	20	3300 ± 126.21**
10	3	3163 ± 316
12	3	3820 ± 454
14	5	3502 ± 300
16	3	4908 ± 380
18	3	10193 ± 213
20	3	7958 ± 692

* 1 unit is that amount of inhibitor required to decrease the activity of a preparation of pancreatic ribonuclease by 50% under conditions described in Experimental Section (p. 50)

** The results are expressed as the mean ± S.E. of the mean of duplicate experiments for each animal.

Table 16 : Level of RNase-inhibitor in Liver of Pregnant Rats expressed as a percentage of Level in corresponding Control Rats.

Day of Pregnancy	No. of Animals	% of RNase-inhibitor in non-pregnant rat	
		per g liver	per liver
10	3	106 ± 10.24*	130 ± 12.67
12	3	116 ± 21.52	149 ± 36.79
14	5	107 ± 10.67	127 ± 14.00
16	3	153 ± 22.02	194 ± 31.23
18	3	298 ± 15.24**	364 ± 10.35**
20	3	246 ± 6.23**	331 ± 2.62**

* Each observation represents the Mean ± S.E. of the mean as determined in duplicate sets of experiments for each experimental animal.

Statistical significance of the difference between pregnant and non-pregnant rats. (Student's t-test), ** $p < 0.01$.

Table 17 : Effect of pregnancy on level of Acid Ribonuclease Activity in Rat Liver.

Day of Pregnancy	No. of Animals	Units per g tissue
Non-pregnant	24	983 ± 20.16*
10	3	1086 ± 63
12	3	1118 ± 40
14	5	1059 ± 76
16	3	1142 ± 99
18	5	1315 ± 46.50
20	5	1266 ± 14.87

* Results are expressed as the Mean ± S.E. of the mean of duplicate experiments for each animal.

Table 18 : Alterations in liver Acid RNase activity between the 10th and 20th days of pregnancy in the rat.

Day of pregnancy	No. of Animals	% of control of Acid RNase activity	
		specific activity*	total activity*
10	3	105 ± 4.15**	128 ± 3.50
12	3	107 ± 10.42	129 ± 4.26
14	5	114 ± 14.35	133 ± 17.80
16	3	119 ± 15.27	151 ± 18.16
18	5	139 ± 6.85***	176 ± 13.47+
20	5	135 ± 7.41***	180 ± 10.07***

* Specific activity = Units per g liver per 30 min

Total activity = Units per whole liver in g

Units of activity is expressed in absorbance units (A_{260})

for detail definition see text.

** Each observation represents the mean ± S.E. of the mean as determined in duplicate sets of experiments for each experimental animal.

Statistical significance of the difference between pregnant and non-pregnant rats. (Student's t-test)

+ $p < 0.02$

*** $p < 0.01$

Table 19 : Effect of pregnancy on level of acid phosphatase activity of rat liver.

Day of Pregnancy	No. of Animals	$\times 10^3$ ug p-Nitrophenol per mg tissue
Non-pregnant	21	1632 ± 28.17*
14	5	1303 ± 94.78
16	5	1427 ± 73.21
18	5	1085 ± 54.99
20	6	1176 ± 80.49

* Results are expressed as the Mean ± S.E. of the mean of duplicate experiment for each animal.

Table 20 : Liver Acid phosphatase Activity in Pregnant Rats expressed as a Percentage of Control level.

Day of Pregnancy	No. of Animals	% of control of acid phosphatase	
		specific activity*	total activity*
Non-pregnant	21	100	100
14	5	83 ± 6.17**	101 ± 9.87
16	5	86 ± 5.93+	113 ± 10.44
18	5	67 ± 3.54***	88 ± 5.30***
20	6	75 ± 5.76***	93 ± 6.37***

* Specific activity = ug p-nitrophenol per mg liver per 15 min

Total activity = ug p-nitrophenol per whole liver.

** Each observation represents the mean ± S.E. of the mean as determined in triplicate sets of experiment for each experimental animal.

Statistical significance of the difference between pregnant and non-pregnant rat (Student's t-test).

