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RAPID RECOVERY OF STARVATION
DAMAGED RNA BY REFEEDING

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

RAPID RECOVERY OF STARVATION

DAMAGED RNA BY REFEEDING

CHAPTER I

INTRODUCTION

Nutritional status has been reported by several workers to markedly alter the composition and morphology of the liver. The type of diet consumed has been shown to influence the amounts of the various cellular constituents, including DNA, RNA, proteins, lipids, carbohydrates, etc. (References documenting these observations will be discussed later.) The extent of these alterations varies with the type of diet and the length of time it is fed. If the diet is deficient in one or more essential nutrients the effects of the deficiency may be repaired by providing the lacking ingredients.

The DNA content per liver, and thus the number of cells, has been reported to remain constant in adult rats during starvation. (Kosterlitz, 1947; Davidson, 1947; Thompson, 1953; Enwönwu, 1971; Hayashi and Kazmierowski, 1972). Although Hirsch and Hiatt (1966) reported a 10% to 25% decrease in liver DNA after 5 days of starvation, subsequent studies from the same laboratory (Edleman, Hirsch, Hiatt and Fox, 1969) showed that this apparent decrease in liver DNA was due to the presence in the liver extract of a diphenylamine reacting material which was

not DNA. These results show that starvation does not lower the liver DNA content. There are conflicting reports about the effect of starvation upon the amount of DNA per liver cell. (Fukuda and Sibatani, 1953; Conrad and Bass, 1957; Harrison, 1953). The amount of DNA per weight of liver changes according to the shifts in extracellular and intracellular water volume, (Harrison, 1953). Montecuccoli, Novello and Stripe (1971) reported that DNA synthesis was markedly reduced in rats starved for 3 days or fed a protein-free diet for 7 days. In view of the facts that DNA content per liver remained constant and the rate of DNA synthesis was reduced during starvation, it may be concluded that the rate of DNA degradation was also reduced during starvation.

Severe starvation (Watson et al. 1973; Wittman, Lee and Miller, 1969; Sox and Hoagland, 1966; Fleck, Sheperd and Munro, 1965) or the removal of protein from the diet (Pronczuk, Rogers and Munro, 1971; Gaetani et al. 1969; Wunner, Bell and Munro, 1966; Fleck, Sheperd and Munro, 1965) or feeding of grossly unbalanced amino acid mixtures, Pronczuk, Rogers and Munro, 1971) have been reported to shift the hepatic polysomal profiles from heavier to lighter aggregates. Polysomal disaggregation resulting from an overnight fast (Sidransky et al. 1968; Wunner, Bell and Munro, 1966; Fleck, Sheperd and Munro, 1965) or due to the lack of an essential amino acid (Park et al. 1973; Murty and Sidransky, 1972; Pronczuk, Rogers and Munro, 1971; Sidransky et al, 1967) was restored to normal within one hour of refeeding an amino acid mixture in the first case and refeeding the deficient amino acid in the second case. There are, however, contradicting reports as to which one

of the essential amino-acids is most important in restoring the polysomal profiles. Tryptophan alone was reported to restore the polysomal profiles in rats (Wunner, Bell and Munro, 1966) and mice (Murty and Sidransky, 1972; Sidransky et al. 1967). McGown and Richardson (1973) however, reported that omission of tryptophan or methionine from an amino acid mixture used to perfuse rat livers resulted in polysomal disaggregation. It seems that the most limiting amino acid for the species concerned would be most effective in restoring the polysomal profiles.

Because of the rapidity of polysomal recovery (1 hour) after administration of tryptophan to overnight fasted mice, most workers believed that the recovery was independent of newly synthesized RNA and was probably due to closer spacing of ribosomes on unchanged mRNA. However, Murty and Sidransky (1972) from their studies involving actinomycin D and ribonculease digestion techniques, have reported a marked increase in the amount of mRNA after tryptophan administration to fasted mice. Therefore, they concluded that the polysomal aggregation was dependent upon new mRNA synthesis.

Exogenous amino acids are not the only curative factor able to restore polysomal profiles to normal since protein free diets have also been shown to cause the reaggregation of polysomes in starved animals. Refeeding a protein-free diet for 8 hours (Webb et al. 1966) or glucose alone for 10 hours (Wittman et al. 1969) restored the polysomal profiles of starved rats. These studies emphasize the role of energy in restoring the polysomal profiles. The time taken to restore the polysomal profiles varies with the source of energy since Wittman

et al. (1969) found that feeding glucose to starved rats restored the polysomal profiles within 10 hours, while fat did not. Similar results were obtained by Watson et al. (1973) who found that refeeding of a high sucrose diet to 7-day starved rats restored the polysomal profiles within 24 hours, while refeeding of high-fat diet took 48 hours. From these observations, it appears that the lack of either amino acids or energy can cause disaggregation of polysomal profiles. Although the above studies show that either amino acids or energy can cause the reaggregation of polysomes from starved animals, it is clear that the recovery will be only temporary if both energy and protein are not made available. If the animal is supplied with amino acids alone, it can use them both as source of protein and energy. On the other hand, if it is supplied with energy alone, it will be able to maintain normal polysomal profiles only as long as it's body amino acid pools are not depleted. After the depletion of body amino acid pools, the polysomes will again be disaggregated. It must also be pointed out that in the studies mentioned above, where amino acids restored the polysomal profiles within one hour of administration, the animals were starved only overnight, while in the studies where the energy sources took several hours to restore the polysomal profiles, the rats were starved for 3-7 days.

The mode of action by which glucose restores polysomal profiles is still not well understood. Wittman et al. (1969) suggested that glucose was acting through induction of insulin, since insulin alone or with glucose stimulated hepatic protein synthesis and polysomal reaggregation in hypophysectomized or diabetic animals. However, admini-

stration of insulin to fasted or fasted-refed rats was without effect. Polysomal profiles from 3-day starved rats, could, however, be restored by insulin within 10 to 30 minutes after injection when supplemented with glucose (Wittman and Miller, 1970).

Several studies have been carried out investigating the relationship of ATP supply and polysomal aggregation. (Stewart and Farber, 1967; Oler et al. 1969; Van Venrodij et al. 1970, 1972; Freudenberg and Mager, 1971). Oler et al. (1969) suggested that ATP deficiency was not the trigger for polysomal disaggregation. Freudenberg and Mager (1971) have postulated that polysomal disaggregation is due to AMP, ADP accumulation. Van Venrodij et al. (1972) using ascites tumor cells in culture have shown that the presence of glucose in the medium is essential for polysomal aggregation. However, it is not clear if glucose acts by raising the ATP level or by lowering the AMP, ADP levels or by some other metabolic function.

It has been reported that when a rat is starved (Davidson, 1947; Thompson, 1953; Enwönwu, 1971) or fed a protein-deficient diet (Kosterlitz, 1947; Munro et al. 1953; Enwönwu, 1970) an immediate and extensive loss of RNA from the liver occurs for about 2 days, after which a new and lower plateau is reached. Starvation of adult rats for 5 - 7 days has been reported to decrease the RNA and protein content of the liver to 50% of normal (Kosterlitz, 1947; Petermann and Hamilton, 1958; Wilson and Hoagland, 1967). During starvation, the contents of nuclear RNA, ribosomal RNA and soluble RNA decreased exponentially with loss of body weight and at approximately the same relative rates. Starvation also decreased the levels of ribonucleotides in liver cells

(Onishi, 1970). Munro et al. (1964) and Munro (1968) reported that the fractional rate of rat liver RNA degradation correlated with the ribosomal subunit population and that the subunit population was most abundant during the period of most rapid RNA loss during starvation. Rizzo and Webb (1969) reported a linear and inverse relationship between the rate of ribosomal synthesis and the concentration of nonfunctional monomeric ribosomes. Munro (1953) reported that the dietary availability of amino acids, rather than calories, was the major factor in the regulation of liver RNA content.

Refeeding of a complete diet to starved rats was found to repair deficits in RNA and protein content within several days (Laird et al. 1955). Garza et al. (1970) reported that refeeding a high-carbohydrate (89% sucrose) protein-free diet for 48 hours to 48-hour starved rats did not change hepatic RNA content from that found at the starvation level. Refeeding of high protein (89% casein) carbohydrate-free diet for the same period, on the other hand, restored the RNA content to normal levels.

The rate of degradation and synthesis of rat liver RNA has been studied by several workers and a half-life of 5 days with a turnover time of 7.2 days has been reported for rRNA of adequately fed rats (Gerber et al. 1960; Loeb et al. 1965; Wilson and Hoagland, 1967; Hirsch and Hiatt, 1966; Blobel and Potter, 1968). Hadjiolov (1966) however, reported a half-life of 40 hours for rRNA. It is not clear why Hadjiolov's data gave such a low half-life for rRNA. Enwönwu et al. (1971) estimated a half-life of 30 hours for rRNA during the first 2 days of starvation. In the second phase of starvation, however, the

half-life of rRNA was about 7 days.

Wilson and Hoagland (1967) using actinomycin D ,found that rat liver cytoplasm contained mRNA species with an apparent half-life ranging from 3 to 80 hours. Murty and Sidransky (1972) using actinomycin D doses which would selectively allow the labelling of mRNA ,or by using doses of ribonuclease which would selectively digest mRNA ,found that during 0.5 to 3 hours after injection of ^{14}C-orotic acid, the specific activity of mRNA of free polyribosomes was on an average of 2-3 times higher than that of mRNA of membrane-bound polyribosomes, over the entire period of labelling.

Hirsch and Hiatt (1966) concluded that the loss of RNA that occurred during starvation was due to an increase in ribosomal RNA degradation as well as to a reduction in the rate of RNA synthesis. Clark et al. (1957) reported that the rapid loss in liver RNA content during the first 24-48 hours on a protein deficient diet was due to accelerated RNA breakdown rather than to reduction in its rate of synthesis. Enwönwu et al. (1970) found that animals habituated to a protein-deficient diet underwent reductions in rates of both synthesis and degradation of RNA.

Hayashi and Kazmierowski (1972) observed that during fasting proportionately greater amounts of radioactivity were incorporated into non-ribosomal RNA (mRNA). Wilson and Hoagland (1966) found that refeeding of animals starved for 4-5 days led to gradual replenishment of cytoplasmic polysomal aggregates in 8-12 hours. During this period the total rRNA content increased two-fold, i.e. reached normal values, whereas non-ribosomal RNA was still 55% below normal. They also re-

ported that microsomes and ribosomes from the livers of rats starved for one week incorporated amino acids less actively *in vitro* than preparations from the livers of rats that were starved for the same period and then refed for 10-15 hours. But if the ribosomes were treated with puromycin to strip off mRNA and nascent proteins, equal amounts of polyphenylalanine were synthesized upon addition of polyuridylic acid by those ribosomes from either starved or starved-refed rats. From these studies it may be generalized that severe starvation causes a marked reduction in the RNA content of the liver and also changes the half-life of liver RNA. Ribosomes from starved animals do not appear to be defective in protein synthesis.

Starvation (Onishi, 1970) and protein depletion (Clark and Jacob, 1972) has been reported to decrease the levels of RNA polymerase. Shaw and Fillios (1968) reported that over a 42 day period the activity of DNA-dependant RNA polymerase was significantly higher in the liver nuclei of the rats fed a low-protein (5%) diet, and significantly lower in the liver nuclei of rats fed a high-protein (40%) diet, when compared with the liver nuclei from controls (20%). An increase in template efficiency of endogenous DNA in rats given low protein diets may be due to loss of deoxyribonucleohistones. However, over a prolonged period of protein depletion the relatively higher levels of the RNA polymerase diminished in the low-protein fed groups. Vesley and Cihak (1970) reported a significant increase in the DNA-dependent RNA polymerase activity in the livers of rats 3 hours after tube-feeding a complete amino acid mixture or tryptophan alone when compared to rats tube-fed a tryptophan-devoid diet. These results suggest that both prolonged starvation

and prolonged amino acid deficiency may decrease the levels of RNA polymerase.

