AN ABSTRACT OF THE THESIS OF

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in Insect Physiology (Major) presented on November 28, 1972 (Date)

Title: RIBOSOMES AND PROTEIN SYNTHESIS IN THE FAT BODY OF LEUCOPHAEA MADERAE (FAB.)

Abstract approved: Victor J. Brookes

Several procedures for isolating ribosomes from the fat body of female cockroaches are described and evaluated. The addition of deoxycholate or Triton X-100 to the homogenizing medium rather than to the postmitochondrial supernatant was an important factor in improving the yield of ribosomes.

The fat body ribosomes were characterized and found to behave differently than ribosomes isolated from the tissues of other animals. The majority of the particles were heavy aggregates, and the ribosomes appeared to be relatively stable to RNase treatment. Increasing the KCl concentration from 25 mM to 250 mM resulted in some dissociation of heavy aggregates. The principal properties of the cell-free protein synthesizing system were also studied. The system did not require GTP and ATP-generating systems. RNase caused an unexpected stimulation of incorporation of amino acid into protein.

Ribosomes and Protein Synthesis in the Fat Body of *Leucophaea maderae* (Fab.)

by

Fu-meei Yeh

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1973

APPROVED:

Redacted for Privacy

Professor of Entomology
in charge of major

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Date thesis is presented November 28, 1972

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ACKNOWLEDGEMENTS

The author wishes to express her deep appreciation to Dr. V. J. Brookes for his patient guidance of the research and the preparation of the manuscript. Without his help this course of work would have been impossible.

The author is also most grateful to Dr F. P. Conte for his kind discussions and permission to use his facilities. Thanks are also due to Dr. G. S. Beaudreau for his helpful suggestions and a supply of ^{3}H-rRNA (E. coli) and to Dr. M. Felton for providing ^{3}H-Qβ phage.

The research was financed by a grant from the National Science Foundation to Dr. V. J. Brookes.

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RIBOSOMES AND PROTEIN SYNTHESIS IN THE FAT BODY OF LEUCOPHAEA MADERAE (FAB.)

I. INTRODUCTION

Secretions from the corpora allata (CA) stimulate the development of the ovaries in a large number of insect species. Present evidence suggests that these glands control ovarial maturation in species that (a) do not carry large reserves of nutrient material through metamorphosis; (b) undergo repeated cycles of egg maturation; or (c) have a period of reproductive diapause (15, 17, 66, 70).

Röller and his associates (59) succeeded in identifying methyl trans, trans, cis-10-epoxy-7-ethyl-3, 11-dimethyl-2, 6-tridecadienoate (JH) as an agent that can substitute for the CA in the metamorphic functions controlled by the endocrine gland. Many analogs and mimics of this substance are also biologically active (66). Juvenile hormone (JH) and analogs are also effective in initiating ovarial development (8).

Under the influence of the secretion of the CA or JH, yolk protein is deposited in membrane-bound vesicles in the cytoplasm of the oocyte. During a reproductive cycle one or more proteins appear in the blood, the "specific female proteins," which are probably yolk proteins or precursors of yolk proteins. Telfer (71) showed that in the cecropia silkmoth, female specific proteins were

removed from the blood and taken into the oocyte by pinocytosis. Several types of evidence suggest that this process of yolk deposition may be widespread in insects (70).

Most of the yolk protein in the eggs of the cockroach, Leucophaea maderae, consists of a high molecular weight lipoglycoprotein ($S_{20, w}$ = 28S). This protein seems to be assembled somewhere in the follicle by the aggregation of 3 identical subunits (14). The subunit ($S_{20, w}$ = 14.5S) is manufactured in the fat body following treatment of the female by JH or one of its analogs. When fat body, isolated from females treated with hormone, is incubated in a tissue culture medium, the 14.5S component is secreted into the medium together with a number of other proteins (7). If the medium contains ^{14}C-leucine, as much as 50 percent of the label incorporated in the total secreted protein is found in the 14.5S component (Brookes, unpublished). This suggests that under the influence of JH much of protein synthesis is directed toward the synthesis of this one protein.

This work was started with the expectation of eventually isolating from the fat body a system that would synthesize yolk protein. Such a system would permit the study of hormone action at a subcellular level. Several methods have been used to isolate ribosomes from fat body and when the ribosomes were incubated with the appropriate soluble factors, a small amount of labeled

amino acid was incorporated into protein. The fat body ribosomes were characterized and found to behave differently than ribosomes isolated from the tissues of other animals.

II. HISTORICAL REVIEW

Early History of Action of Corpora Allata (CA) in Reproduction

The role of the CA in the stimulation of insect reproduction was first demonstrated by Wigglesworth in 1936. The hormonal function of CA was early recognized as affecting the incorporation of yolk into the oocytes in Rhodnius (75). Allatectomy and reimplantation of glands in many species have shown that with the exception of the orders Phasmida and Lepidoptera, the CA secretion plays an important part in stimulating egg maturation in most adult insects studied (17, 74). The secretion of the CA acts to promote either the synthesis of yolk protein, the incorporation of yolk into the developing oocyte, or both of the processes, in many different species. For some species, neurosecretion from the pars intercerebralis is also required in egg maturation (17, 31).

Evidence that JH secreted by the CA plays a key role during egg development is provided by the following experiments. Farnesol injected into allatectomized mature female Schistocerca reduced yolk resorption caused by allatectomy and stimulated yolk deposition in oocytes (32). Isolated abdomens of female Leucophaea initiated nearly normal egg maturation following implantation of active CA or application of JH or its analogs (8).

Female Specific Protein

The involvement of female specific proteins in yolk formation is supported by the following observations (70): 1) In insects thus far studied, the concentration of female protein decreases when yolk is being formed. 2) The concentration of female blood protein rises significantly during the period of yolk formation in ovariectomized females. 3) The high level of female protein in the egg is due to the uptake from the blood. Female specific proteins associated with yolk protein have been identified immunologically in Leucophaea, Hyalophora, Rhodnius, Sarcophaga, etc. (17).

Most of the yolk protein in mature eggs of L. maderae consists of a single species with a sedimentation coefficient of 28S. This protein is a trimer and is formed during yolk deposition by the aggregation of a subunit with a sedimentation coefficient of 14.5S (14). The 14.5S component predominates during the early stages of development and isnearly absent in the mature egg. On the basis of immunology and sedimentation characteristics, the 14.5S yolk protein is identical to a component synthesized in the fat body. In Leucophaea, therefore, the female specific protein is presumably synthesized in the fat body under the influence of JH, and released into the blood, then taken into the insect oocyte. During deposition, this protein aggregates to form the major yolk protein (7, 9, 14).

Female specific proteins are taken into the oocyte by pinocytosis. In the cecropia moth, Telfer (71) showed that the blood protein migrated intercellularly to the surface of the oocyte, and entered the cytoplasm in the form of droplets. These droplets coalesced to form the yolk spheres. Similar conclusions were drawn from electron microscopic studies in Aedes (60), P. americana (1) and Leucophaea (62).

Ribosomes and Protein Synthesis in a Cell-free System

General Systems

The study began with the important investigations of Zamecnik and his colleagues from 1950 onwards. These workers established the first system able to synthesize proteins in the absence of intact cells, identified the microsome (the ribosome) as the site of protein synthesis, discovered tRNA, and established the requirements for ATP and GTP in this system (79). Many important findings related to protein biosynthesis have been obtained since then. All protein systems require the following participants: adequate amounts of 20 amino acids commonly found in protein; more than 20 but fewer than 64 tRNA molecules; at least 20 amino acid-activating enzymes; enzymes involved in initiation, elongation, and termination; structurally and functional competent ribosomes; mRNA; ATP; GTP; appropriate concentrations of Mg^{2+} and K^{+} (or NH_4^{+}). The most

extensive studies of cell-free systems are those from E. coli (23, 53) rabbit reticulocytes (10, 58), and rat liver (4, 32, 53, 73). Bacterial systems and bacterial ribosomes, chiefly from E. coli, have been the most widely studied. Since organisms differ greatly in their biology and physiology, detailed differences in processes between bacteria and eukaryotes do exist. However, on the whole, the requirements for protein synthesis are common to all systems.

Ribosomes are the site of protein synthesis. It is well known that several ribosomes held together by a mRNA strand (polysomes) are active in protein synthesis, while monosomes are far less active (23, 54, 58, 73). Various treatments result in degrading polysomes to smaller particles, monosomes and/or small portions of light ribosomes. Although ribosomes of higher organisms, both animals and plants, are similar to those of bacteria in chemical and physical characteristics and in the role they play in protein synthesis, they differ in several respects. The bacterial ribosome consists of a 70S particle that readily separates into two subunits 50S and 30S in solutions devoid of Mg^{2+}. The ribosome of eukaryotes is larger, with a sedimentation coefficient of 80S and dissociates into 60S and 40S subunits. Complete dissociation occurs in solutions that are not only devoid of Mg^{2+} but also have a high concentration of K^{+} (0.5-1.0 M) or in solutions that are alkaline (pH 7.5 to 8.5). More protein is associated with the eukaryote ribosome than with that of

bacteria. Purified 80S ribosomes contain RNA and protein in roughly equal proportion and there is no intrinsic lipid or carbohydrate component. In many cases, treatment with DOC increases the RNA/protein ratio to 1.2 to 1.5. Several species of RNA are obtained from the ribosomes following treatment with SDS. In eukaryotes the 60S particle gives rise to 28 to 30S and 5 to 7S rRNA, while 18 to 19S rRNA is derived from the 40S particle. The 50S of the bacterial ribosome is composed of 23S and 4S rRNA, and the 30S particle is made up of 16S rRNA (48, 67).