+ $p < 0.05$

*** $p < 0.01$

3.8 Effect of Pregnancy on Level of Acid Phosphatase Activity in Rat Liver.

Since acid RNase is believed to be mainly lysosomal in origin, the effect of pregnancy on acid phosphatase another enzyme found in lysosomes was studied in order to see if the rise in the former enzyme could be correlated to an increase in lysosomes in pregnancy. Tables 19, 20 and figure 14 show that in contrast to acid RNase, the level of acid phosphatase activity tends to decrease in concentration as pregnancy proceeds.

3.9 Summary of enzyme changes found in livers of pregnant rats.

The pattern of changes in alkaline and acid RNase, acid phosphatase and RNase-inhibitor observed during pregnancy are shown in figure 14. Activities were calculated per g tissue and expressed as a percentage of values in corresponding control rats.

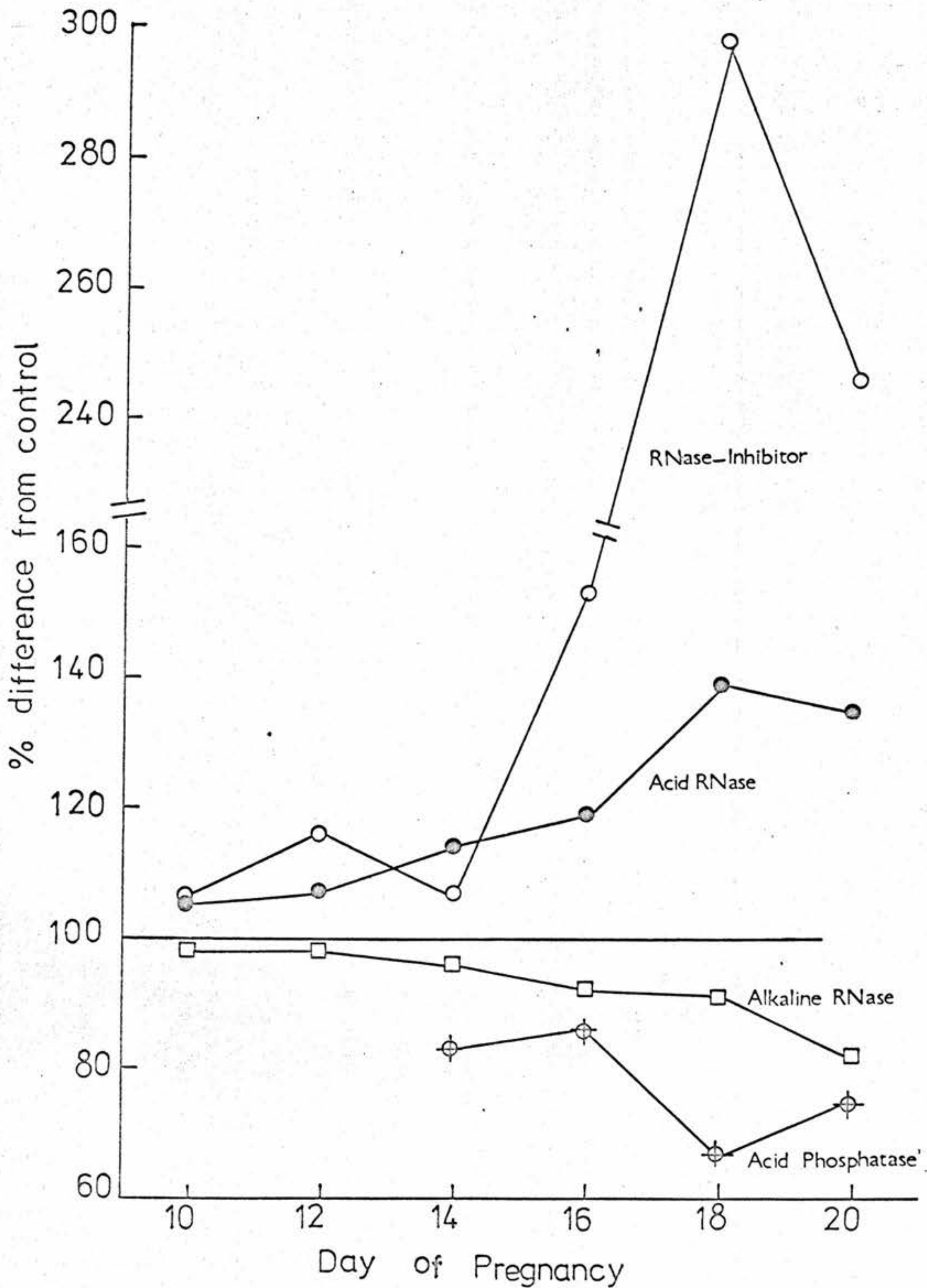


Figure 14: The changes of Acid and Alkaline RNase, RNase-Inhibitor and Acid phosphatase activities during the last week of gestation in the Rat when results are expressed as percentage difference from control.