Moris and Lamirande (1964) observed the presence of a ribonuclease in the microsomal fraction of rat liver and Gavard and Lamirande (1972) reported the complete purification of an alkaline ribonuclease (pH 7.2 to 8.8) from rat-liver microsomes. Arora and Lamirande (1971) found that ribosomes from starved animals were more susceptible to autodegradation as compared to ribosomes from control animals. From these studies they concluded that starvation caused an increased activity of ribosomal ribonuclease. This was, however, in contradiction to the earlier reports of Sox and Hoagland (1966) who had claimed no change in the level of ribosomal ribonuclease during starvation. Hird et al. (1964) showed that free ribosomal subunits were rich in endogenous ribonuclease. Sheppard et al. (1970) reported that the enzyme activity of acid-stable ribonuclease was lower in fed rats as compared to fasted rats. Allard et al. (1957) reported a decrease of 35% and 28% in the levels of acid (pH 5.5) and alkaline (pH 7.5) ribonucleases of rat liver homogenate after 7 days of starvation. Onishi (1970) observed that free and latent alkaline ribonuclease activities were maintained up to 20-25% body weight loss, but then decreased to 50% of the normal level when the weight loss reached 40-45% of the initial weight. Acid ribonuclease activity and the activity of alkaline ribonuclease inhibitor were also reported to decrease with loss of body weight. These studies indicate that the levels of some cellular ribonucleases change with starvation. Some of the ribonucleases are increased, others are decreased and still others remain unchanged with starvation.

Polyacrylamide gel electrophoresis patterns of ribosomal proteins showed no differences between 4-5 day starved and starved-refed ribosomes (Sox et al. 1966) and between starved-refed and normal ribosomes. (Wilson, Hill and Hoagland, 1967).

One can find little in the literature concerning the events taking place at the RNA level regarding the disaggregation of polysomes with starvation and their rapid recovery after refeeding. We do know that polysomal disaggregation during starvation is accompanied by a decrease in liver RNA content, however, we don't know if the disaggregation is due to the lack of rRNA and/or mRNA or to defective mRNA and/or rRNA. Ribosomes from the starved animals after stripping off their nascent proteins and mRNA were as efficient in in vitro protein synthesis as ribosomes from the control animals (Wilson and Hoagland, 1966). Ribosomes from starved animals are more susceptible to autodegradation when compared with ribosomes from the control animals (Arora and Lamirande (1971). We do not know if this is due to increased fragility of rRNA or due to the lack of some structural ribosomal proteins or endoplasmic reticular membranes. There are contradicting reports about the levels of ribosomal ribonucleases during starvation. The reaggregation of the polysomal profiles after refeeding of starved animals could be due to the repair or de novo synthesis of the structural constituents of polysomes. Some of the factors that could be involved in polysomal reaggregation are mRNA, rRNA, structural ribosomal proteins, endoplasmic reticular membranes, ribonuclease inhibitor, etc.; however, we don't know which one(s) of these factors is involved.

Keeping all these problems in view, the present studies were

undertaken to investigate the following aspects of polysomal disaggregation and reaggregation.

1) Changes in the RNA profiles after different periods of starvation.

2) The period of time required to restore the RNA profiles to normal, after refeeding either a high carbohydrate (HC) or a high fat (HF) diet.

3) Change in the level of ribosomal ribonuclease during starvation and the possible relationship between the changes in RNA profiles and ribosomal ribonuclease level.

4) Comparison of the RNA content of the livers of starved rats with livers of starved-refed rats to see if a correlation exists between RNA content and the RNA profiles.

5) The relative loss during starvation and the relative increase after refeeding in the individual species of RNA (i.e. mRNA, rRNA).

CHAPTER II

MATERIALS AND METHODS

Diet Materials

A standard laboratory chow diet was used to raise the animals from the weanling stage until placed on starvation. A high carbohydrate or a high fat diet was used during the refeeding periods after starvation. The compositions of these diets are shown below.

Compositions of rat diets

Ingredient	High sucrose (HS)		High fat (HF)		Stock diet[1]	
	g	Kcal	g	kcal	Source	g
Casein	20.00	80.0	32.92	131.7	Protein	24.27
Sucrose[2]	69.90	279.6	-	-	CHO	56.23
Lard[3]	-	-	51.36	462.2	Fat	4.15
Corn oil[4]	5.00	45.0	8.32	74.9	Fiber	4.86
Salt mix	4.00	-	5.72	-	Ash	7.78
Vitamin premix	0.50	2.0	0.76	3.0		
Vit. A,D,E[5]	17.71 mg	-	25.6mg	-		
Choline Cl	0.10	-	0.16	-		
Methionine	0.50	-	0.76	-		
	100.00	406.6	100.00	671.8		359.4

Composition of diets(continued)

	HS	HF	Stock diet
Kcal/g diet	4.0	6.6	3.6
Non protein energy : Protein energy, kcal	4.0	4.0	2.7
CHO energy: Protein energy, kcal	3.5	0.02	2.3
Fat energy: Protein energy, kcal	0.6	4.0	0.4

[1] "Rat/Mouse Diet" of Teklad inc., Monmouth, Ill., a list of ingredients including minerals and vitamins was supplied by the manufacturer, together with an "average analysis" on which the above data are based.

[2] C&H Sugar, California and Hawaiian Sugar Co., San Francisco, Calif.

[3] Silverleaf (oxygenated), Swift & Co., Chicago, Ill.

[4] Mazzola, Corn Products, Inc., New York, N.Y.

[5] Mixture of 5 mg vitamin A palmitate (250,000 USP units/g), 0.6 mg vitamin D_2 (500,000 USP units/g) and 20 mg vitamin E succinate.

Reagents

The following chemicals, of reagent or analytical grade, were purchased from Fisher Scientific Company, Fair Lawn, New Jersey: sodium chloride, sodium acetate, sodium hydroxide, potassium chloride, trichloroacetic acid, magnesium chloride, orcinol, xylene, and liquified phenol.

Toluene, scintillation grade, and naphthalene were obtained from Eastman Kodak Company, Rochester, New York.

P-dioxane, ethylene glycol monoethylether and isoamyl alcohol

were obtained from Baker Chemical Company, Phillipsburg, New Jersey.

Sodium dodecyl sulfate (SDS) and polyvinyl sulfate (PVS) were purchased from K and K Laboratories, Inc., Plainview, New York.

Pure U.S.P. ethyl alcohol was obtained from U.S. Industrial Chemicals Company, New York.

Orcinol and deoxycholate (DOC) were obtained from Sigma Chemical Corporation, Saint Louis, Missouri.

^{32}P as $H_3{}^{32}PO_4$ in HCl-free water was purchased from New England Nuclear, Boston, Mass.

Yeast RNA and ribonuclease A were obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

Instruments and Equipment

Centrifugations were carried out with a Sorvall automatic refrigerated centrifuge, RC2-B, Ivan Sorvall Inc., Norwalk, Connecticut and with a Spinco Model L2-65B ultracentrifuge, Beckman Instruments Company, Spinco Division, Palo Alto, California. The water bath used was a Metabolyte water-bath shaker with temperature control, Model G77, New Brunswick Scientific Company, Inc., New Brunswick, New Jersey. Spectrophotometric measurements were made using a Gilford Model 2400 from Gilford Instrument Laboratories, Inc., Oberlin, Ohio. Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer, Model 3214.

Methods

Preparation of the Animals

Male, albino rats of the Holtzman and Sprague-Dawley strains

were raised on laboratory chow diet with free access to food and water. They were caged separately in a room with controlled temperature and light periods. After the animals had reached a weight of about 350-400 grams, groups of six rats were put on a 5-7 day starvation period. During this period they had free access to water. These rats were then refed with either a high sucrose (HS) or a high fat (HF) diet for 4, 8, or 24 hours before sacrifice. Rats which ate less than 3 grams during the first 2 hours of refeeding were rejected. All rats were killed between 8:30 a.m. and 9:15 a.m. to minimize diurnal variations.

Preparation of total RNA

Rats were killed by decapitation with a guillotine and livers were removed immediately, wiped clean, weighed and dropped into liquid nitrogen. The frozen livers were lyophylized for 24 hours and crushed into a fine powder in a cold room. Normally, liver powder obtained from three rats was pooled for the extraction of RNA. Ribonucleic acids were extracted as described below. Unless otherwise specified, all of the following processes were carried out at a temperature close to $0^{o}C$.

Cold sodium acetate buffer 0.02 M, pH 5.2, containing 200 µg/ml of PVS (in the amount of 3 ml per gram of wet liver) was added to the powdered liver and stirred with a magnetic stirrer in an ice bath for 5 minutes. An equal volume of cold water-saturated phenol was then added and stirred again for 10 minutes. The phases were separated by centrifugation at 27,000 x g for 10 minutes in a Sorvall centrifuge at a temperature of $4^{o}C$. The aqueous phase, containing the RNA, was

harvested with a Pasteur pipette. The interphase and phenol phase were given a second cold extraction with the original volume of acetate buffer. The aqueous phases from the two cold extractions were pooled. The residual RNA left after the second cold extraction was extracted using the original volume of acetate buffer, but now containing 0.5% SDS and was heated up to 63°C in a boiling water bath. The mixture was cooled immediately in an ice bath. The aqueous phase was harvested after centrifugation. Fresh phenol was used for a second hot extraction and the aqueous phase again isolated. The aqueous phases from two hot extractions were pooled.

The hot and cold aqueous phases were then deproteinized separately by three extractions (by stirring for 5 minutes in an ice bath) with 0.5 volume of phenol, followed by two extractions with 0.5 volume of chloroform-isoamyl alcohol (4:1 v/v).

Precipitation of RNA

Ribonucleic acids were precipitated by adding 0.25 volume of cold 2.5 M NaCl and 2.5 volumes of ethanol, pre-chilled to -20°C. The ethanol mixture was kept at -20°C overnight.

Recovery of RNA

Ribonucleic acids were recovered by centrifugation in a Sorvall centrifuge at 27,000 x g for 15 minutes. The ethanol was discarded. The tubes were wiped dry of ethanol and the RNA pellet was dissolved in 2 ml of cold water. The optical density of the RNA was determined at 260, 280, and 232 nm to make sure it was free of proteins. Additional washings with chloroform-isoamyl alcohol were given, if the

optical density at 232 nm was more than half of the optical density at 260 nm. As a criteria of purity, the optical densities of yeast RNA at 232, 260 and 280 nm were determined and the optical density at 260 was found to be twice that of the optical density at 280 or 232 nm.

Gradient Fractionation of RNA

Convex isokinetic sucrose gradients (15 to 32.8%) were made according to Noll (1967). About 200 µg of RNA was layered on the gradient and centrifuged at 2°C for 10 and 1/2 hours at 198,759 x g using an SW 40 Ti rotor. The optical density patterns of these gradients were monitored at 260 nm by displacing them from the bottom with 50% sucrose solution and passing them through a 5 mm continuous flow-through cell, using a Gilford spectrophotometer.