Insect Systems

Ribosomes or microsomes that are active in protein synthesis have been isolated from flies [Drosophila adults (19), Calliphora larvae and pupae (63), Musca larvae, pupae, adults (37, 45) and eggs (21, 22)], beetles [Tenebrio pupae (34, 35)], crickets [Acheta adults (40)], and moths [Bombyx larval silk glands (43)]. Most of these preparations must be considered as crude since the requirements for amino acids, ATP and GTP were usually not absolute. The addition of supernatant fractions derived from insect tissues often proved to be inhibitory (40, 63) or of no value (35), but fractions from rat liver (40, 63), rabbit liver (34, 35), or chick embryo (40) were stimulating. The inhibitory effects of phenol oxidases, commonly found in insect blood and tissues, were overcome in

some instances by phenylthiourea (34, 45). Considered as a whole, the requirements for protein synthesis in insects are not significantly different from those of other animals.

The profiles of ribosomes or microsomes extracted from tissues or whole bodies of most of the species mentioned above have been analyzed by density gradient centrifugation (5, 13, 21, 27, 28, 37, 41, 43, 46, 63, 68, 72). In general these profiles are what might be expected based on findings with tissues from other animals. The profiles usually display a prominent monosome peak and aggregates of various sizes. Ishaaya and Chefurka (37) also described a heavy fraction in extracts of houseflies that appeared to be mostly membranes. The ribosomes of Acheta are unusual in that most of this material was identified as monosomes and disomes. The absence of heavier aggregates in Acheta was attributed to disruption during preparation although the ribosomes were resistant to RNase (40).

The capacity of the ribosomes for protein synthesis varies with the stage of development. Protein synthesis is greatest with ribosomes from two- and five-day-old Calliphora pupae (63). During the late larval stage most of the ribosomes were found in heavy aggregates (greater than trisomes) and the incorporation of amino acids was associated with those aggregates. During pupal and pharate adult stages, the ribosomes were mainly monosomes and

most of the amino acid activity incorporated was associated with disomes and trisomes (63).

An unusual quality of the ribosomes from some species of insects is resistance to RNase; the ribosomes of Musca (72) and Drosophila (68) were stable to both high and low concentrations of the enzyme. Stability to low concentration of RNase was found with ribosomes from the Acheta (40). However, very small amounts of RNase degraded the polysomes of Calliphora, but to what state of aggregation was not reported (63). Treatment of resistant ribosomes from Musca with trypsin and then RNase reduced heavy ribosomes to monosomes (72). A similar result was reported with Drosophila by Stafford et al. (68) but was not confirmed by the work of Boshes (5).

The ribosomes from specific tissues of insects have been isolated and identified in only a few instances. Nascent fibroin was found in association with very large aggregates (420S) of ribosomes from the silk gland of Bombyx mori. Ribosomes from the midgut of B. mori (27, 28) and from the eggs of the housefly (21) showed a typical profile with aggregates ranging from monomers to pentamers. Midgut ribosomes were stable to RNase but those from eggs were degraded by the enzyme. Ribosomes from the salivary glands of Chironomus tentans have also been isolated and analyzed (13). Most of the ribosomes are in the form of aggregates that are very sensitive

to endogenous RNase but less to enzyme that was added to the preparation.

Studies of ribosomal RNA (rRNA) from different species of insects have shown a range of $S_{20, w}$ values for each of the three types of rRNA as follows: 4 to 8S, 16 to 18S, and 27 to 28S (2, 20, 21, 25, 26).

The Effect of Hormones on Nucleic Acid Metabolism

In Vertebrates

A large amount of evidence suggests that developmental hormones act on target cells by affecting the metabolism of RNA either at the level of transcription or translation (24, 38, 69). Closely related to the action of juvenile hormone is that of estrogen on the oviduct and liver of yolk producing vertebrates. Although the action of estrogen on target tissues is more diverse than the action of juvenile hormone in adult insects appears to be, the vertebrate hormone induces the synthesis of specific proteins associated with the developing oocyte. These proteins are precursors of lipovitellin, formed in the liver (29, 61) and ovalbumin synthesized in the oviduct (55). Estrogen also acts on the uterus and here its function is quite complex resulting in the eventual growth of the organ and the differentiation of certain types of cells (76). No comparable action of juvenile hormone has yet been recorded in adult insects. The

earliest effect of estrogen on the uterus is the synthesis of specific proteins detectable in the cytosol and resulting apparently from the earlier synthesis of a new mRNA (24, 38). In a series of studies with chick oviduct, it was demonstrated that estrogen can act on the target tissue to promote the accumulation of a specific mRNA, the ovalbumin mRNA. The presence of this mRNA was shown to be estrogen dependent, and, when added to a cell-free system, the mRNA directed the synthesis of the major oviduct protein, ovalbumin (51, 55, 57).

In Insects

In the blowfly Calliphora, Karlson, Sekeris, and their colleagues demonstrated that the synthesis of specific mRNA, which carries the information for the enzyme DOPA-decarboxylase, was induced in vivo by ecdysone (39, 77). In a further experiment, RNA extracted from the nuclei of the epidermis of insects at the appropriate developmental stages caused the formation of DOPA-decarboxylase in a cell-free rat liver system (64). This presumptive enzyme product was characterized only by its catalytic activity. Direct evidence for cell-free synthesis of DOPA-decarboxylase by this specific mRNA is still lacking. Various studies are surveyed and summarized by Wyatt (77) that indicate that specific changes of the puffing patterns are induced by ecdysone or JH in the chromosomes of larval salivary

glands. It was found that puffing involved RNA synthesis as indicated by incorporation of uridine-^{3}H. However, it is not known whether puffing involves the synthesis of new mRNA. In their studies of Tenebrio molitor pupae, Ilan and Ilan (36) claimed that the control of gene expression by JH was at the translational level and involved the production or activation of certain tRNA's and activating enzymes. Hormonal induction of vitellogenin synthesis has been studied in L. maderae (7, 8). Since the induction is inhibited by actinomycin (16), it is possible that this induction is controlled at the gene level.

III. MATERIALS AND METHODS

Reagents

The following is a list of the reagents used in the course of this work, the supplier and the abbreviations used in the text.

Supplier	Reagent	Abbreviation
Sigma, Mo.	Adenosine-5'-Triphosphate (Disodium salt)	ATP
	Guanosine-5'-Triphosphate (Sodium salt)	GTP
	2-Phosphoenolypyruvic Acid (Potassium salt)	PEP
	Pyruvate Kinase	---
	Bovine Serum Albumin	BSA
	Bovine Pancreatic Ribonuclease Type X-A, 100 Kunitz unit per mg protein	RNase
	Dithiothreitol	DTT
	β-Mercaptoethanol	BME
	Sodium Deoxycholate	DOC
New England Nuclear Corp., Mass.	L-(^{14}C)-leucine, 262 mc/m mole uniformly labeled	---

Supplier	Reagent	Abbreviation
New England Nuclear Corp., Mass.	Aquasol	---
Packard Instrument Co., Ill.	2, 5-bis-(2-(5-tert-Butyl-benzoxazolyl))-Thiophene	BBOT
	Triton X-100	---
Pharmacia, Piscatoway, N. J.	Sephadex G-100	---
	Blue Dextran 2000	---
Schwarz/Mann, N. Y.	Sucrose (Ribonuclease free)	---
Fisher Scientific Co.	Sodium Lauryl Sulfate, U. S. P.	SDS

R^3-rRNA (Escherichia coli) was a gift from Dr. Beaudreau, Department of Agriculture Chemistry. Tritiated Qβ phage was kindly provided by Dr. M. Felton, Department of Microbiology.

Solutions

The following solutions were used in the present studies:

1. Medium A: 0.025 M KCl, 0.05 M Tris-HCl (pH 7.6 at 20°C), 0.01 M $MgCl_2$, 0.006 M BME.

Medium A was treated with 1 mg/ml macaloid and centrifuged at

35,000 g for 30 minutes before use.

2. Medium B: 0.05 M KCl, 0.01 M Tris-HCl (pH 7.6 at 20° C), 0.01 M $MgCl_2$, 0.004 M DTT.

Where necessary, Triton X-100, or a freshly prepared solution of DOC were added to the above media to a final concentration of 1 percent. In some experiments the concentration of KCl was increased to 0.25 M.

3. TKM solution: 0.05 M Tris-HCl (pH 7.6 at 20° C), 0.025 M KCl, 0.005 M $MgCl_2$.
4. Incorporation buffer: 0.075 M sucrose, 0.045 M KCl, 0.05 M Tris-HCl (pH 7.6 at 20° C), 0.0025 M $MgCl_2$, 0.003 M BME.