4. Discussion

4.1 Alteration in Liver Weight and Liver RNA content in the Pregnant Rat.

In the present work an increase in liver weight was observed after the tenth day of pregnancy. There was a further increase in liver weight between the 14th and 16th day of pregnancy and the liver remained at this higher level until the 20th day of pregnancy (Table 6). This pattern of alteration in liver weight between the tenth and twentieth day of pregnancy is similar to that found previously by Clark, Steele & Goodlad (1971). In a study of liver DNA during the 10th to 20th day of pregnancy in rats fed a similar diet to those in the present study, Morrison (1966) found no significant alteration from the fourteenth day in either the total amount of DNA per liver or in the amount of DNA per nucleus during the final few days of pregnancy. It may therefore be concluded that the increase in liver size from the sixteenth day onwards is due to an increase in cell size rather than to an increase in cell number.

The increase in concentration of liver RNA, apparent from the 16th day of pregnancy (Table 7), is also in keeping with the findings of other workers (Campbell et al., 1949, Brunt, 1966). From the sixteenth day onwards the RNA concentration increased progressively (Fig. 11). The nature of this extra RNA is a matter of interest. Goodlad & Lumsden (unpublished

results) compared the intracellular distribution of RNA in livers of non-pregnant and 18- day pregnant rats by differential centrifugation. Their results are shown in Table 21. It is apparent that the excess RNA laid down in pregnancy is in the microsomal fraction and therefore presumably ribosomal in origin.

Table 21 : Distribution of RNA in subcellular fractions of liver of the pregnant rat.

Component	mg RNA/g liver		Significance (pregnant v control)
	Pregnant	Control	
Nuclei	2.1 ± 0.5*	1.6 ± 0.1	N.S.
Mitochondria	1.9 ± 0.3	2.0 ± 0.2	N.S.
Microsomes	6.8 ± 0.7	4.1 ± 0.7	< 0.01
Non-particulate fraction	0.9 ± 0.1	0.7 ± 0.1	N.S.

* Values are the mean ± S.E. of 4 pregnant and 4 non-pregnant rats.

4.2 Turnover of Ribosomal RNA in Liver of the Pregnant Rat.

The increase in liver RNA content during the last week of gestation in the rat could arise from an increased rate of synthesis, a decreased rate of breakdown or a combination of the two. In view of the finding of Goodlad & Lumsden that the increase in liver RNA during pregnancy was associated with the microsomal fraction (Table 21), it was decided to carry

out experiments to determine the turnover of ribosomal RNA during the stage of pregnancy when the marked increase in liver RNA occurred.

Blobel and Potter (1966) found that cytoplasmic RNA constitutes about 95% of the total RNA in liver. There is little doubt that the bulk of liver RNA occurs in the ribosomes (Hoagland, 1960; Yčas, 1963). Cell fractionation studies such as those carried out by Goodlad and Lumsden tend to underestimate the amount of RNA associated with the ribosomes. Nuclear and mitochondrial fractions are contaminated with ribosomal aggregates and ribosomes themselves may breakdown under the conditions of homogenisation giving rise to an increase in the RNA content of the cell sap or cytosol. In an attempt to obtain a more accurate value for the amount of ribosomal RNA in liver, Hirsch (1967) employed isotopic dilution techniques to compensate for the destruction of RNA during its isolation from tissue components. He concluded that in rat liver 85 - 95% of the cellular RNA is in fact found in the ribosomes and about 7% is 4S or transfer RNA. When rats were fasted for a period of time such that the total liver RNA content was reduced by 50%, the amount of RNA associated with the ribosomes was still 85 - 95% of the total amount present (Hirsch 1967; Hirsch and Hiatt 1966). The 28S and 18S RNA components of the ribosomes have not been found to occur free in the cytosol.