Isolation of ribosomes for extraction of rRNA

Ribosomes from the livers of starved or starved-refed rats were isolated according to Egly et al. (1972). Livers were rinsed in 0.25 M sucrose, blotted, weighed and homogenized in 2 volumes of pH 8.0 buffer (TEA 20 mM; KCl 50 mM; MgAc 4 mM; sucrose 0.25 M). The homogenates were centrifuged at 20,000 x g for 15 minutes. The post-mitochondrial supernatant layers were diluted 2.5 times with TEA buffer and centrifuged at 78,000 x g in a No. 30 rotor for 143 minutes. The pellet from 25 gm liver tissue was suspended in 12 ml of TEA buffer and diluted to 36 ml with the same buffer. Deoxycholate was added to a final concentration of 1%. Four and one-half ml of the DOC treated homogenate were layered on top of a discontinuous gradient, comprised of 2 ml each of 1.7 and 1.3 M sucrose and centrifuged at 105,000 x g

for 17 hours at 2°C in a No. 40 Ti rotor.

The ribosomes were found as a pellet at the bottom of the tube, while the interphase between 1.3 and 1.7 M sucrose contained particles referred to as informosomes by Egly et al. (1972). Ribonucleic acids from the ribosomal pellets were extracted, purified and fractionated as previously described for total RNA.

Isolation of ribosomes for ribonuclease estimations

Ribosomes were isolated according to the method of Tashiro and Siekevitz (1965). The livers were homogenized in 2.5 volumes of cold solution A (see Appendix) with eight up and down strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 x g for 15 minutes. The postmitochondrial supernatant was centrifuged at 105,000 x g for 2 hours. The microsomal pellet from 10 grams liver tissue was suspended by homogenization in 10 ml of solution B (see Appendix) and DOC was added to a final concentration of 0.5 %. The suspension was centrifuged at 105,000 x g for 90 minutes. The ribosomal pellet was rinsed twice with solution C (see Appendix), suspended by homogenization in 10 ml of solution C and centrifuged at 13,500 x g fo 10 minutes. Magnesium chloride was added to the supernatant layer to a final concentration of 0.05 M. Five minutes later it was centrifuged at 6,000 x g for 10 minutes. The pellet contained purified ribosomes which were suspended in 10 ml of solution C and stored at 4°C overnight.

Isolation of microsomal RNA

Microsomes isolated as described above were used in some of

the experiments to extract microsomal RNA.

Extraction of Microsomal RNA by the use of Pronase

A 2 mg/ml solution of Pronase in solution B was incubated at 37°C for 3 hours to remove any ribonuclease contamination. This solution was filtered and kept frozen until used. The microsomal pellets from one rat liver were suspended in 10 ml of sodium-acetate (pH 5.2) buffer. Five-tenths ml of 10 % SDS (final concentration of SDS, 0.5%) and 0.5 ml of the Pronase (2 mg/ml) solution (final concentration of Pronase 100 μg/ml) were then added to the microsomal suspension and the mixture was gently stirred for 30 minutes at room temperature. Ribonucleic acids were then extracted in two cold (0°C) extracts, using sequentially 10 ml and 5 ml of cold water-saturated phenol. The rest of the procedure was essentially the same as described for total RNA, except that hot extraction was not used.

Assay for Ribonuclease

The method of Gavard and Lamirande (1972) was used with some modifications. The incubation system consisted of 200 μmoles of Tris-HCl buffer, pH 8.0 ; 100 μmoles of ethylenediamine-tetraacetate (EDTA), pH 7.0 ; 1 mg of RNA and 0.5 ml sample of ribosomes in a total volume of 5 ml (see Appendix for details). The tubes were incubated at 37°C for 1 hour. One ml aliquots were removed and the reaction was stopped by adding 2.0 ml of cold 5 % $HClO_4$. After centrifugation at 10,000 x g for 10 minutes the optical density at 260 nm was monitored in order to determine acid-soluble nucleotides. In another series of experiments, ^{32}P-labelled rRNA, isolated from the livers of starved and normal rats

was used as substrate for ribosomes isolated from starved and control rats. In these experiments radioactivity released into the acid-soluble fraction was used as an index of ribosomal ribonuclease activity.

Determination of Buoyant Density of Ribosomes

Ribosomes isolated by the method of Tashiro and Siekevitz (1965) were fixed in formaldehyde (2 % final concentration) for 2 hours in an ice bath. Four to five OD_{260} units of the fixed ribosomes were layered on top of a linear cesium chloride gradient (density range 1.25 to 1.65) and centrifuged at 199,000 x g for 16 hours at 10°C, using an SW 56 Ti rotor. Optical density was monitored at 260 nm by displacing the gradient from the bottom with 57 % cesium chloride and passing it through a 5 mm continuous flow-through cell using a Gilford spectrophotometer. Aliquots of 2 drops were collected and the densities were determined by weighing 100µl fractions out of every fifth aliquot.

Quantitative Estimation of Ribosomal RNA and Protein

The amount of ribonuclease was expressed on the basis of mg of rRNA and mg of ribosomal protein. For this purpose rRNA was estimated by using the orcinol reaction of Mejbaum (1939) with yeast RNA as a standard. Protein was quantitated by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Studies involving the rate and amount of RNA synthesized after refeeding

In order to estimate the rate of RNA synthesis during the

time of refeeding, ^{32}P as phosphoric acid in HCl-free water was injected intraperitonially 22 hours or intravenously 75 minutes prior to sacrifice. Food was offered for 4 or 8 hours before killing. To find the specific activity of the RNA, 4 OD_{260} units of RNA solution were placed on a filter paper strip, washed sequentially with cold 10 % TCA, cold 5 % TCA, cold ethanol plus ether (50:50) and cold ether. The ether was allowed to evaporate and the strips were put into 10 ml volumes of toluene scintillation fluid (composition given in Appendix) and counted. In experiments with ribonuclease, 0.5 ml aliquots were taken directly for counting using 10 ml volumes of Bray's counting mixture.

In experiments where the specific activities of individual RNA species were determined, 0.6 ml aliquots from the gradients were collected into separate tubes and 2 mg of bovine serum albumin were added as a carrier. The RNA-albumin mixture was precipitated with cold 10 % TCA, followed by washing with cold 5 % TCA. The pellets were dissolved in 0.5 ml of ammonium hydroxide and counted in 10 ml volumes of Bray's counting mixture. (Composition of this counting mixture given in Appendix).

Statistical Analysis of the Data

Comparison among different groups was made by using Student's t test. Different values of P (0.001 to 0.05) were used to test the significance of the difference among different groups. The actual values of P used to test the significance of differences are given in the footnotes of the tables Values of P higher than 0.05 were considered to represent non-significant (NS) differences.

CHAPTER III

RESULTS

Period of Starvation and RNA Profiles

The first experiment was designed to see if starvation caused any change in the RNA profiles. Rats were starved for 5 or 7 days and the liver RNA was extracted. About 65 % of the total RNA was extracted at 0°C and the residual RNA was extracted by heating (63°C). No differences were found between the optical density profiles of the RNA extracted at 0°C from continuously-fed, 5-day starved or 7-day starved rats. Ribonucleic acid profiles from continuously-fed and 7-day starved rats are shown in Figures 1a and 1b, respectively. Figure 2 shows the profiles of the RNA extracted by heating from the livers of 5 and 7-day starved rats. No differences were found in the profiles of the RNA extracted with heat after 5 days of starvation when compared with continuously-fed rats. After 7 days of starvation, there was a marked reduction in the amount of RNA sedimenting at 18S and 28S with a concomitant relative increase in the amount of RNA sedimenting below 18S. Thus, this experiment showed that the RNA extracted by heating from the livers of 7-day starved rats was degraded. No degradation of the RNA extracted by heating from the livers of 5-day starved rats or the RNA extracted at 0°C from the livers of both 5 and 7-day starved rats was noticed.

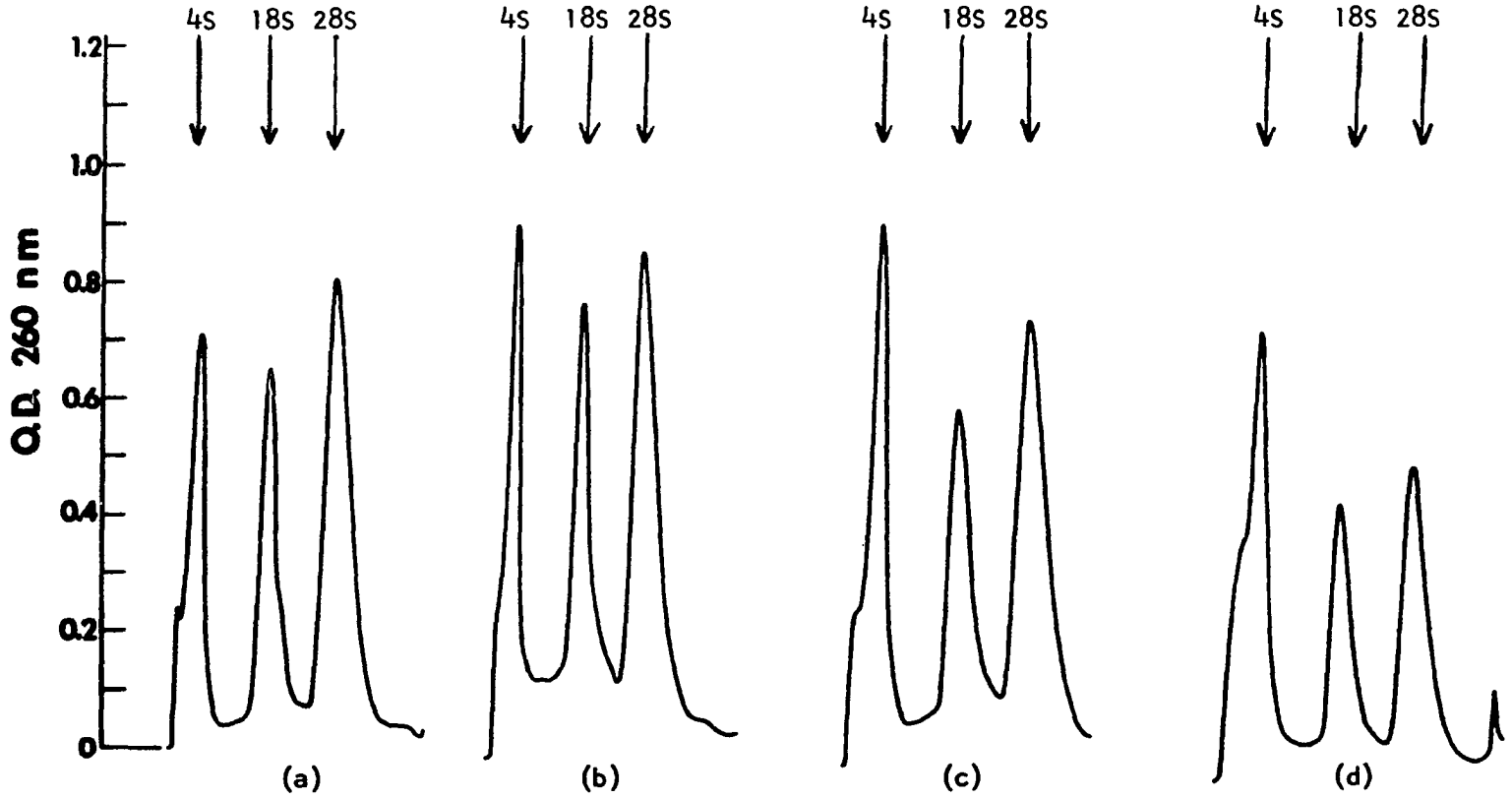

Figure 1. Optical density profiles of total rat liver RNA extracted at 0°C.

(a) Represents 6 continuously chow diet fed rats.

(b) Represents 9 seven-day starved rats.

(c) Represents 11 seven-day starved 4 hours HS diet refed rats.