Animals

All of the animals that were used in the experiments to be described were young mated female of Leucophaea maderae in the vitellogenic phase of oocyte maturation. The development was assessed by measuring the length of the basal oocytes with an ocular micrometer in a dissecting microscope. The details of rearing were described by Chambers and Brookes (11).

Preparation of Ribosomes

Before dissection the female cockroaches were anesthetized with carbon dioxide and then chilled on ice for at least 30 minutes;

all further operations were carried out at 0-5° C, except where noted. Fat body was quickly dissected out and placed in several volumes of ice-cold 0. 25 M sucrose medium A or B. The collected fat bodies, equivalent to two or three insects, were then rapidly transferred to the freezer. Particular attention was given to obtaining fat bodies with a minimum of contamination from other tissues such as eggs, gut, etc. The procedure from dissection to homogenization lasted as long as two hours when large numbers of cockroaches were dissected. Fat bodies, weighing about 6. 4-14 gm, were homogenized with 8 to 10 strokes in 3 volumes medium A or B containing 0. 25 M sucrose in a pre-chilled, loose-fitting lass homogenizer. The homogenate was centrifuged for 10 minutes at 17, 000 $g_{(max)}$. DOC or Triton X-100 was added prior to homogenization or added to the postmitochondrial supernatant (PMS) depending upon the preparation. An aliquot of 4. 5 to 5. 3 ml of PMS was layered over a discontinuous gradient consisting of 2. 2 ml of 0. 5 M and 2. 3 ml of either 1. 5 or 2. 0 M sucrose solution in medium A or B. The gradients were then centrifuged for 2. 5 or 4 hours in a Spinco no. 65 rotor at 40, 000 rpm (101, 000 $g_{ave.}$). Following centrifugation the supernatant was carefully poured off and the pellet was quickly rinsed with distilled water and stored at -20° C as a pellet. Generally the procedure described above was standard. Ribosomes prepared in this way were used in profile analysis and amino acid incorporation

experiments.

Preparation of High-Speed Supernatant Factors and Microsomes

Microsomes were prepared by centrifuging 7 to 9 ml of PMS in 0.25 M sucrose modified medium A (0.004 M DTT instead of 0.006 M BME) for 1 hour at 40,000 rpm in a no. 65 rotor. pH 5 enzymes, following the procedures of Hoagland et al. (33), were precip itated from the resulting supernatant fraction by adjusting the pH to 5.1. This precipitate was washed with cold distilled water once and dissolved in 1/10 original volume of incorporation buffer. A column of Sephadex G-100 (1 cm diameter; void volume: 4 ml) was prepared and equilibrated with incorporation buffer. The post-microsomal supernatant was placed at the top of the column. The column was eluted with incorporation buffer. A fraction of the same volume as the original sample was collected after collecting the void volume (4). The pH 5 and G-100 Sephadex fraction were normally used immediately after preparation.

Amino Acid Incorporation

The standard incorporation mixture, 0.5 ml, contained: 6 mM $MgCl_2$; 35 mM Tris-HCl (pH 7.6); 100 mM KCl; 3 mM BME; 0.1 mM each of 19 unlabeled L-amino acids excluding leucine; 2 mM ATP;

0.5 mM GTP; 8 mM PEP; 20 μg pyruvate kinase; 50 μl pH 5 fraction (approximately 1.2-1.77 mg protein); 0.15 ml G-100 Sephadex fraction (approximately 1.5 mg protein or 0.06 mg RNA); 0.25 μc ^{14}C-L-leucine (containing about 323000 cpm/0.954 n mole/0.25 μc); and ribosomes.

The mixtures were prepared in flasks at 0-5° C and the reaction was started by transferring them to a water bath at 30° C. Thirty seconds were taken as zero time. For measuring incorporation kinetics, samples of 50 μl were removed at indicated times and pipetted onto Whatman 3 MM filter paper discs, which were then transferred after 10 seconds into a beaker containing ice-cold 10 percent TCA (0.1 M leucine). The discs were processed as described by Mans and Novelli (49). The radioactivity was determined in a Liquid-Scintillation Counter (Parkard Tri-Carb Spectrometer, Model 3310) with 10 ml Toluene-BBOT (4 g BBCT/l Toluene) as the scintillation liquid. Settings were as follows: Gain, 20 percent; Discriminator, 50-1000; Time, 10 min.

All results have been corrected for counts found in zero time controls. Although I attempted to add ^{14}C-L-leucine and ribosomes just before the beginning of the incubation and to standardize the conditions used, the zero time values tended to be somewhat variable (between 3 and 58 cpm above background/vial). Furthermore, it was observed that counts of zero time controls increased once ribosomes

were added to the incubation medium, even at 0° C for 5-10 minutes. In the experiments described, the concentration of ribosomes per 0.5 ml varied from 0.006-1.66 mg rRNA. The data have been presented as cpm/mg RNA.

Sedimentation Profile of Ribosomes in Sucrose Gradients

The frozen pellets were suspended in TKM solution. One-ml samples were then layered on the top of 22 ml, 0.3-1 M, linear sucrose gradient containing TKM solution. Five-ml of 60 percent sucrose TKM solution was used as a cushion beneath the sucrose gradient. Gradients were centrifuged for 2 or 4 hours at 25,000 rpm in a SW 25.1 rotor in a Model L2 or L65B Spinco ultracentrifuge.

After centrifugation, the gradients were removed from the top by displacement with 65 percent sucrose that was pumped into the bottom of the tube, forcing the effluent to pass through a drop counter to a fraction collector. Thirty eight drops, approximately 1 ml, were collected for each tube and the absorbances at 260 nm and 280 nm were measured on a Gilford 2000 Recording Spectrophotometer. For assay of radioactivity, 0.5 ml of each 1 ml fraction was dispersed in 14.5 ml Aquasol solution. Counting was performed on a Parkard Tri-Carb Scintillation Counter.

Analytical Methods

RNA was estimated by the procedure of Fleck and Munro (18) and Munro and Fleck (52) using $E^{1\%}_{1\ cm}$ = 312 (32 μg/ml) = 1.00 O. D. Protein was determined according to Lowry et al. (47) using BSA as standard.

IV. RESULTS

Isolation of Microsomes and Ribosomes

The first attempts to isolate ribosomes from fat bodies followed the procedures established by Wettstein et al. (73). These resulted in a poor yield of RNA and a low level of amino acid incorporation. More successful were modifications of procedures previously used for insects. These procedures include the chilling of the insects prior to dissection and inclusion of a reducing agent in the homogenizing medium.

In Table 1 are described some of the characteristics of ribosomes obtained by a variety of isolation procedures. Microsomal preparations (Expt. 8) produced the highest recovery of RNA. The only comparable recoveries were obtained when the fat bodies were homogenized in medium B with Triton X-100 and the PMS was centrifuged through 1.5 M sucrose (Expt. 4 and 5). The recoveries were much lower when PMS was centrifuged through 2 M sucrose, and the recoveries from medium A were essentially the same as those from medium B (Expt. 2 and 6). Kaulenas (40) using DOC, and Boshes (5) using Triton X-100 found that the addition of the detergent to the homogenizing medium rather than to the PMS was an important factor in improving the yield of ribosomes. The same results were obtained

Table 1. The effect of the isolation procedure on the characteristics of ribosomes obtained from adult female cockroaches, Leucophaea maderae.

Experiment #	Preparation					Number of determination	RNA content of ribosomes recovered per 1 gram wet weight fat body (mg), ±S.E.	Protein content of ribosomes recovered per 1 gram wet weight fat body (mg), ±S.E.	Weight ratio RNA/protein ±S.E.
	Homogenization medium	KCl concentration in the medium (M)	Detergent and stage of detergent addition	Sucrose concentration in bottom layer (M)	Centrifugation time (hour)				
Ribosomes									
1	A	0.025	DOC PMS	2.0	4	5	0.0561 ±0.0161	0.0823 ±0.0428	0.7534 ±0.2875
2	A	0.025	DOC H	2.0	4	3	0.1771 ±0.1029	0.3542 ±0.0932	0.4710 ±0.1114
3	A	0.25	DOC H	2.0	4	1	0.1118	0.3865	0.2893
Ribosomes									
4	B	0.05	Triton X-100 H	1.5	2.5	4	0.4090 ±0.0844	0.9709 ±0.3046	0.4338 ±0.0421
5	B	0.05	Triton X-100 H	1.5	4	3	0.3757 ±0.0264	1.1253 ±0.2696	0.3522 ±0.1196
6	B	0.05	Triton X-100 H	2.0	4	8	0.1619 ±0.0858	0.4541 ±0.2451	0.3861 ±0.1867
7	B	0.25	Triton X-100 H	2.0	4	2	0.1076 ±0.0375	0.3437 ±0.0361	0.3082 ±0.0452
Microsomes	Modified								
8	A	0.025	---	0.25	1	5	0.4422 ±0.1213	---	---

Abbreviation: PMS, postmitochondrial supernatant; H, homogenate; S.E. standard error of the mean.

with DOC in the present work (Expt. 2), and in the isolation of medium with Triton X-100 the detergent was routinely added to the homogenizing medium.