It thus appears that in the case of liver at any rate, the total RNA in a sample gives the best approximate measure of the amount of ribosomal RNA present. In the present experiments, therefore, an estimate of the radioactivity of ribosomal RNA was obtained from the product of the specific activity of RNA in isolated ribosomes and the total RNA content of the liver at various times after rats were injected with ^{3}H-orotic acid.

All studies on the turnover of a tissue component using radioactive precursors suffer from the defect that when the labelled components break down, the radioactive products of its catabolism may be re-incorporated into the particular component by normal synthetic pathways. Consequently, turnover times observed tend to be longer than the actual turnover time. The more rapid the rate of loss of radioactivity from a tissue component, the more the apparent turnover time must approach the true turnover time. With careful choice of labelled precursor, re-utilisation of radioactivity arising from the catabolic processes can be reduced to a minimum. Thus ^{14}C-guanidino-arginine is the precursor of choice for the study of liver protein turnover (Stephen and Waterlow, 1966). ^{14}C-arginine released by catabolism in this case is diluted with the amino acids of the urea cycle, radioactivity is removed from the system as urea and little is available for reincorporation into protein.

In studies on the turnover of ribosomal RNA and protein in rat liver using ^{3}H-orotic acid and ^{14}C-guanidino arginine, Hirsch and Hiatt (1966) found that both these ribosomal constituents had a biological half-life of 5 days. Since it is accepted that turnover times of protein in liver observed employing ^{14}C-guanidino-arginine as labelled precursor are not far removed from actual turnover times, these workers assumed that since the half-life they observed for the turnover of ribosomal RNA was similar to that for ribosomal protein the value of 5 days for the biological $t_{0.5}$ of ribosomal RNA must be a good estimate of the true $t_{0.5}$ for this component and that little re-utilisation of label arising from the breakdown of labelled RNA must have occurred. Enwonwu and Munro (1970) confirmed the similar rates of turnover of ribosomal RNA and protein using the same labelled precursors. Blobel and Potter (1968) however, found that in rats given a single injection of ^{3}H-orotic acid, the rate of loss of label from nuclear RNA paralleled the loss from the acid-soluble pool and concluded that significant re-utilisation of the radioactivity had occurred. However, it must be pointed out that nuclear RNA is a heterogeneous mixture and only a small proportion of the molecular species synthesised in the nucleus reaches the cytoplasm (Darnell, Philipson, Wall, Adesnik, 1971) and, moreover, as observed in the present work (Table 11) the radioactivity of the acid-soluble

nucleotides constitutes only a small fraction of that of the total acid-soluble pool (Tables 10 and 11). It is thus felt that on the basis of available evidence ^{3}H-orotic acid is a satisfactory precursor for investigations on ribosomal RNA turnover.

In the present work, from the second day following the injection of ^{3}H-orotic acid, the plot of log total radioactivity of ribosomal RNA was linearly related to the number of days after the injection (Fig. 12). The biological half-life of ribosomal RNA in the liver of the pregnant rat was calculated to be 7.81 days equivalent to a turnover time of 11.27 days and a daily fractional degradation rate of 8.87%. Since the total RNA content in the pregnant rat liver showed a gain of 5.82% per day, the fractional rate of synthesis must be higher than the rate of degradation by this amount, i.e. 14.69% per day. Ribosomal RNA in the liver of the non-pregnant control rat had a biological half-life of 5.68 days, equivalent to a turnover time of 8.20 days and thus a daily fractional degradation rate of 12.20%. Since the liver of the non-pregnant rat neither gained nor lost RNA (Fig. 12), the fractional rate of synthesis thus remained the same as its daily fractional degradation rate.

The biological half-life of 5.68 days calculated for the control rat is slightly higher than the value of about 5 days reported in the literature for normal rats (Hirsch

and Hiatt, 1966; Omura, Siekevitz and Palade, 1964; Enwonwu and Munro, 1970). This discrepancy might well be due to the differences in sex of animals used for the present study or the manner of feeding employed. During prolonged starvation the rate of RNA degradation in rat liver is enhanced whereas the fractional rate of RNA synthesis is decreased (Hirsch and Hiatt, 1966). In another study, Enwonwu and Munro (1970), showed that animals habituated to a diet deficient in protein undergo a reduction in the rate of both synthesis and degradation of ribosomal RNA. From the present study, the elevated RNA content in the liver of the pregnant rat during the later stages of gestation was accompanied by a rate of synthesis of 6.21 mg RNA per day whereas in the control the daily amount of RNA synthesised was 3.47 mg RNA.