(d) Represents 8 seven-day starved 4 hour HF diet refed rats.

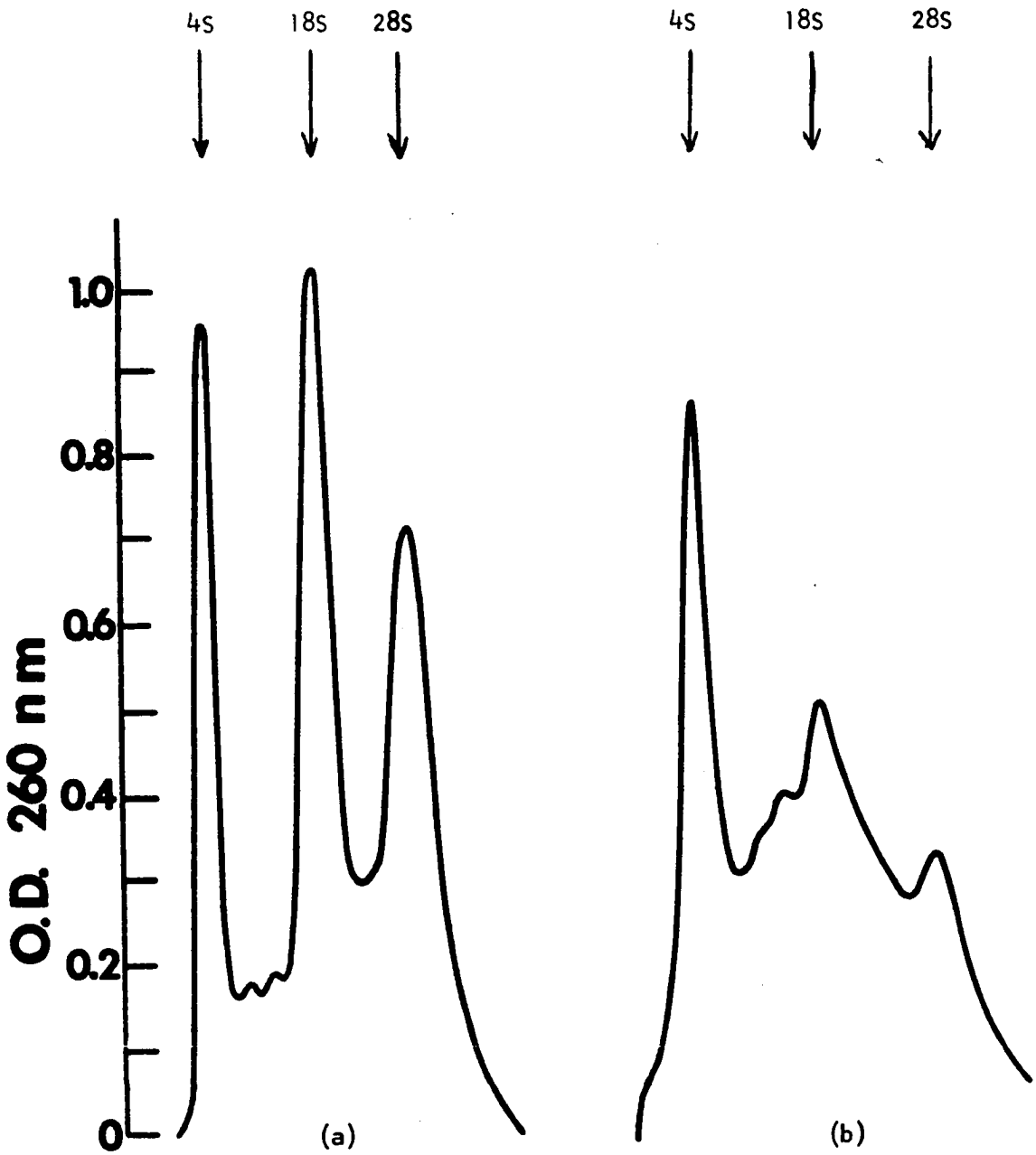

Figure 2. Optical density profiles of total rat liver RNA extracted by heating to 63°C.

(a) Represents 3 five-day starved rats.

(b) Represents 9 seven-day starved rats.

Recovery of RNA profiles after refeeding the 7-day starved rats

The next series of experiments were planned to find the period of time required to restore the RNA profiles to normal after refeeding. In our earlier studies with polysomal profiles we compared the HF versus HS diets, hence both these diets were again compared in regard to their ability to restore the RNA profiles. Seven-day starved rats were refed either HF or HS diet for 4 or 8 hours. The effects of refeeding these diets on RNA profiles are shown in Figures 1 and 3. Figure 1 shows the profiles of the RNA extracted at 0°C from 4 hour HS or HF diet refed rats in comparison to the RNA profiles of control and 7-day starved rats. Perusal of Figure 1 reveals no differences among the profiles of RNA extracted at 0°C from the control, 7-day starved and starved-refed rats. Four hour refeeding of the HS diet restored the profiles of the RNA extracted by heating to normal (Figure 3b). Figure 3c shows that 4 hour refeeding of HF diet to the 7-day starved rats caused a marked increase in the amounts of RNA sedimenting at 18S and 28S, and a decrease in the amount of RNA sedimenting below 18S, as compared to the profiles of heat extracted RNA from the livers of 7-day starved rats (Figure 2b). These profiles (Figure 3b) were, however, not entirely normal. Eight hour refeeding of the HF diet to the 7-day starved rats was required to restore the RNA profiles entirely to normal (Figure 3d).

Starvation and Ribosomal RNA

In the earlier experiment when RNA from the livers of 7-day starved rats was extracted by heating a marked degradation of the RNA sedimenting at 18S and 28S along with an increase in the amount of RNA

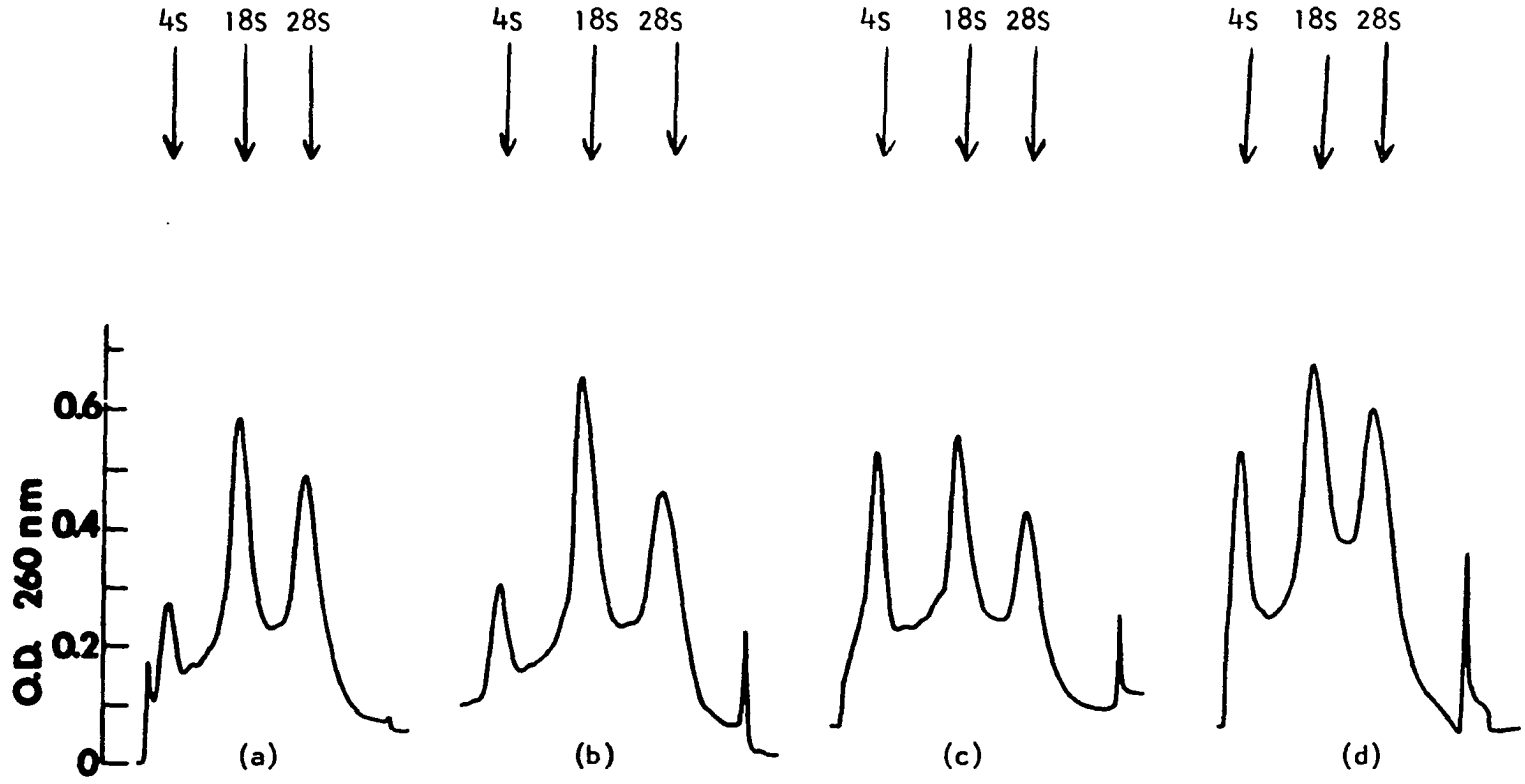

Figure 3. Optical density profiles of total rat liver RNA extracted by heating to 63°C.

(a) Represents 6 continuously chow diet fed rats.

(b) Represents 11 seven-day starved 4 hour HS diet refed rats.

(c) Represents 8 seven-day starved 4 hour HF diet refed rats.

(d) Represents 5 seven-day starved 8 hour HF diet refed rats.

sedimenting below 18S was noticed. This suggested that the ribosomal RNA from 7-day starved animals was degraded on extracting with heat. Therefore, in the next series of experiments, RNA was extracted from isolated ribosomal fractions. Ribosomes from starved and refed animals were isolated by a procedure involving DOC, and rRNA was extracted at 0 and 63°C. The profiles of the 0°C extracted rRNA from the ribosomes of starved and 4 hour, HS diet, refed rats are shown in Figure 4. In contrast to the earlier experiments with total RNA, there were marked differences in the profiles of rRNA extracted at 0°C from the ribosomes of starved (Figure 4a) and refed animals (figure 4b). The 18S and 28S peaks of the 7-day starved rats were markedly lower than that of the 4 hour, HS diet, refed rats. The profiles of the heat-extracted rRNA from the ribosomes of starved and 4 hour HS diet refed rats are shown in Figure 5. There were no differences in the rRNA profiles of starved and refed rats. The rRNA profiles from both starved and refed rats were markedly degraded. No 18S and 28S peaks existed and all of the rRNA was found to sediment below 18S. These results showed that rRNA from refed rats was also degraded, although to a lesser extent, than the rRNA from starved rats. The degradation of rRNA extracted at 0°C was probably due to prolonged DOC treatment and will be discussed later.