Protein was a major contaminant of all the preparations and the ratio of RNA to protein was lower than has been found in ribosomes from other animals including insects. The amount of contaminating protein was reduced in ribosomal preparations from crickets (40), and the wings and legs of chick embryos (44) by homogenizing in a medium containing KCl in concentrations as high as 0.24 M. In experiments 3 and 7 of Table 1 the fat body ribosomes were prepared in media containing 0.25 M KCl, or 5 to 10 times that of media ordinarily used. The higher concentration of KCl did not improve the yield or the purity of the ribosomal preparation but there was some effect on sedimentation behavior and amino acid incorporation which will be described in a subsequent section.

Sedimentation Characteristics

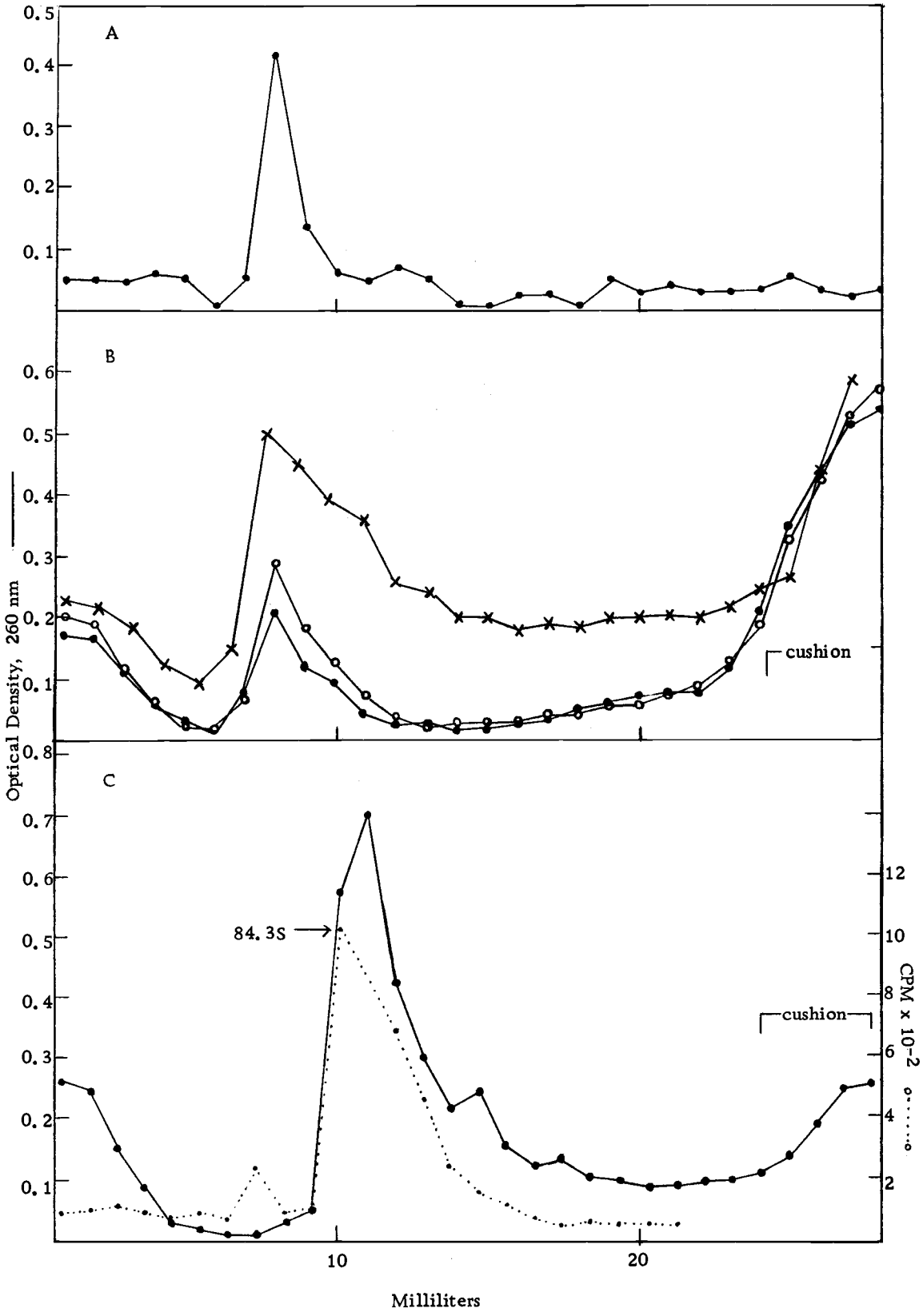
A large number of preparations isolated by procedures shown in Table 1 were analyzed by sucrose density gradient centrifugation under conditions that were supposed to show the polysomal aggregates commonly found in animal tissues. All of these preparations contained a light material sedimenting near the top of the gradient which was largely protein, a peak in the monosome region, and a low level of absorbance in the rest of the gradient. A typical profile is shown in Figure 1A. As much as 90 percent of the total material

Figure 1. Characteristics of fat body ribosomes in sucrose gradients. Ribosomes were analyzed on linear gradients composed of 0.3 to 1 M sucrose in TKM. In some experiments, 5 ml of 60 percent sucrose was placed at the bottom of the tube as a cushion. One ml samples were added to the surface of the gradients. Centrifugation was for 2 or 4 hours at 25,000 rpm in a Spinco SW 25.1 rotor.

A. Ten OD_{260} units were analyzed. Centrifugation was for 2 hours without a cushion.

B. The sample contained 0.208 mg RNA. Centrifugation was for 2 hours without a cushion. ●–● Fresh ribosomes prepared 24 hours prior to centrifugation. o-o Fresh ribosomes 5 minutes after incubation at 37° C. x-x Ribosomes were analyzed 3 months after preparation.

C. Same ribosomes as in 1B. The ribosomes were analyzed 1 month after preparation. The ribosomes were centrifuged for 4 hours together with 5 μl of H^3-Qβ virus (approximately 1500 cpm).


A
B
C
Optical Density, 260 nm
cushion
84.3S
cushion
CPM x 10-2
Milliliters
10
20

placed on the gradient was recovered as a pellet at the bottom of the tube. When a sucrose solution of high density was placed at the bottom of the gradient, the progress of the sedimenting material was retarded as shown in Figure 1B. Despite the cushion, a pellet was always obtained which accounted for 30-70 percent of the total fraction.

The ratio of the absorbance (OD_{260}/OD_{280}) of the fraction near the top of the gradient was less than 1, suggesting that this material was largely protein. The single peak that was found in the first third of the gradient had a ratio of 1.8-1.9 and it sedimented together or one tube ahead of the marker, Qβ virus $S_{20,w} = 84.3$ S (56). The material in the rest of the gradient and in the pellet had a ratio of 1.1-1.5 which suggests nucleic acids associated with protein.