Pregnancy would thus appear to alter both the rate of synthesis and degradation of liver ribosomal RNA. Glucocorticoid treatment of rats also causes a marked increase in liver RNA which is associated with a decrease in the rate of degradation and an increase in the rate of synthesis (Ottolenghi and Barnabei, 1970). These workers (Barnabei and Ottolenghi, 1968) found that the increased rate of synthesis was accompanied by an increase in the activity of liver RNA polymerase activity. Since a similar increase in synthesis of ribosomal RNA occurred in the latter stages of

pregnancy, it is felt that an investigation of the effect of pregnancy on RNA polymerase activity would be profitable, in particular the enzyme specifically involved in the synthesis of ribosomal RNA (Weaver, Blatti and Rutter, 1971). Liver alkaline RNase activity was found to be depressed in liver of cortisol-treated rats (Ottolenghi and Barnabei, 1968) and the level of alkaline RNase-inhibitor was increased. The level of ribonuclease activity was therefore investigated in livers of pregnant rats.

4.3 Alteration in RNase activity in the Liver of the Pregnant Rat.

There is much information in the literature to support the view that in tissues which are actively growing or which have a high rate of protein synthesis, alkaline RNase activity decreases and the level of alkaline RNase-inhibitor is increased, whereas in circumstances in which a catabolic state predominates, alkaline RNase activity is higher and the level of the RNase-inhibitor is lower than normal.

Shortman (1962) found that in partially hepatectomised rats, there was an early decrease in alkaline RNase activity and that the level of the RNase-inhibitor was increased at 12 hr and reached a maximum level of 139% of normal at 48 hr after the operation. Rahman, Cerny and Peraino, (1969) also found that alkaline RNase activity was decreased in regenerating liver between 8 and 24 hr after partial hepatectomy.

Evidence of an association between low levels of alkaline RNase, high levels of inhibitor and increased tissue growth are not restricted to liver. Imrie and Hutchison (1965) showed that when adrenocorticotrophin was administered to rats, latent alkaline RNase was reduced, the concentration of inhibitor was increased and a general increase in RNA content in the adrenals occurred at 18 hr after the injection. When lymphocytes were transformed into blast-like cells by treatment with phytohaemagglutin, Kraft and Shortman (1970) observed that this morphological change to a cell with intense biosynthetic activity was accompanied by an increase in the level of RNase-inhibitor while the total amount of alkaline RNase remained unaltered.

Phagocytes which are characterised by having a high catabolic activity, have a high level of RNase activity while the concentration of the inhibitor is at a low level compared with cells of other tissues such as thymus and liver (Kraft and Shortman 1970a). In livers of rats deprived of dietary protein and calories, there is a massive breakdown of the endoplasmic reticulum as well as disaggregation of polysomes and loss of protein synthetic capacity. Enwonwu and Sreebny (1970) have reported that the activity of free alkaline RNase in these animals is much increased, due to a marked decrease in the level of ribonuclease inhibitor (Enwonwu and Sreebny, 1971).

From evidence such as the above, Shortman has proposed that the ratio between the amount of alkaline RNase and alkaline RNase-inhibitor in a tissue is one of the factors which regulate the level of cytoplasmic RNA by controlling RNA degradation (Kraft and Shortman, 1970b). The results found in the present work in the liver of the pregnant rat would appear to substantiate this view. During the period of marked increase in liver RNA (Table 7) there is a decrease in the rate of RNA catabolism (Fig. 12), a decrease in the level of alkaline RNase activity and a marked increase in the level of RNase-inhibitor (Fig. 14). However, at present little is known concerning the mechanism of ribosome degradation. Ribosomal RNA presumably is dissociated from the protein component of the ribosome as a first step and the secondary structure (Cox, 1966) of rRNA would seem to make it relatively resistant to attack by ribonucleases currently recognised. Obviously clarification of this degradation process is required before the above evidence implicating the RNase/RNase-inhibitor ratio as a regulator of say ribosomal RNA breakdown may be regarded as any more than circumstantial.