In the first experiment, the RNA extracted from the livers of 7-day starved rats at 0°C was not degraded while the RNA extracted by heating was degraded. In this experiment using DOC, there was degradation of the rRNA extracted both at 0°C and 63°C, from the livers of both starved and starved-refed rats. These results suggested : (1) The extent of degradation of RNA was dependent upon how drastic the

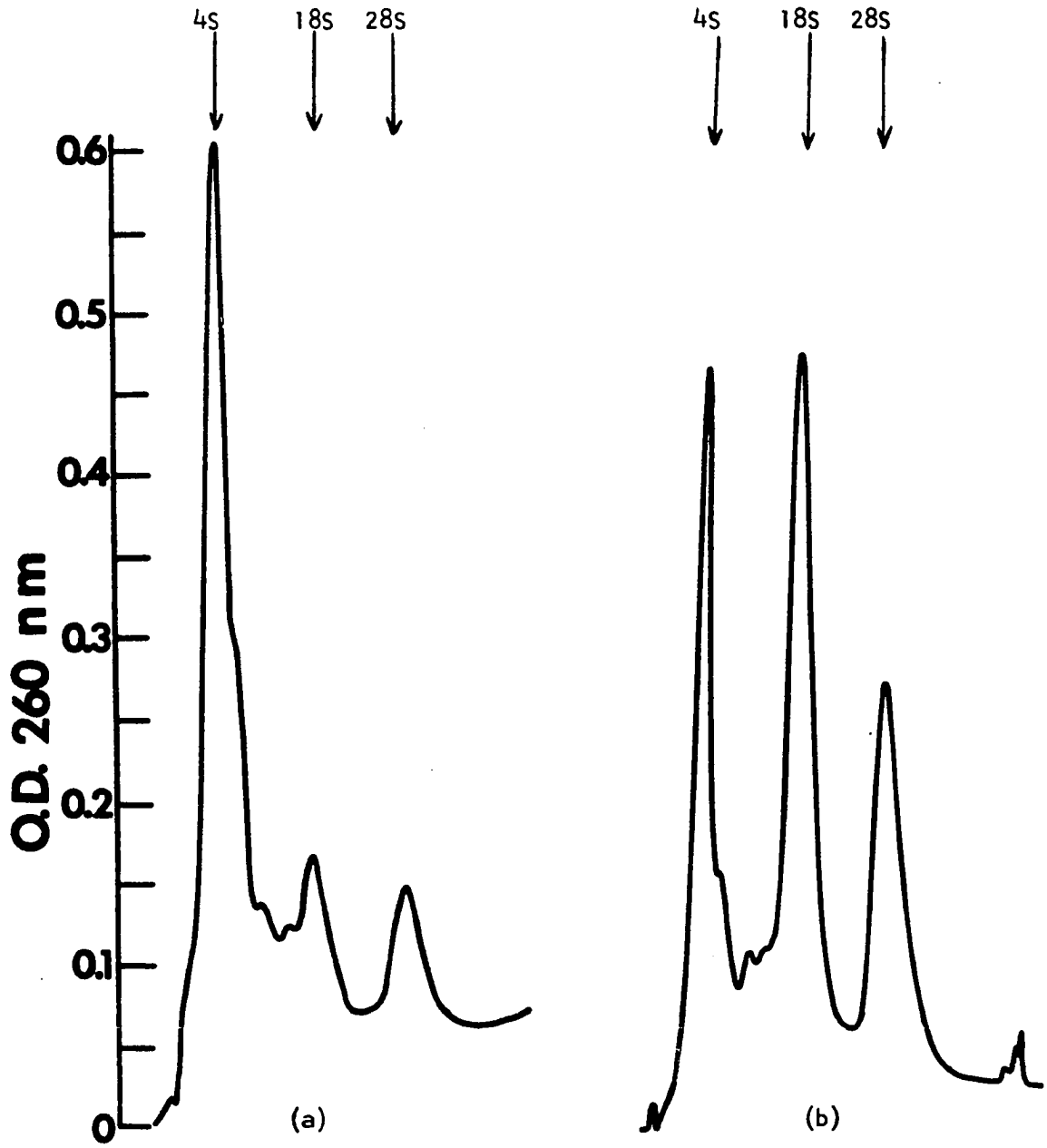

Figure 4. Optical density profiles of the rRNA extracted at 0°C from the DOC-treated ribosomes of rat liver.

(a) Represents 6 seven-day starved rats.

(b) Represents 8 seven-day starved 4 hour sucrose diet refed rats.

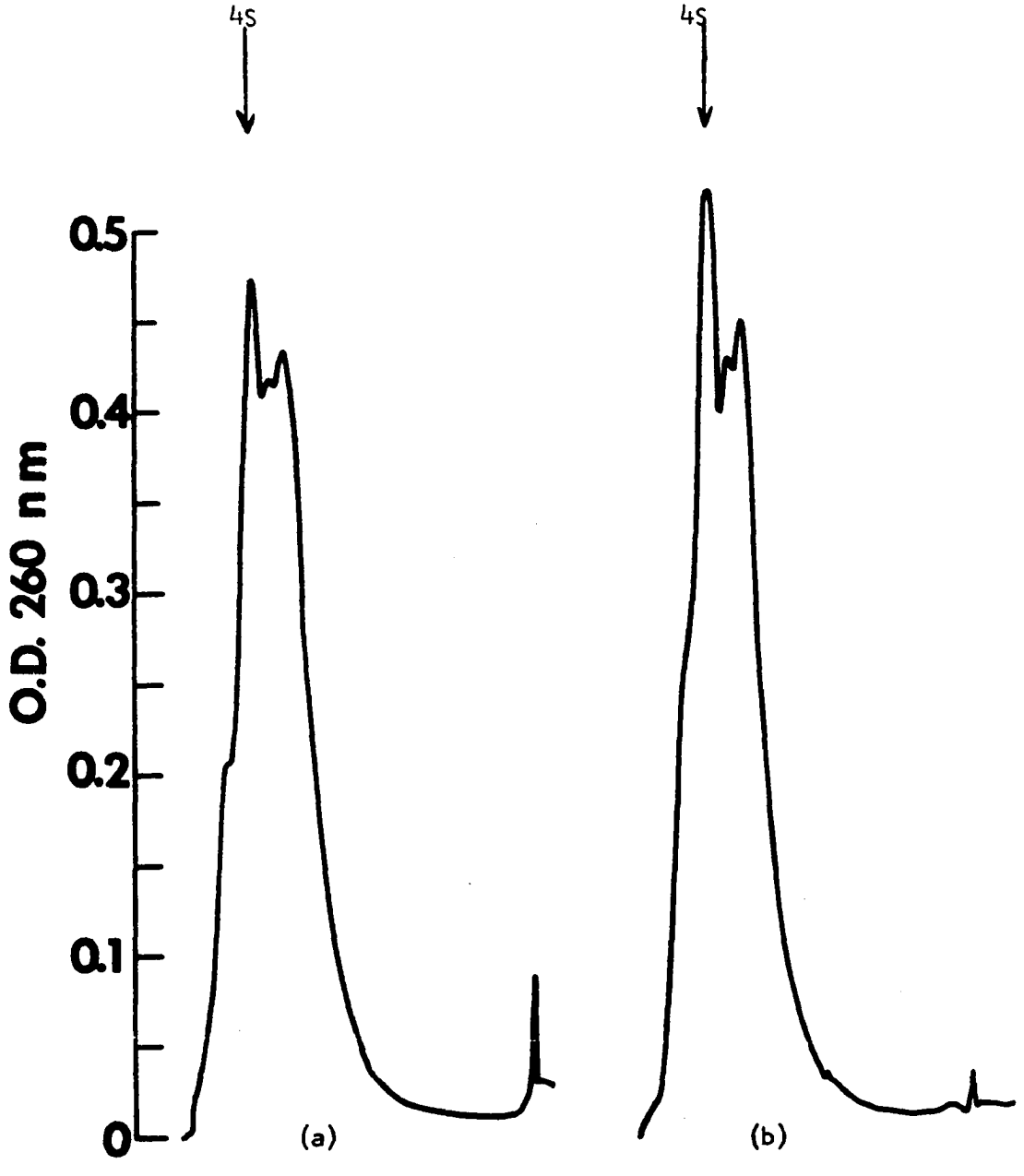

Figure 5. Optical density profiles of the rRNA extracted by heating to 63°C from the DOC-treated ribosomes of rat liver.

(a) Represents 6 seven-day starved rats.

(b) Represents 8 seven-day starved, 4 hour HS diet refed rats.

extraction conditions were, and (2) the RNA from the livers of starved rats was more susceptible to degradation as compared to the RNA from the livers of continuously fed or starved-refed rats.

Starvation and Ribosomal Ribonuclease

Arora and Lamirande (1971) reported results contradicting those of Sox and Hoagland (1966) claiming that the increased autodegradation of ribosomes from starved animals was due to an increased level of ribosomal ribonuclease during starvation. In an attempt to resolve this controversy and to see if the degradation of RNA from the livers of starved animals could be attributed to an increased activity or amount of ribosomal ribonuclease, the next series of experiments were planned to examine the activity of ribosomal ribonuclease in the liver ribosomes of starved and normal animals. Table I shows the results when yeast RNA was used as a substrate for the ribosomal ribonuclease. Ribosomes from the livers of starved animals were found to be at least two fold more susceptible to autodegradation (i.e. when incubated without yeast RNA) than ribosomes from the livers of normal animals. When incubated with yeast RNA as a substrate, the ribosomes from the livers of starved animals again showed the release of significantly higher amounts of acid-soluble nucleotides as compared to the ribosomes from the livers of normal animals. These values represented the sum of autodegradation and endogenous ribonuclease activity. However, if the autodegradation values were deducted from the total, the level of endogenous ribonuclease was found to be the same in the ribosomes from the livers of both normal and starved animals.

TABLE I

RIBOSOMAL RIBONUCLEASE ACTIVITY MEASURED BY RELEASE OF ACID-SOLUBLE NUCLEOTIDES FROM YEAST RNA

Ribosomes	Number of animals	Per mg of ribosomal RNA			Per mg of ribosomal protein		
		a Autodegradation + Endogenous RNase	b Autodegradation	c Endogenous RNase	d Autodegradation + Endogenous RNase	e Autodegradation	f Endogenous RNase
Normal	7	1.57 ± 0.11	0.34 ± 0.06	1.32 ± 0.11	3.67 ± 0.24	0.73 ± 0.10	2.92 ± 0.22
7 day starved	7	1.97 ± 0.09	0.75 ± 0.08	1.31 ± 0.08	4.79 ± 0.22	1.82 ± 0.21	2.94 ± 0.11

Each of the values represents the mean $\pm$ standard error of the mean of OD_{260} units of acid soluble nucleotides released from yeast RNA, during 1 hour of incubation with ribosomes. Figures within each column are compared. For columns c and f the difference is non-significant, for column a $0.01 < P < 0.02$, for columns b and d $0.001 < P < 0.005$ and for column e $P < 0.001$.

Since yeast RNA is not the natural substrate for ribosomal ribonuclease. these results could not be taken as conclusive evidence to rule out the increased ribonuclease level during starvation. Therefore, in the next series of experiments ^{32}P-labelled rRNA isolated from the livers of starved and normal rats was used as a substrate for the ribosomes from the livers of starved and normal rats. The radioactivity released into the acid-soluble fraction was taken as an index of ribonuclease activity. The results shown in Tables 2 and 3 revealed no significant differences in the CPM of ^{32}P released into the acid-soluble fraction by the ribosomes from the livers of starved and normal rats. These results support the earlier findings, when yeast RNA was used as a substrate for ribosomal ribonuclease. The ribosomal ribonuclease activity per mg of rRNA or ribosomal protein was not increased during starvation, even though the ribosomes from the livers of starved animals were at least two-fold more susceptible to autodegradation as compared to ribosomes from the livers of normal rats.