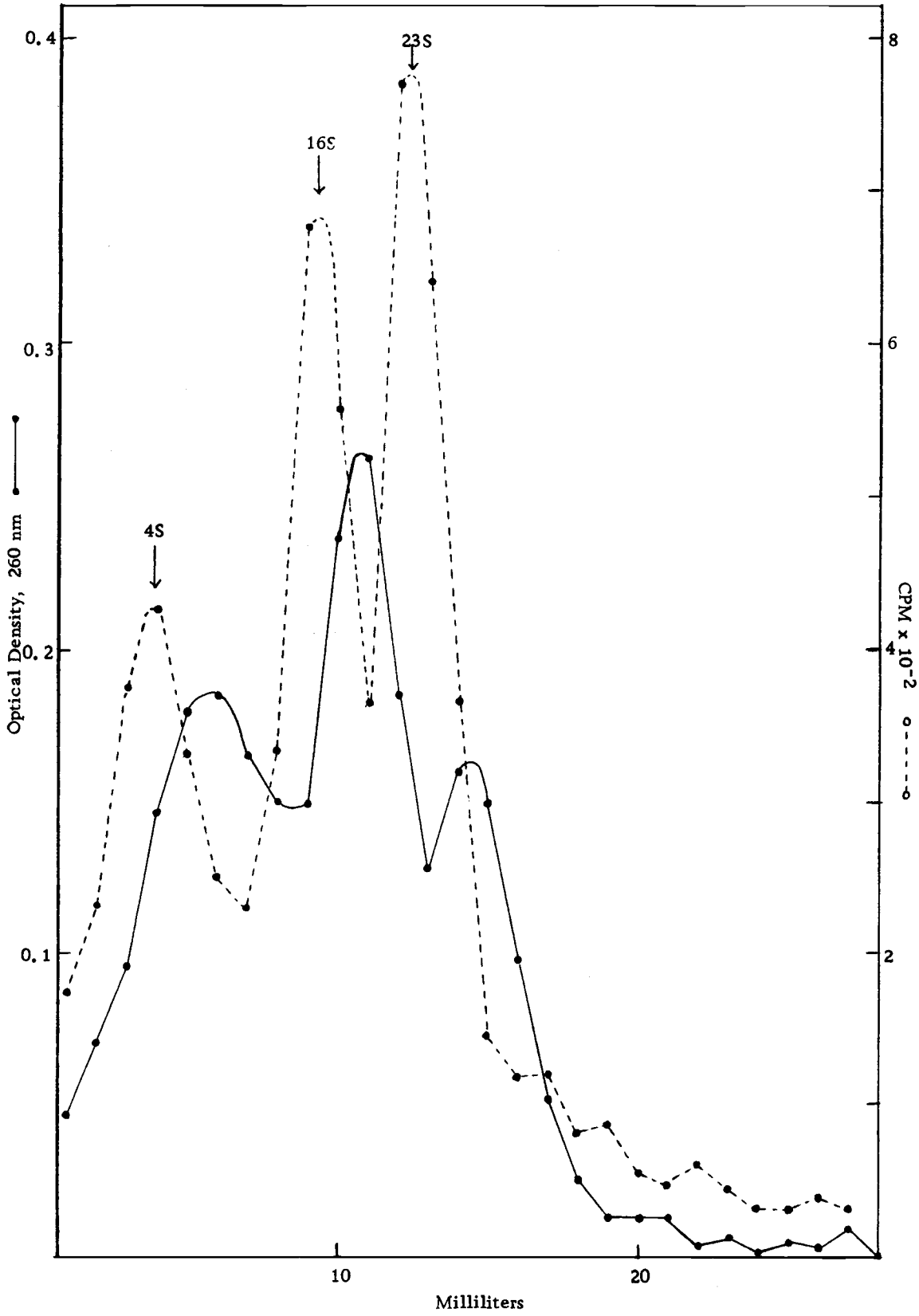
The polysome profiles shown in Figure 1B and 1C were made with the same sample of ribosomes at three intervals after the preparation, during which time the ribosomes were stored at -20° C. In 1B are the profiles of ribosomes 24 hours and 3 months after preparation. Also shown in Figure 1B is the profile of 24 hour ribosomes that were incubated for 5 minutes at 37° C. The profile of Figure 1C was made one month after preparation. Because the duration of centrifugation was 2 hours in Figure 1B and 4 hours in Figure 1C, the two profiles are not directly comparable. However, the results do show that the effect of prolonged storage is the

loosening of the pellet so that more material is found in all fractions of the gradient. Unfortunately, the recoveries in the pellet were not recorded in any of the experiments except the one with the 3 month ribosomes. In this experiment only 8.5 percent of the RNA added to the gradient was recovered in the pellet. After incubation at 37° C for five minutes there appeared to be a slight increase in the monosome fraction. This increase was more pronounced in another experiment to be described later.

The ribosomal character of the material in the pellet was established by an experiment in which the pellet after profile was suspended in 0.5 percent SDS in 5 mM Tris-HCl buffer (pH 7.6) and incubated for 5 minutes at 37° C. This material was centrifuged as described in Figure 2 together with ribosomal RNA of Escherichia coli. The result was a cytoplasmic RNA profile showing 3 peaks with sedimentation values of approximately 6S, 18·6S, and 27S. The ratio of the 18S:27S was the reverse of that usually found in animal tissues and bacteria. This may be the result of an incomplete extraction of the RNA from the pellet.

Ribosomes from some animal tissues aggregate with proteins during preparation. To circumvent this, higher concentrations of KCl are used in the homogenizing and suspending media. Considering the possibility that such aggregation may account for the heavy pellet in fat body preparation, ribosomes were prepared and

Figure 2. Sedimentation characteristics of ribosomal RNA. The pellet obtained after centrifugation for 2 hours as described in Figure 1 was suspended in 1 ml 5 mM Tris buffer containing 0.5 percent SDS and heated for 5 minutes at 37° C. A 0.5 ml sample was layered on 27.5 ml 15-30 percent sucrose linear gradients made up with 5 mM Tris buffer and centrifuged at 4° C for 17 hours in a SW 25.1 rotor at 25,000 rpm together with 10 μl of H^3-RNA from E. coli (approximately 44,000 cpm). The tubes were analyzed for absorbance at 260 nm and assayed for radioactivity as described in "Materials and Methods." Sedimentation values were assigned according to the procedure of Martin and Ames (50) for comparing an unknown peak to a known standard with similar sedimentation properties.

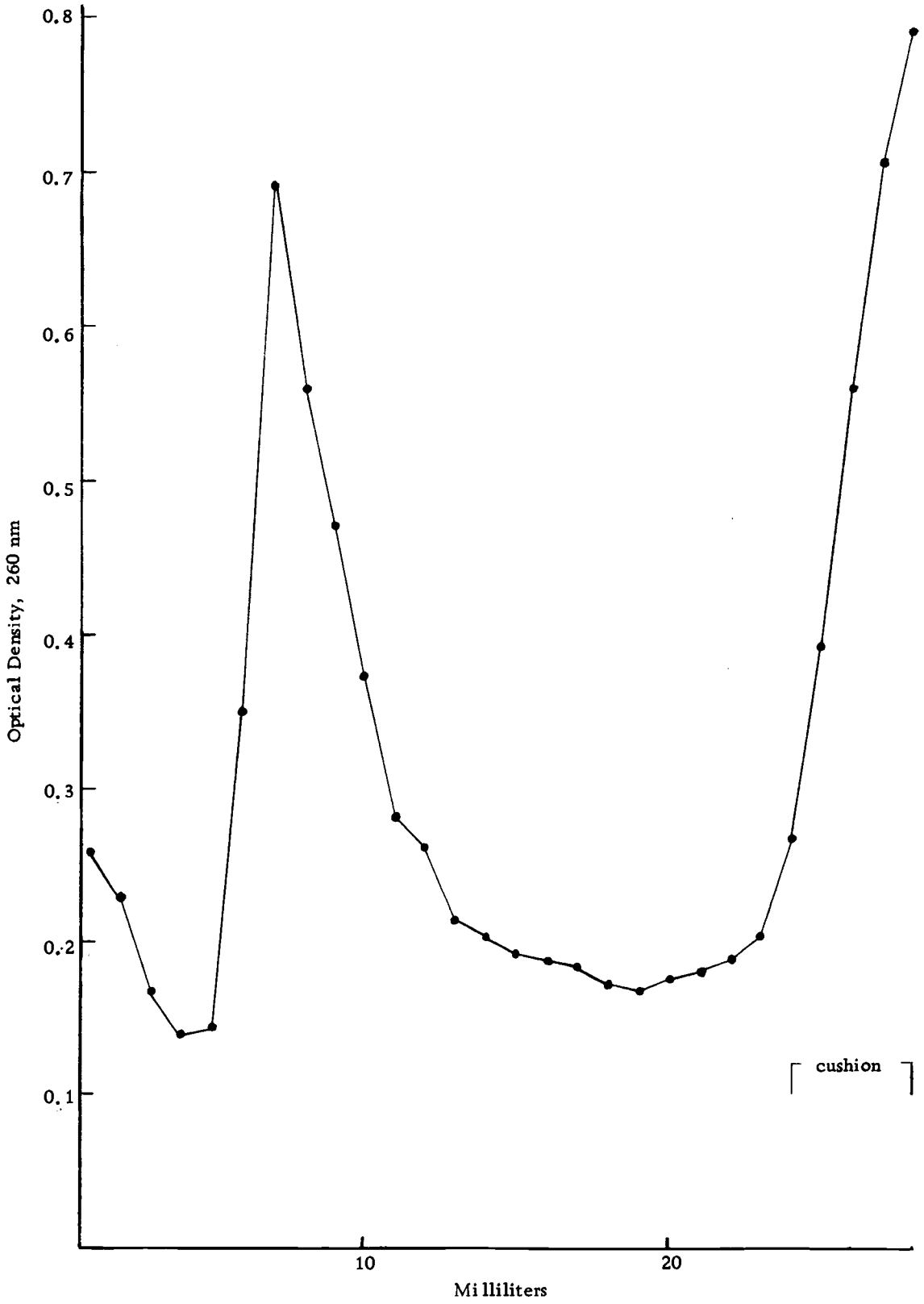

0.4
0.3
0.2
0.1
23S
16S
4S
Optical Density, 260 nm
8
6
4
2
CPM x 10-2
10
20
Milliliters

suspended in buffer containing 0.25 M KCl. An example of profiles prepared in this way is shown in Figure 3. Most of the sample appeared in the gradients; (in the experiment of Figure 3), only 3.6 percent was recovered in the pellet. Profiles of ribosomes prepared in the higher salt buffers show a larger monosome peak than the ribosomes prepared in the usual buffer containing 0.025 or 0.05 M KCl.

Ribosomes prepared in medium B were analyzed in the usual sucrose gradient with cushion but with 1 M KCl and no Mg^{2+}. After 2 hours of centrifugation, the profile showed one large fraction with a peak at tube five. The rest of the gradient including the cushion contained very little material absorbing at 260 nm and there was little pellet. When high salt ribosomes were centrifuged under the same conditions but for 8 hours, the results were as shown in Figure 4. The profile contained a small fraction near the top of the gradient, two large peaks in the center, and a small peak in the cushion. The absorbance ratios (260 nm/280 nm) were 0.7, 1.9, 2.0, and 1.3, respectively. About 12.5 percent of the total RNA of the sample was recovered in the pellet. No markers were included in this experiment, but based on the work of others, the major peaks are assumed to be ribosomal subunits.