Another possibility which should be considered is that the RNase/RNase-inhibitor system may play some importance in determining the nature of the messenger RNA molecules available for translation (Artman and Engelberg, 1964). From the work of Jordan and Morgan (1969), mentioned in the introduction,

it may be inferred that the increase in protein synthesis occurring in livers of pregnant rats is not a general one but is apparently directed towards the production of specific proteins. Whether the inhibitor could preferentially prevent the degradation of the corresponding messenger RNA molecules must also await further understanding of the processes of intra-cellular RNA breakdown. There is nevertheless some evidence associating ribonuclease inhibitor with the stability of polysomes in general. Thus the stability of isolated polyribosomes was shown to be increased when they were incubated with the high-speed supernatant of a liver homogenate (Takahashi, Mase and Sugano, 1966; Bont et al. 1967; Blobel and Potter, 1966). Livers of rats maintained on a protein-free diet were found to have a higher amount of monosomes and dimers relative to polysomes than did livers of rats fed a normal diet and at the same time, the concentration of liver RNase-inhibitor was shown to be decreased (Warner, Knopf and Rich, 1963; Wunner, Bell and Munro, 1966; Gaetani, Massotti and Spadoni, 1969). In the liver of the pregnant rat there is also evidence that the relative proportion of polysomes to monosomes plus dimers is increased above normal levels on the 18th day of pregnancy (Goodlad and Lumsden, unpublished results).

The reason for the gradual increase of acid RNase before parturition remains obscure. This gradual increase in acid

RNase activity is similar to the behaviour of this enzyme in regenerating liver (Shortman, 1962). In lactating mammary gland (Slater, 1961) gradual increase in acid RNase activity was observed which accompanied an increase in RNA content. If it is accepted that acid RNase is lysosomal in origin (De Duve, 1969), one might also have expected to find an increase in liver acid phosphatase activity during the later stages of pregnancy. However, the present study showed this was not so. It is of interest that in foetal, new born and young rats, liver acid RNase activity was significantly higher than in adult rats, while acid phosphatase activity was relatively independent of age (Rahman, Cerny and Peraino, 1969). Hence, acid RNase might have a bimodal distribution, one fraction being located in the acid phosphatase-containing lysosomes and another fraction in some other subcellular particle which increases during pregnancy and development. From studies using zonal gradient centrifugation Rahman, Howe, Nance and Thomson (1967) claimed that rat liver lysosomes are heterogeneous with respect to enzyme content. These workers found that acid phosphatase and cathepsin C showed a similar distribution and therefore were presumably located in one class of particle whereas acid RNase and cathepsin D activities sedimented together but in a different zone from acid phosphatase. The finding, in the liver of the pregnant rat, that acid phosphatase activity was depressed whereas acid

RNase was increased before full term might appear to support this assumption. However, the apparent difference in distribution of the acid hydrolases may be a reflection of different proportions of hepatocytes and reticuloendothelial cells in livers of normal and pregnant rats. Recently, Lentz and DI Luzio (1971) concluded that the average rat liver hepatocyte contains more protein, nucleic acid and hydrolase activities than the average reticuloendothelial cell on the basis of total activity. A study of ribonuclease activity in the different liver cell types might therefore yield an explanation of the difference in behaviour of the two acid hydrolases.

5. Summary.

1. In the pregnant rat fed a restricted amount of diet, liver weight was found to be increased after the tenth day of gestation. A further increase was apparent on the sixteenth day.

2. Liver RNA increased disproportionately from the sixteenth day of pregnancy.

3. The biological $t_{0.5}$ for ribosomal RNA in livers of 15 to 20-day pregnant and non-pregnant rats was found to be 7.81 and 5.68 days respectively. Corresponding turnover times for degradation were 11.27 days in the pregnant and 8.20 days in the non-pregnant rat. The daily fractional degradation rate of liver RNA was found to be 8.87 and 12.20% respectively for pregnant and non-pregnant rats. The rate of synthesis of ribosomal RNA in livers of pregnant and non-pregnant rats was estimated to be 6.21 and 3.47 mg per day, respectively. The increase in RNA content of the pregnant rat is therefore due both to an increase in the rate of synthesis and decrease in the rate of catabolism of ribosomal RNA.

4. Total liver alkaline RNase activity was decreased towards the end of pregnancy. The level of RNase-inhibitor on the other hand was markedly increased.

5. Liver acid RNase activity increased and liver acid phosphatase decreased during the later stages of pregnancy in the rat.

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