Feeding Regimens and Liver RNA Concentrations

The recovery of RNA profiles after refeeding starved animals could be due to de novo RNA synthesis or the synthesis or repair of some other cellular constituents, such as structural ribosomal proteins or endoplasmic reticular membranes etc, which shield the RNA from the drastic extraction conditions and thus reduce the amount of degradation. To determine whether the recovery was due to de novo RNA synthesis, the liver RNA concentrations under these feeding regimens were investigated and are presented in Table 4. The difference between

TABLE 2

RIBOSOMAL RIBONUCLEASE ACTIVITY AS MEASURED BY THE CPM OF ^{32}P RELEASED FROM NORMAL RAT LIVER RIBOSOMAL RNA

Ribosomes	Number of Animals	Per µg of ribosomal RNA	Per µg of ribosomal protein
Normal	5	46.88 ± 2.39	113.22 ± 6.01
7 day starved	5	46.39 ± 3.17	113.39 ± 4.05

Each of the values represents the mean $\pm$ the standard error of the mean of the CPM of ^{32}P released from normal rat liver rRNA, during 1 hour of incubation with ribosomes. Figures within each column are compared and are not significantly different.

TABLE 3

RIBOSOMAL RIBONUCLEASE ACTIVITY AS MEASURED BY THE CPM OF ^{32}P RELEASED FROM STARVED RAT LIVER RIBOSOMAL RNA

Ribosomes	Number of Animals	Per µg of ribosomal RNA	Per µg of ribosomal protein
Normal	5	51.1 ± 3.95	123.35 ± 9.93
7 day starved	5	54.13 ± 2.71	144.60 ± 10.25

Each of the values represents the mean $\pm$ the standard error of the mean of the CPM of ^{32}P released from 7 day starved rat liver rRNA during 1 hour of incubation with ribosomes. Figures within each column are compared and are not significantly different.

TABLE 4

MICROSOMAL RNA CONCENTRATIONS OF RAT LIVER
FOLLOWING VARIOUS DIETARY MANIPULATIONS

Group	Number of Animals	Initial body weight gm	Body weight after starvation gm	Body weight at sacrifice gm	Liver weight gm	mg RNA per liver a	mg RNA per gm liver b	mg RNA per 100 gm final body weight c
Normal	6	364±3	-	-	11.0±0.5	23.33±1.04	2.14±0.09	6.43±0.30
5 day starved	4	360±3	279±4	-	6.6±0.2	13.60±0.12	2.06±0.02	4.90±0.08
7 day starved	4	361±5	263±1	-	5.8±0.3	11.37±1.26	1.95±0.13	4.31±0.46
8 day starved	3	373±2	245±6	-	4.1±0.7	9.75±2.12	2.36±0.11	3.97±0.78
4 hour HS diet refed	5	369±3	269±3	278±3	6.8±0.5	13.37±1.52	1.98±0.16	4.50±0.59
4 hour HF diet refed	5	363±4	266±6	272±9	7.2±0.2	12.56±1.02	1.64±0.09	4.20±0.18
8 hour HF diet refed	5	365±4	267±4	280±4	6.7±0.3	13.20±0.36	1.92±0.08	4.54±0.15

TABLE 4

continued

Refed groups were all 7-day starved prior to refeeding. Figures in the table represent the mean ± the standard error of the mean. Figures within each column are compared under columns a and c, the normal group is significantly different from all other groups at P <0.001. All groups under column b are not significantly different.

the RNA content among 5, 7 and 8 day starved rats were non-significant when tested with Student's t test. However, when these differences were tested with the least squares method, the best fit line had a slope of - 5.2, which was significant at $P<0.05$. This analyses showed that between the 5th and 8th day of starvation the daily loss in liver RNA content was still significant.

The amount of RNA per total liver or per 100 gm final body weight was not significantly different among the 7-day starved or starved-refed groups ; however, all groups were significantly lower than the normal. When the RNA concentrations were expressed per gm liver, the differences in the RNA concentrations among all groups were non-significant. Seven day starvation decreased the liver RNA content to about 50 % of normal. The RNA content was about 60 % of normal after 5 days of starvation or 4 hours after refeeding the 7-day starved rats. These results did not show any correlation between the changed RNA profiles and RNA content, suggesting that the recovery of RNA profiles did not involve the de novo synthesis of rRNA but that it may involve the de novo synthesis of mRNA or the synthesis or repair of the other factors listed earlier.

Use of ^{32}P to determine the amount and type of RNA Synthesized during different dietary regimens

The amount of RNA newly synthesized during refeeding was investigated by injecting ^{32}P into rats, 22 hours before sacrifice. Table 5 shows that the specific activity of liver microsomal RNA was significantly lower for the 7-day starved group than the normal or refed groups. The specific activity of the normal group was about three times higher than the starved group while those of the refed groups

TABLE 5

SPECIFIC ACTIVITY OF MICROSOMAL RNA, 22 HOURS AFTER INTRAPERITONIAL INJECTION OF ^{32}P 0.5 mCi/100 gm BODY WEIGHT

Group	Number of Animals	RNA extracted at 0°C CPM per mg x 10^{-4}	RNA extracted by heating to 63°C CPM per mg x 10^{-4}
Normal	4	12.60 ± 0.99	14.21 ± 1.41
7 day starved	3	4.66 ± 0.08	4.20 ± 0.04
7 day starved 4 hour HS diet refed	4	25.48 ± 1.40	29.35 ± 2.75
7 day starved 4 hour HF diet refed	4	29.97 ± 1.78	33.61 ± 3.58

The figures in the table represent the mean $\pm$ the standard error of the mean. Figures within each column are compared. Under both the columns the starved group is significantly different than all other groups at $P < 0.001$. The difference between 4 hour HS or HF diet refed groups is non-significant under both the columns.

were 6 times higher than the starved. The differences between the specific activities of the groups fed either HS or HF diet being non-significant. During the same period (22 hours) the incorporation of ^{32}P into microsomal RNA per total liver was about five times greater in the normal group than in the starved group. Four-hour refeeding of the 7-day starved rats caused about six fold increase in the total amount of ^{32}P incorporated into liver microsomal RNA. The differences between the groups fed either HS or HF diet were again non-significant (Table 6).

The RNA concentrations of the starved and starved-refed groups were not significantly different (Table 4), but the specific activity and total incorporation of ^{32}P for the refed groups were about 6 times higher tham those of the starved group. These results suggested that changed RNA profiles did not involve the RNA species with a slow turnover rate but may have involved some rapidly turning-over RNA species. This required the short-term labelling of RNA and investigation of the distribution of radioactivity under individual species of RNA. A valid comparison of the distribution of radioactivity under individual species of RNA among different groups could not be made until the optical density profiles from all the groups were similar (i.e. there was no degradation of 18S and 28S peaks). An extraction procedure which would allow the complete extraction of RNA without drastic extraction conditions such as heat and DOC was required. This was achieved by the use of an extraction procedure involving pre-treatment of the microsomal fraction with Pronase. In this procedure all of the RNA was extracted at $0^{o}C$ (without heat and DOC). The optical density and radioactivity profiles of microsomal RNA extracted from the livers of different groups, 75 mi-

TABLE 6

INCORPORATION OF ^{32}P INTO MICROSOMAL RNA PER WHOLE LIVER, 22 HOURS AFTER INTRAPERITONIAL INJECTION OF 0.5 mCi ^{32}P/100 gm BODY WEIGHT

Group	Number of Animals	RNA extracted at 0°C CPM x 10^{-5}	RNA extracted by heating to 63° CPM x 10^{-5}
Normal	4	17.59 ± 2.69	8.72 ± 0.98
7 day starved	3	3.84 ± 0.02	1.85 ± 0.03
7 day starved 4 hour HS diet refed	4	24.73 ± 1.09	10.03 ± 0.47
7 day starved 4 hour HF diet refed	4	23.54 ± 2.39	7.57 ± 0.81

The figures represent the mean $\pm$ the standard error of the mean. Figures within each column are compared. Under both the columns the starved group is significantly different from all other groups at $P < 0.001$. The difference between the 4 hour HF or HS diet refed groups is non-significant under both the columns.

nutes after intravenous injection of ^{32}P are shown in Figure 6. The optical density profiles of microsomal RNA did not show any major differences among the different groups. However, there was a small peak between the 4S and 18S regions of the starved and refed animals which was missing in the normal animals. In all groups of animals the incorporation of radioactivity was the highest in the 4S peak, followed by 4S-18S, 18S and 28S. The relative distribution of the radioactivity among individual RNA species 75 minutes after intravenous injection of ^{32}P, 0.3 mCi/100 gm body weight, is presented in Table 7. The specific activity of all species of microsomal RNA from the 8 hour, HF diet, refed group was significantly higher than that of the corresponding species of the starved group. The differences between the starved group and the normal were non-significant. Table 8 shows that during refeeding the synthesis of all species of RNA was markedly increased but the highest increase was in the 4S-18S and 18S region (normally called mRNA region). To calculate the total amount of the newly synthesized RNA, results in Table 9 are presented as total incorporation of ^{32}P per liver RNA of each kind, and a comparison is made among different groups in Table 10. The differences between the starved and normal group were not significant, while the refed group had 2.3 times more total incorporation of ^{32}P into total microsomal RNA as compared to the starved group. The relative increase in the amount of mRNA after refeeding being again higher than in the amount of rRNA. These results showed that refeeding of the starved rats increased the synthesis of both mRNA and rRNA, but the synthesis of mRNA was turned on to a greater extent.

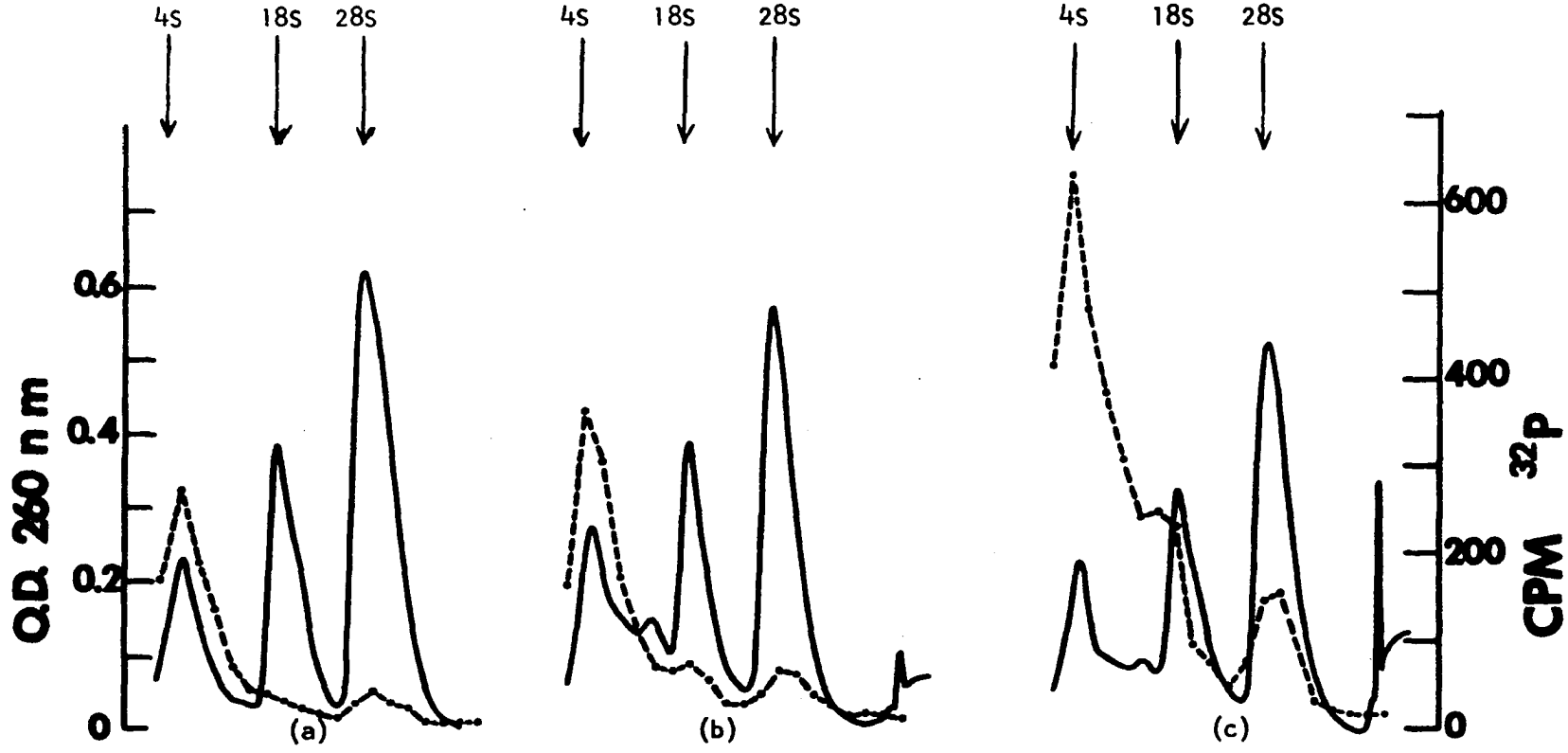

Figure 6. Optical density and radioactivity profiles of rat liver microsomal RNA extracted from the microsomes pretreated with Pronase ; ——— represents the optical density, -------- represents the radioactivity.