Figure 5 shows the result of an experiment in which the effects of exogenous and endogenous RNase on the ribosomes were tested.

Figure 3. The effect of KCl on the sedimentaion characteristics. Ribosomes were isolated in Buffer B containing Triton X-100 and 0.25 M KCl 4 days prior to analysis. Aliquots containing 0.25 mg RNA were analyzed as described under Figure 1 except that the gradient was 0.25 M with respect to KCl.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical Density, 260 nm
cushion
10
20
Milliliters

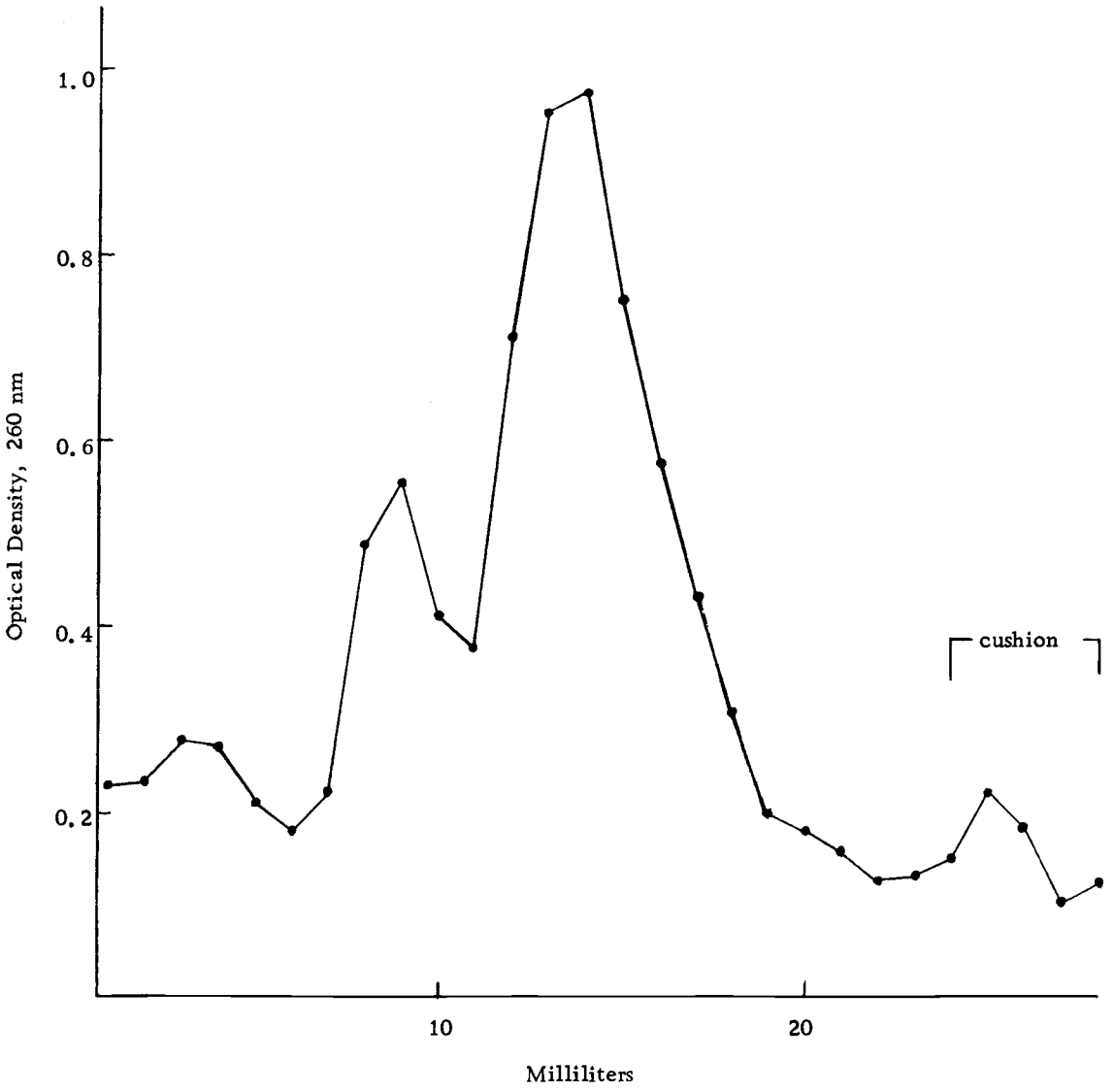


Figure 4. Sedimentation characteristics of ribosomal subunits. Ribosomes (0.39 mg RNA) were suspended in 1 ml of 0.05 mM Tris buffer, 1 M with respect to KCl. They were analyzed as described under Figure 1 except that the gradient contained 1 M KCl, no Mg^{2+} and the time of centrifugation was 8 hours.

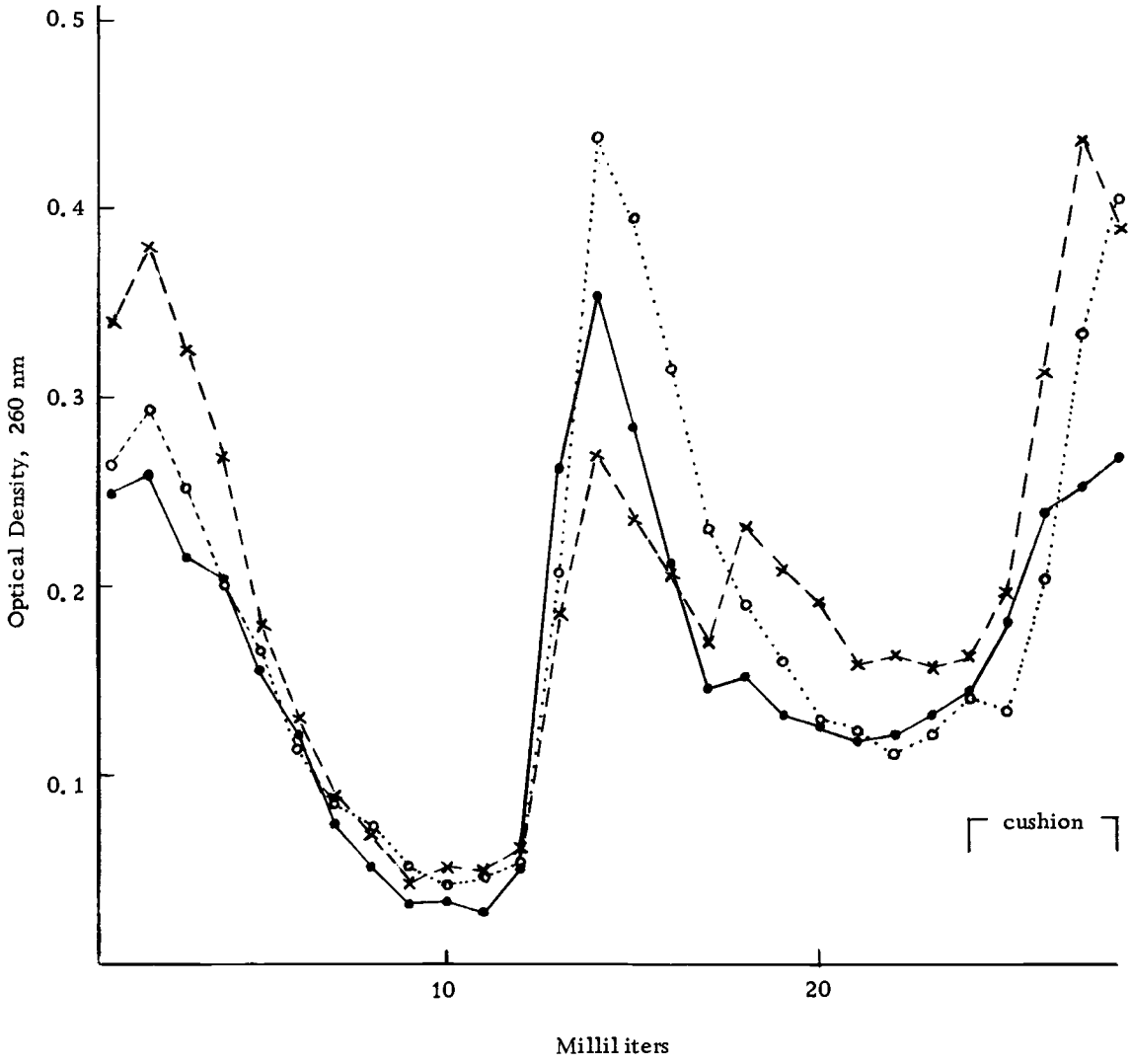


Figure 5. The effect of RNase on the sedimentation characteristics of fat body ribosomes. Ribosomes were prepared 1 week before analysis. The procedures followed were identical to those of Figure 1. Each gradient received 0.62 mg RNA and was centrifuged for 4 hours. •-• control; o-o incubated for 5 minutes at 37°C before centrifugation; x-x incubated for 5 minutes at 37°C with 0.0115 μg RNase before centrifugation.