(a) Represents 7 continuously chow diet fed rats.

(b) Represents 9 seven-day starved rats.

(c) Represents 6 seven-day starved 8 hour fat diet refed rats.

TABLE 7

THE RELATIVE DISTRIBUTION OF RADIOACTIVITY AMONG INDIVIDUAL SPECIES OF RNA, 75 MINUTES AFTER INTRAVENOUS INJECTION OF 0.3 mCi ^{32}P/100 gm BODY WEIGHT

Group	Number of animals	CPM put on the gradient	CPM in individual peak regions			
			4S region	4S-18S region	18S region	28S region
		a	b	c	d	e
Normal	3	1372±207 (100%)	696±73 (50.7%)	296±78 (21.6%)	151±29 (11.0%)	201±40 (14.7%)
7 day starved	4	1691±244 (100%)	876±136 (51.8%)	334±49 (19.8%)	201±36 (11.9%)	248±29 (14.7%)
7 day starved 8 hour fat diet refed	3	3725±308 (100%)	1543±43 (41.4%)	931±149 (25.0%)	648±120 (17.4%)	551±9 (14.8%)

Figures represent the mean ± standard error of the mean. Figures without parenthesis within each column are compared. For all the columns the difference between starved and normal groups is non-significant. The difference between starved and refed groups under columns a and e is significant at $P < 0.005$ and under columns b, c, d, at $P < 0.01$. Figures within parenthesis represent the percentage distriubtion of radioactivity, assuming 100 percent for column a.

TABLE 8

COMPARISON OF THE AMOUNTS OF ^{32}P INCORPORATED INTO INDIVIDUAL SPECIES OF MICROSOMAL RNA 75 MINUTES AFTER INTRAVENOUS INJECTION OF ^{32}P, 0.3 mCi/100 gm BODY WEIGHT

Group	Total microsomal RNA	4S region	4S-18S region	18S region	28S region
Starved/normal	1.23	1.26	1.13	1.33	1.23
8 hour refed/starved	2.20	1.75	2.79	3.22	2.22

These ratios were calculated from the data given in Table 7.

TABLE 9

INCORPORATION OF ^{32}P INTO INDIVIDUAL SPECIES OF MICROSOMAL RNA PER TOTAL LIVER, 75 MINUTES AFTER INTRAVENOUS INJECTION OF 0.3 mCi ^{32}P/100 gm BODY WEIGHT

Group	Number of animals	Total microsomal RNA CPM x 10^{-5} a	4S CPM x 10^{-5} b	4S-18S CPM x 10^{-5} c	18S CPM x 10^{-5} d	28S CPM x 10^{-5} e
Normal	3	1.48±0.11	0.80±0.02	0.31±0.06	0.16±0.02	0.21±0.03
7 day starved	4	0.97±0.18	0.50±0.13	0.18±0.03	0.12±0.03	0.15±0.03
7 day starved 8 hour HF diet refed	3	2.21±0.18	0.91±0.03	0.55±0.09	0.38±0.07	0.33±0.01

Figures represent the mean ± standard error of the mean. Figures within each column are compared. For all the columns the difference between starved and normal groups is non-significant. The difference between starved and refed groups under columns a, c and e is significant at $P < 0.005$, under column d at $P < 0.01$ and under column b at $P < 0.05$.

TABLE 10

COMPARISON OF THE AMOUNTS OF ^{32}P INCORPORATED INTO MICROSOMAL RNA PER TOTAL LIVER, 75 MINUTES AFTER INTRAVENOUS INJECTION OF 0.3 mCi ^{32}P/100 gm BODY WEIGHT

Group	Total Microsomal RNA	4S	4S-18S	18S	28S
Starved/Normal	0.65	0.62	0.59	0.72	0.71
Refed/Starved	2.29	1.82	3.00	3.31	2.15

These ratios were derived from the data given in Table 9.

Buoyant Densities of the Ribosomes

The buoyant densities of ribosomes were determined to see (1) if the ribosomal preparations were pure ribosomes, (2) if there was any gross change in the RNA to protein ratio of the ribosomes from starved animals. The buoyant densities of the ribosomes from starved and control rats were found to be 1.518 ± 0.013 gm/cm^3 and 1.510 ± 0.005 gm/cm^3 respectively. The difference in the buoyant densities was statistically non-significant. These results showed that the ribosomal preparations were pure and there was not any gross change in the RNA to protein ratio of ribosomes during starvation. However, these results do not necessarily exclude the changes in the individual ribosomal proteins. Two dimensional disc-gel electrophoresis studies need to be conducted to see if one or more of the structural ribosomal proteins are missing or exist in a lower concentration during starvation.

CHAPTER IV

DISCUSSION

Starvation and Liver RNA Content

The microsomal RNA content of the liver was reduced to 59,49 and 42 % of the normal levels after 5, 7 and 8 days of starvation respectively. Similar losses in liver RNA content were reported by Kosterlitz (1947) ; Petermann and Hamelton (1958) ; Wilson and Hoagland (1947) who found that starvation for 5-7 days reduced the RNA content to 50 % of normal. Refeeding HS diet for 4 hour or HF diet for 8 hour to the 7-day starved rats did not cause a significant increase in the liver RNA content. This is in marked contrast to Wilson and Hoagland (1966) who reported a complete recovery of the rRNA within 8-12 hours of refeeding the chow diet to the 4-5 day starved animals ; however, this is probably due to the difference in the ribosomal isolation procedure . In their procedure, the RNA recovered as rRNA was less than 55 % of total RNA. These workers also observed that after 8-12 hours of refeeding the non-ribosomal RNA was still 55 % below normal. It is probable that they were recovering only a part of the ribosomes. Garza et al. (1970) did not get any increase in RNA content after 48 hour refeeding of a protein free, high-carbohydrate diet to the 48 hour starved rats, while refeeding a high protein, carbohydrate-free diet for the same period restored the RNA content to normal. These studies emphasize the fact that

in connection with the recovery of RNA after starvation, the length of starvation and the type of diet refed should not be ignored.

Starvation and RNA profiles

Some of the factors that could lead to increased degradation of RNA during starvation are briefly discussed as follows.

Role of Ribosomal Ribonuclease

An increase in the ribosomal ribonuclease activity or amount during starvation is the most likely cause for the increased degradation of rRNA during starvation. This possibility was first investigated by Sox and Hoagland (1966) who found no differences in ribonuclease activity between ribosomes from livers of starved and control animals. Because they had used a substrate (polyuridylic acid) which was not the natural substrate for ribosomal ribonuclease, their conclusions were considered doubtful by other workers. Arora and Lamirande (1971) reported results contradicting those of Sox and Hoagland (1966). From their studies on autodegradation of the ribosomes from the livers of starved and control rats, they concluded that the level of ribosomal ribonuclease increased during starvation, and that this caused increased autodegradation of the ribosomes from the livers of starved animals. When we used the techniques of Arora and Lamirande (1971) our results agreed in part with their's. We found that ribosomes from the livers of starved animals were at least two times more susceptible to autodegradation when compared to ribosomes from the livers of control animals. However, we do not agree that the increased autodegradation of ribosomes from the livers of starved rats is due to increased activity of ribosomal ribonuclease. Three different

substrates (yeast RNA, rRNA from starved and rRNA from normal rat livers) for the ribosomal ribonuclease were used and no change in the level of ribosomal ribonuclease per mg of ribosomal RNA or ribosomal protein during starvation was found. Since the results obtained by the use of rRNA from rat liver or yeast RNA as a substrate for the ribosomal ribonuclease activity were the same, this implies that even if the yeast RNA is not the natural substrate for ribosomal ribonuclease it can be used as a substrate for the ribosomal ribonuclease.

Since our results did not show any change in the activity of ribosomal ribonuclease during starvation, the increased autodegradation of the ribosomes from the livers of starved rats may be due to the RNA in these ribosomes being more easily accessible to the ribosomal ribonuclease. The increased access of the ribosomal ribonuclease to rRNA during starvation may be due to some changes in the orientation or unfolding of RNA itself or to the lack of some protective structural ribosomal proteins. Blobel and Potter (1967) observed that free ribosomes were more susceptible to enzymic degradation than membrane-bound ribosomes. Starvation was reported to damage the endoplasmic reticular membranes, thus decreasing the availability of ribosomal binding sites on the membranes and leading to an increase in the amount of free ribosomes (Tomi et al. 1961). Hird et al. (1964) reported that free ribosomal subunits were rich in endogenous ribonuclease. Munro et al. (1964) and Munro (1968) reported that during starvation the fractional rate of RNA degradation correlated with the ribosomal subunit population, which was most abundant during the most rapid RNA loss during starvation. In view of these reports and our results, we feel that the

increased autodegradation of the ribosomes from the livers of starved rats is not due to increased ribonuclease activity but may be due to the lack of binding sites in the endoplasmic reticular mambranes and an increase in the ribosomal subunit population.

The autodegradation of the ribosomes will also depend upon the amount of ribonuclease inhibitor. An inhibitor of alkaline ribonuclease is found in the cell cytoplasm. During starvation (Onishi 1970) and protein deficiency (Gaetani et al. 1969) the level of the inhibitor for alkaline ribonuclease was reported to decrease to a greater extent than the decrease in the alkaline ribonuclease, thereby freeing some of the latent ribonuclease to attack RNA. The increased degradation of RNA in this case would be due to the lack of ribonuclease inhibitor rather than to an increase in the level of ribonuclease. Since the ribosomal ribonuclease is an alkaline ribonuclease and has properties similar to that of the cytoplasmic ribonuclease, it is possible that the cytoplasmic inhibitor of the alkaline ribonuclease may inhibit the ribosomal ribonuclease. It may, therefore, be possible that the increased degradation of the RNA from the livers of the starved animals may be due to a decrease in the amount of the ribonuclease inhibitor. However, since the assay for the autodegradation was carried out in the absence of cytoplasm, it is unlikely that the increased autodegradation of the ribosomes from the livers of the starved animals was due to the lack of cytoplasmic inhibitor of ribonuclease. Since the cytoplasmic ribonuclease inhibitor is found to be associated with the cytoplasmic alkaline ribonuclease, it may, therefore, be possible that such an inhibitor is also found to be associated with the ribosomal ribonuclease and the decrease in this inhibitor during starvation

may be responsible for increased autodegradation of ribosomes from the livers of starved rats. Our results have shown that the ribosomal ribonuclease can use exogenous substrate and based on these results we found no change in the level of ribosomal ribonuclease during starvation. However, to completely rule out the possibility of an increase in the ribonuclease level during starvation, studies involving the solubilized enzyme need to be conducted.