A ribosome preparation was divided into three parts of 1 ml each. The first served as a control, the second was incubated for 5 minutes at 37° C, and the third was incubated for 5 minutes after the addition of 0.0115 μg of RNase. After centrifugation the profile was analyzed and the pellet recovered for an estimation of the RNA. In the control tube, the pellet contained 72 percent of the RNA placed on the gradient. The pellet from incubated ribosomes contained 59 percent, and that from ribosomes treated with RNase contained 53 percent. The effect of incubation was to increase the amount in all three fractions: the top of the gradient, the monosome fraction, and the material in the cushion. Treatment with RNase reduced the size of the monosome fraction. The other fractions were increased including those that appeared between the monosomes and cushion. In a number of experiments, larger amounts of RNase were added to ribosomes (10 μg/ml). Profiles were analyzed but the recoveries in the pellet were not measured. The principal effect of the higher concentration of RNase was to destroy the monosome fraction and substantially increase the light fraction. Large amounts of material appeared in the cushion.

Amino Acid Incorporation

Table 2 shows the results of experiments in which the incorporation of of ^{14}C-leucine into protein was measured in cell-free

Table 2. Amino acid incorporation into protein by different ribosomal preparations.

Group #	Ribosomal Preparation		Input ribosomes concentration (mg 4-RNA)	Input pH 5 enzymes concentration (mg protein)	Weight ratio pH 5 enzymes/ r-RNA	^{14}C-leucine * incorporated per mg RNA (30 min.) cpm
	Medium	Conditions				
I	A	DOC,PMS 0.025 M KCl 2 M sucrose 4 hour	0.201 0.134 0.067 0.0624	1.558 1.558 1.558 3.50	7.7 11.5 23.0 55.9	877.12 1123.14 1234.14 3177.0
	Modified** A	0.025 M KCl 0.25 M sucrose 1 hour	1.4 0.8032	3.50 1.558	2.5 1.9	940.2 854.3
II	A	DOC,H 0.05 M KCl 2 M sucrose 4 hour	0.12	1.196	10.0	2805.4
	B	Triton X-100, H 0.05 M KCl 1.5 M sucrose 2.5 hour	1.6664 0.8332 0.5176 0.2 0.1	1.396 1.396 1.396 1.474 1.474	0.7 1.4 2.3 7.4 14.7	320.27 461.48 464.90 2312.25 2813.0
		Triton X-100, H 0.05 M KCl 2 M sucrose 4 hour	0.134 0.079	1.350 1.408	10.0 17.85	3747.8 3610.8
	B	Triton X-100, H 0.25 M KCl 4 hour, 2 M sucrose	0.064	1.408	22	3844.5

Abbreviations and methods are the same as Table 1. * Incubation mixtures as in "Materials and Methods." Concentration of ribosomes and pH 5 enzymes as indicated. Each value is the mean of duplicate samples, the variation being within ±10 percent. ** See microsome preparation in "Materials and Methods."

systems containing different preparations of ribosomes. Group 1 and 2 are not directly comparable because different lots of leucine were used.

Both groups of experiments do show that the various preparations of ribosomes were active in the incorporation of amino acid into protein. However, the activity was not proportional to the concentration except at low concentration of RNA. When the RNA concentration is compared to that of the pH 5 fraction there is some suggestion that the pH 5 fraction may be a limiting factor. If it is, then the effect is greater at high concentrations of RNA than at low and the effect may also depend upon the way in which ribosomes were prepared. As shown in Table 3 ribosomes in medium B with Triton X-100 were no more active than the controls when combined with twice as much pH 5 and Sephadex fractions were added. Ribosomes prepared in media with an elevated concentration of KCl showed a much higher activity in the presence of greater amounts of soluble factors. The activity was reduced by the absence of soluble factors and was entirely dependent on the presence of the ribosomes. Experiments were also performed in which ATP, GTP and the generating system were omitted from the reaction mixture. The results obtained were variable but overall, these components were not essential to the reaction.

Figure 6 shows the results of a time course study of the

Table 3. Effect of various additions and omissions on amino acid incorporation in vitro by two different ribosomal preparations.

Incubation medium	Standard ribosomes	High-salt ribosomes*
Complete	100	100
Minus pH 5 enzymes	40.75 ±16.43**	---
Minus G-100 Sephadex fraction	45.68 ±12.16**	---
Minus pH 5 enzymes and G-100 Sephadex fraction	18.22	21.04
Double pH 5 enzymes and G-100 Sephadex fraction concentration	101.33	148.54
Minus ribosomes	0 or trace	0 or trace

*Ribosomal preparation the same as standard ribosomes except 0.25 M KCl instead of 0.50 M KCl during isolation procedures.

**Mean values shown ±standard error of mean (at least 3 duplicates). The incorporation tubes contained the standard mixture described in "Materials and Methods" with the indicated addition or omission. The incubation time was 30 minutes. Duplicate samples corrected for zero time.

Figure 6 Time course of the incorporation of ^{14}C-leucine into protein in a cell-free system. The flasks contained 0.064 mg RNA (standard ribosomes) or 0.079 mg RNA (ribosomes prepared in 0.25 M KCl) in 0.5 ml standard incubation medium at 30° C, 50 μl samples were taken at the intervals shown and counted as described in "Materials and Methods." Each point on the graph is the average of duplicate samples.

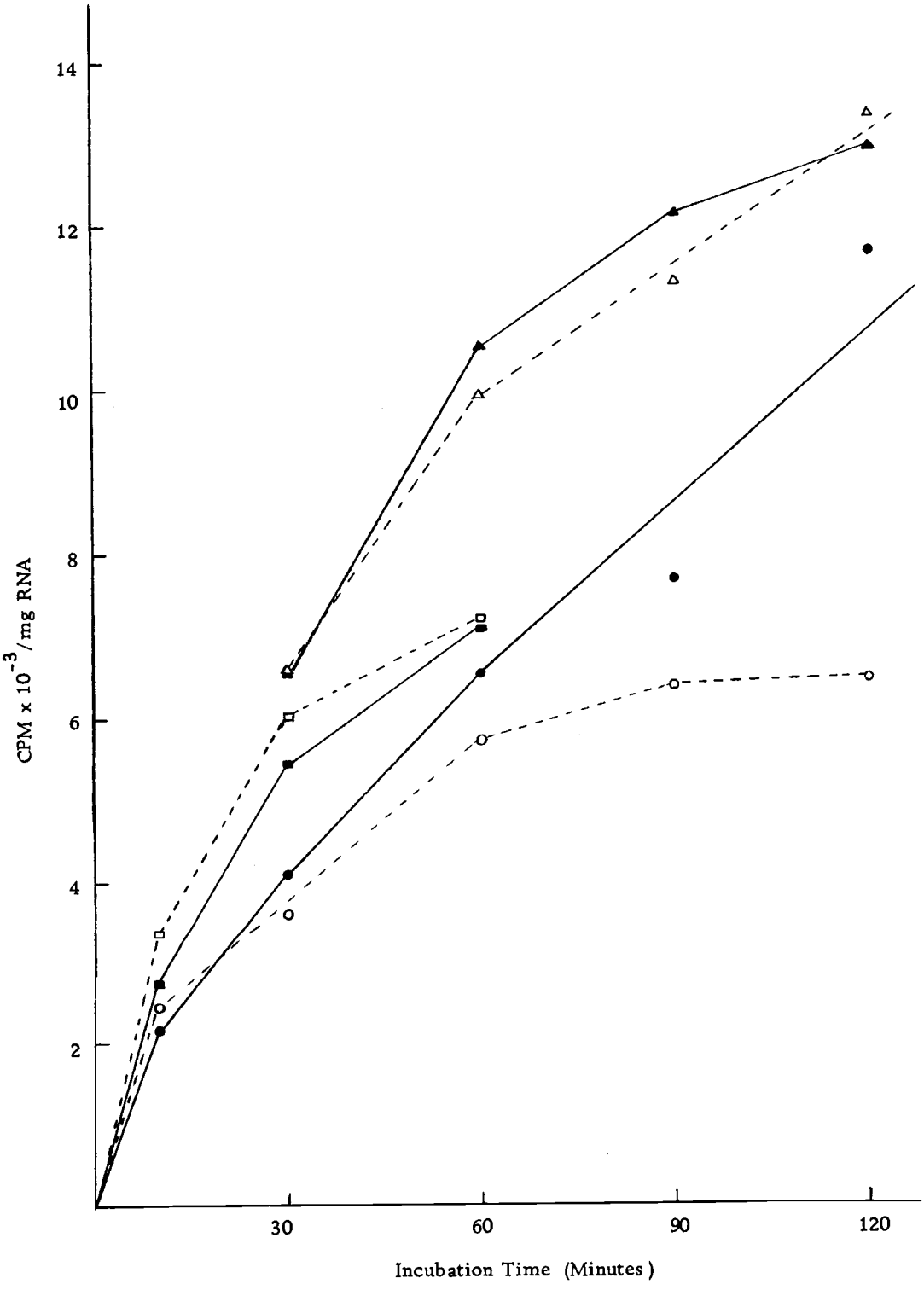
A. Standard Ribosomes: o △ □

B. Ribosomes prepared in 0.25 M KCl: • ▲ ■

(o or •) control

(△ or ▲) 9.2 μg RNase was added 20 minutes after incubation.