Extraction conditions and RNA degradation

The extent of RNA degradation varied depending upon how drastic the extraction conditions were. Heating to 63°C was drastic enough to cause degradation of the RNA from starved animals, while extraction under the same conditions did not cause the degradation of RNA from the continuously fed or starved-refed groups. Heat could cause the degradation of RNA in several ways such as unfolding the RNA molecules and thus making them more accessible to the hydrolyzing agents or by breaking the phosphodiester bonds of the RNA backbone, etc. As mentioned earlier, during starvation there is an increase in ribosomal subunit population and there are less binding sites in the endoplasmic reticular membranes for the ribosomes. It is possible that ribosomal subunits are more susceptible than the whole free ribosomes, which in turn are more susceptible than membrane-bound ribosomes to heat degradation. The ribosomal subunit population is less in continuously-fed than in the starved animals and more of the ribosomes from continuously-fed animals are mambrane bound, and are, therefore, less susceptible to degradation with short-time heating (2-3 minutes). However, prolonged heating (15 to 20 minutes) does cause the degradation of RNA from the continuously

fed animals as well.

When DOC was used the RNA extracted at 0°C was also degraded from both starved and refed groups, although the extent of degradation was more in the starved group than in the refed group. Sugano et al. (1967) claimed that the increased degradation of polysomes on treatment with DOC was due to the release of ribosomal ribonuclease from microsomes by DOC. We agree with these workers regarding the fact that treatment of microsomes with DOC causes RNA degradation. However, we don't think that the degradation of RNA after DOC treatment of ribosomes was necessarily due to the release of ribonuclease from the microsomes. Since these workers did not examine the amount of latent ribonuclease released after DOC treatment, their results do not rule out the other possible effects of DOC which could increase the RNA degradation. In our studies, we did not find any difference in the ribonuclease level between starved and continuously fed groups, although the RNA from the starved group was more degraded than the RNA from the refed group after DOC treatment of the microsomes. We, therefore, think that DOC may be interacting with ribosomes or with endoplasmic reticular membranes and thus exposing the RNA to the ribonuclease. Since the RNA in the ribosomes of the starved animals is comparatively poorly shielded, the interaction of DOC may more rapidly expose the RNA to the ribonuclease in the starved group, while in the refed group the exposure of RNA to the ribonuclease may be comparatively slow. This explanation can be supported by the fact that interaction between DOC and endoplasmic reticular membranes is a stoichiometric reaction (Staehelin et al. 1963). Because in the starved group there are less of the endoplasmic reticular membranes and

thus the same concentration of DOC may cause more degradation of the RNA in the starved than in the refed group. Changes in the conformation of ribosomes during starvation also need to be investigated to see if the RNA in the ribosomes from the starved animals is more exposed and thus easily accessible to the ribonuclease as compared to the RNA in the ribosomes from the continuously fed animals.

On the basis of the following observations, we think that the RNA from the livers of the starved animals is more susceptible to degradation than the RNA from the livers of continuously fed or starved-refed animals.

1) The ribosomes from the starved group were at least two fold more susceptible to autodegradation as compared to the ribosomes from continuously fed group. The increased autodegradation of the ribosomes from the starved group was not due to increased ribonuclease activity.

2) The ribosomal RNA extracted at 0°C from the DOC treated ribosomes from the livers of starved rats was more degraded than the rRNA extracted from the DOC treated ribosomes of starved-refed rats.

3) The RNA from the livers of starved animals was degraded when extracted by heating to 63°C, while this treatment did not degrade the RNA from the livers of continuously fed or starved-refed group.

The Net and Relative Amounts of the Individual RNA Species Synthesized during these Dietary Regimens

Twenty-two hours after injection of ^{32}P, the specific activity of the liver microsomal RNA was about 3 times higher in the normal than in the 7-day starved rats. These results contradict those of Hirsch and Hiatt (1966), who found that rats injected with ^{14}C-orotic acid 48

hours before start of starvation and then starved up to 6 days, had a higher specific activity of rRNA over all the 6 days of starvation than the fed rats injected at the same time. Similar reports were made by Hayashi and Kazmierowski (1972) who found a slightly higher specific activity for both free and bound ribosomes from the 48 hour starved rats than those of the normal up to a period of 24 hours after injection of ^{14}C-orotic acid. We agree with them that the total incorporation per liver RNA 22 hours after injection of ^{32}P was more in the fed than in the starved animals. However, we found that the total incorporation in fed animals was about 5 times more than in the starved, whereas they reported about two-fold more incorporation. These differences are probably due to the differences in the length of starvation, and can be accounted for by the fact that during the first two days of starvation the half-life of rRNA was reduced from 5 days to 2 days. Around the 7th day of starvation, the half life of rRNA was increased to 7 days (Enwonwu, et al. 1971). The specific activity as well as the total incorporation per liver microsomal RNA was about 6 times more in the refed than the starved rats.

Seventy-five minutes after injection of ^{32}P the specific activity of microsomal RNA from starved animals was about the same as in the normal animals. This along with the earlier observations that 22 hours after injection of ^{32}P the specific activity of microsomal RNA from the livers of the fed animals was 3 times more than that of starved animals, suggested the rapid turnover or an increased end-labelling of an RNA species in the starved animal. Since the relative incorporation of ^{32}P into mRNA and rRNA in the starved group was about the same as in the

continuously fed group (Table 8) we think that the rapid turnover during starvation involved both mRNA and rRNA. If during starvation, all of the rRNA and mRNA was turned over rapidly, the specific activity of the RNA from the starved group should have been much higher than that of the RNA from the continuously fed group. As the specific activities of the RNA from the starved and continuously fed group after short-term labelling of RNA were not significantly different, the rapid turnover involved only a part of rRNA and mRNA. Messenger RNA from free polysomes was reported to turnover more rapidly than the mRNA from bound polysomes (Tanaka et al. 1970, Sarma et al. 1969, Hayashi and Kamierowski 1972, Wilson and Hoagland 1967) and rRNA from both classes of polysomes was labelled to about the same extent (Tanaka et al. 1970). Forty-eight hour starvation was reported to enhance the labelling of both mRNA and rRNA of free polysomes (Hayashi and Kazmierowski 1972). In view of these observations and our results, we think that during starvation both mRNA and rRNA of free polysomes were rapidly synthesized and degraded.

Refeeding of the starved animals markedly increased the synthesis of both mRNA and rRNA ; however, the synthesis of mRNA was increased to a greater extent than that of rRNA. The greatest increase in ^{32}P incorporation was in the 18S peak. Since the liver RNA contents from the starved and starved-refed groups were not significantly different, it seems that the recovery of RNA profiles after refeeding the starved group was not due to the de novo synthesis of rRNA. Refeeding caused a marked increase in the specific activity of both rRNA and mRNA, the greatest increase was in the mRNA region. It may be that the increased synthesis of mRNA after refeeding leads to the synthesis of some struc-

tural ribosomal or endoplasmic reticular membrane proteins, thus increasing the heat stability of RNA.

The signal findings of this investigation are that the extent of degradation of RNA from the starved animals varies depending upon the period of starvation and the extraction conditions. The increased susceptibility of the RNA to degradation during starvation is not due to an increased ribonuclease level and can be repaired within 4-8 hours of refeeding. The relative incorporation of ^{32}P into mRNA and rRNA during starvation is approximately the same as in continuously fed animals, but the total amount of newly synthesized RNA is markedly reduced during starvation. This may be due to lack of RNA precursors or energy. Refeeding of starved animals increases the syntheses of both rRNA and mRNA but the synthesis of mRNA is turned on to a greater extent than that of rRNA. Further studies need to be carried out to see if there is one or more of the structural ribosomal proteins which is made faster than others during refeeding.

CHAPTER V

SUMMARY

The current studies were undertaken to understand the events at the RNA level leading to polysomal disaggregation during starvation and reaggregation of polysomes after refeeding. Male Albino rats of the Holtzman and Sprague Dawley Strains were used. Rats weighing 350-400 gm body weight were starved for 7 days and then refed either a high carbohydrate or high fat diet for 4 or 8 hours. The changes in the nature and amounts of rRNA and mRNA during these feeding regimens were studied. The possible role of ribosomal ribonuclease in association with these changed RNA profiles was investigated. The following conclusions were made from these studies.

Starvation up to 5 days did not cause any change in the RNA profiles. After 7 days of starvation profiles of the RNA extracted at $0^{o}C$ were not different from the $0^{o}C$ extracted RNA of continuously fed rats. However, the extraction of RNA with heat ($63^{o}C$) from the starved animals caused degradation of the 18S and 28S peaks, which was not found in the continuously fed rats. Four hour refeeding of the HS or 8 hour refeeding of the HF diet to the 7-day starved rats restored the profiles of the heat extracted RNA. Using different procedures of isolation, it was found that RNA from the starved animals was more susceptible to degradation than RNA from continuously fed or starved-refed animals. The

increased degradation of rRNA during starvation was not due to an increase in the activity of ribosomal ribonuclease.

Although, the liver RNA contents of the 7-day starved and starved-refed group were not significantly different, the RNA from the livers of the refed group had markedly higher specific activity than the RNA from the livers of the starved group. Refeeding of the starved animals caused a marked increase in the synthesis of both mRNA and rRNA; however, the relative increase in the amount of mRNA synthesis was greater than the amount of rRNA synthesis. These results suggest that the restoration of RNA profiles after refeeding the starved animal was not due to de novo synthesis of rRNA; it may, however, be due to increased synthesis of mRNA. The increased synthesis of mRNA after refeeding may have caused the synthesis of some structural ribosomal or endoplasmic reticular membrane proteins which may have increased the heat stability of RNA.

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APPENDIX

Solution A		gms/liter
sucrose	0.25 M	85.500
$MgCl_2$	0.005 M	1.017
KCl	0.05 M	3.728
Tris base	0.025 M	3.028

pH was adjusted to 7.6 with 0.1 N HCl

Solution B		
$MgCl_2$	0.001 M	0.203
Tris base	0.05 M	6.055

pH was adjusted to 7.6 with 0.1 N HCl

Solution C		
$MgCl_2$	0.001 M	0.203
Tris base	0.001 M	0.121

pH was adjusted to 7.6 with 0.1 N HCl

Sodium acetate buffer for RNA extraction

Sodium acetate	0.821

pH was adjusted to 5.2 with 0.1 N NaOH

Toluene counting scintillator

Toluene scintillation grade = 1 liter

2,5-Diphenyloxazole (PPO) = 6 grams

1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) = 76 mgs

Bray's Counting fluid

Xylene	500 ml
P - dioxane	1500 ml
Ethylene glycol monoethyl ether	1500 ml
PPO	35 mg
POPOP	1.750 gm
Naphthaleine	280 gm

Ribonuclease incubation system

The incubation system for the ribonuclease assays consisted of :

1 ml of a solution of Tris base 2.422 gm/100 ml pH 8.0.

1 ml of a solution of EDTA disodium salt 3.722 gm/100 ml pH 7.0.

0.2 ml of an RNA solution of 5 mg/ml.

0.5 ml of the ribosomal suspension as source of RNase.

2.3 ml of water was added to make the volume up to 5 ml.