(□ or ■) ribosomes were pre-incubated with 9.2 μg RNase for 10 minutes at 30° C before adding other factors.


14
12
10
8
6
4
2
CPM x 10^{-3}/mg RNA
30
60
90
120
Incubation Time (Minutes)

incorporation of ^{14}C-leucine into proteins in a cell free system. The incorporation proceeded at a gradually diminishing rate for at least 90 minutes. However unexpected results were obtained when ribonuclease was added to the incubation medium. Rather than decreasing the activity, the enzyme increased the rate of incorporation and the effect was more pronounced when added 20 minutes after the incubation than 30 minutes before. Ribosomes prepared in 0.25 M were more active than those prepared in low salt buffer but this effect was not noticeable in the presence of RNase.

V. DISCUSSION

Ribosomes have been prepared from a number of species of insects (5, 13, 19, 21, 22, 27, 28, 34, 35, 37, 41, 43, 45, 46, 63, 68, 72). In comparison with mammalian ribosomes, insect preparations in general suffer from low yield and their protein activity in cell free system is also relatively low. In order to obtain the adequate yields of ribosomes, insect tissues have been homogenized in the presence of detergents (5, 40). Adding DOC to the homogenizing medium of fat body rather than to the PMS also improved the yield. When Triton X-100 was used in place of DOC, there was less variability in the amounts of ribosomes that were extracted.

More ribosomes were recovered when the PMS was centrifuged through 1.5 M sucrose than 2 M sucrose. The lower yields obtained with the higher concentration of sucrose did not represent a purification since the ratio of RNA to protein was the same as in ribosomes prepared in 1.5 M sucrose.

The capacity to incorporate amino acids into protein is one measure that is always used to assess the quality and purity of ribosomes. Ribosomes isolated from fat body by a variety of procedures were active by this standard and there was no activity when ribosomes were omitted from the reaction mixture. However, ribosomes were most active when present in very small quantities and there

was usually a poor correlation between ribosomal concentration and incorporation activity. One possible explanation for this is that other components of the reaction were limiting and the most likely components are those supplied by the pH 5 and Sephadex fractions. The remaining components of the incorporation medium were present in amounts found adequate for protein synthesis in cell free systems prepared from a wide variety of animals tissues. Studies of protein synthesis in other species of insects have usually shown that the so called soluble factors, supplied as a supernatant fraction that remained after removal of the microsomes by high speed centrifugation, are either inhibitory or without a stimulating effect (35, 40, 63). Soluble factors for insect studies have usually been prepared from the tissues of other animals or from E. coli (34, 40, 63). The soluble factors in the present work were obtained from the fat body and were divided into a pH 5 and a high molecular weight (Sephadex) fraction. The addition of both of these fractions was essential to the activity obtained. However, optimal ratio between the concentration of ribosomes and the concentration of soluble factors were not worked out.

Further work on incorporation was discouraged because of uncertainties about the ribosomal preparation. Ribosomes, regardless of the means of preparation always displayed an unusual profile in sucrose gradients with most of the material sedimenting as a

heavy fraction. That this material was ribosomal was shown in two ways: (1) most of the material was reduced to ribosomal subunits by treatment with high concentrations of KCl and (2) the pellet that accumulated during the density gradient centrifugation contained ribosomal RNA.

A survey of the literature showed that the state of ribosomal aggregation, based on profile analysis varies widely depending upon the tissue from which ribosomes are extracted. The vertebrate liver which synthesizes a wide variety of proteins both for intracellular use and for transport, has a complex pattern of ribosomal aggregation with the largest particles containing more than 20 ribosomes per aggregate (54). Reticulocytes which synthesize only one relatively small peptide have ribosomal particles consisting mostly of pentameres (58). Sucrose gradients ranging from 10 to 30 per cent would be expected to retain particles as high as 40 n during a two hour centrifugation at 64, 000 g. Reports describing ribosomal profiles rarely mention the formation of a pellet and one must assume that if pellets occur, they represent a minor fraction of the total sample. In liver, for example, the pellet accounts for 20 percent of the total fraction (73).

The fat body of insects combines the function of the vertebrate adipose tissue and liver. It synthesizes and stores large quantities of fat, protein and glycogen and synthesizes and secretes a variety

of blood proteins (42). Under the influence of juvenile hormone, the fat body of L. maderae also synthesizes a substantial quantity of a specific protein during prolonged periods of vitellogenesis (7). This protein appears to be a multiple of a subunit with a molecular weight of 60,000 to 70,000 (Brookes, unpublished). Using the calculations of Noll et al. (54) to estimate the length of messenger chain that encodes the necessary information for a peptide of this size, and the number of ribosomes such a chain can bind, synthesis of the peptide may take place on an aggregate of 20 ribosomes. Considering the function of the fat body and its response to juvenile hormone, the ribosomal profile should resemble that of liver with possibly a concentration of large particles sedimenting in the lower third of gradients commonly used for profile analysis. Why then do the ribosomes extracted from fat bodies have an atypical ribosomal profile? Since I can find no other report of a similar phenomenon in the literature, the profiles obtained are assumed to be the result of an artifact.

One way in which such an artifact may occur is through the nonspecific binding of the ribosomes to protein. The isolation of ribosomes from collagen-producing tissues of chick embryoes resulted in aggregation attributed to binding of protein to ribosomes. The condition was alleviated by increasing the concentration of K^+ in the isolation medium from 0.12 to 0.24 M. Kaulenas (40) found

that ribosomes isolated from the whole bodies of crickets were recovered in poor yield unless the medium contained 0.25 M KCl. The presence of 0.25 M KCl in the isolating medium used for fat body did not improve the yield but it did reduce the amount of pellet. However, large aggregates were still present in the 60 percent sucrose cushion. The monosome peak was larger and a typical polysome profile was not obtained. Similar results were obtained with polysomes from Drosophila (68). The higher concentration of K^+ improved amino acid incorporation in that the ribosomes were active for a longer period of time.

High concentrations of Mg^{2+} result in the formation of heavy aggregates that centrifuge to the bottom of the tube in density gradients (6, 65). This represents another explanation for the results obtained in the present work. Insect blood is reported to contain high concentrations of Mg^{2+}, as much as 73 meq per liter (12, 78). Unfortunately, the concentration in L. maderae is not known. Assuming that it is high, the Mg^{2+} would very likely be present during the homogenization because the blood clots and is difficult to wash away from the fat body.

Endogenous or exogenous RNase hydrolyses mRNA and reduces polysomes to monosomes or small aggregates (23, 58, 73). At high concentrations the enzyme destroys the monosomes. Concomitantly, amino acid incorporation is also reduced or eliminated. Resistance

of ribosomal aggregates to the action of the enzyme has been reported but the reasons for the resistance are not clear (3, 30, 41, 68, 72). Resistance is sometimes eliminated by prior treatment of ribosomes with trypsin suggesting that ribosomes may be protectively bound to protein (41, 68, 72). Stafford et al. (68) found that in Drosophila resistance depended upon the ionic concentration of the suspending medium and disappeared when the KCl concentration was increased from 0.05 M to 0.1 M. Heavy aggregates brought about by high concentration of Mg^{2+} were reported to be resistant to RNase (65). Fat body ribosomes are at least partially resistant to RNase. At low levels of the enzyme the amount of the pellet was reduced. The remainder of the profile (Figure 5) is reminiscent of one obtained from houseflies where RNase was said to aggregate monosomes (72). The reduction of the monosome peak and an increase in absorbing material in the effluent volume between 18 and 28 ml might suggest some aggregation. However, since there was also a reduction of the pellet, a more plausible interpretation is that the enzyme brought about a general shift from heavy to lighter aggregates and some degradation of the monosomes to material that appeared at the top of the gradient. Higher concentrations of RNase eliminated the monosome peak but some material was still found in the sucrose cushion. A surprising effect of RNase was the enhancement of the amino acid incorporating activity. In all other studies the enzyme

either reduced or eliminated the activity.

In conclusion, the substantial heavy fraction that is present in ribosomal preparations from the fat body of L. maderae probably consists of ribosomes that are largely inactive in protein synthesis. Subjecting the ribosomes to various conditions that reduced the amount of heavy material also improved the capacity to incorporate amino acids. Whether such conditions released ribosomes from nonspecific bonds to protein or interfered with a binding brought about by high concentrations of Mg^{2+} cannot be determined from the data. Some idea as to the cause of aggregation may be obtained by determining the actual amount of Mg^{2+} present in ribosomal preparations and by studying the effects of EDTA, different concentrations of Mg^{2+}, and proteolysis, on ribosomal profiles.

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