STUDIES ON RABBIT RETICULOCYTE RIBOSOMES AND POLYRIBOSOMES AND THEIR RELATION TO HEMOGLOBIN SYNTHESIS

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ABSTRACT

This dissertation is divided into three parts.

The first section is concerned with protein synthesis in cell-free systems from reticulocytes. The sub-cellular reticulocyte fractions, reagents, etc. have been examined for the presence of traces of ribonuclease, using an assay based upon the loss of infectivity of RNA from bacteriophage MS2. This assay is sensitive to 5×10^{-7} γ RNase/ml. In addition, the loss of synthetic capacity of an 80S ribosome on dissociation has been studied, and can be attributed to loss of messenger RNA when the monomer is separated into subunits. The presence of ribonuclease has been shown to be a major cause of polyribosome disintegration during cell-free protein synthesis.

The second section concerns the changes in ribosomes and polyribosomes which occur during the maturation of a reticulocyte into an erythrocyte. With increasing age, the cells lose a large proportion of the ribonucleoprotein, but the percentage of ribosomes present as polyribosomes is only slightly altered. The loss of hemoglobin synthesis on maturation is probably due to both the loss of total ribosomes and to the lessened specific activity of the polyribosomes.

The third section contains analytical ultracentrifugation data on 80S ribosomes, polyribosomes, and ribosomal RNA from reticulocytes. The 60S and 40S subunits, obtained by dissociation of the 80S particle with inorganic pyrophosphate, were also studied. The RNA from reticulocyte ribosomes has been examined under a variety of denaturing conditions,

including dimethyl sulfoxide treatment, formaldehyde reaction and thermal denaturation. From these studies we can conclude that the 28S and 16S RNA's are single polynucleotide chains and are not made up of smaller RNA subunits hydrogen-bonded together.

PREFACE

This thesis is the product of several years of research on ribosomes and polyribosomes from rabbit reticulocytes and their relation to protein synthesis. Since various conceptual as well as technical approaches have been used in these studies, they cannot be successfully integrated into a simple statement of a single problem and its solution. For this reason the dissertation is divided into three sections: Part I on ribosomes in cell-free synthesis systems derived from reticulocytes, Part II on hemoglobin synthesis in cells in various stages of maturity, and Part III on analytical ultracentrifugation of reticulocyte ribosomes, polyribosomes, and ribosomal RNA.

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PART I

Ribosomes and Hemoglobin Synthesis in Cell-free Systems from Reticulocytes

A. Introduction

The scientific progress which has been made in the last few years toward elucidating the mechanisms by which proteins are synthesized in living cells is impressive. From the first primitive systems devised in 1954 to determine the prerequisites for polypeptide synthesis (1), research in this field has evolved to the sophisticated techniques used today to "translate" the DNA code (2). Many experimental approaches have been used to determine the basic events which occur during the synthesis of a polypeptide chain. The resulting scheme for protein synthesis was reviewed by Watson in 1964 (3). In the past two years this scheme has been refined (but not substantially altered) to include new information on peptide chain initiation (4,5), chain termination (6), direction of reading of the messenger RNA (7), peptide bond formation (8), and the primary structure of the amino acid acceptor RNA's (9). The following brief outline of the current view of the mechanism of protein synthesis is presented as a background for the studies in this thesis.

The first step necessary for protein synthesis is the transcription of information stored in the DNA molecules into single stranded RNA. This RNA "message" contains the instructions for the production of one or more specific polypeptides. Then, by a complicated

series of events, this information is translated from the four letter nucleotide "code" into a linear sequence of the twenty amino acids, which in turn determines the three-dimensional conformation, function, and other characteristics of the protein molecule. The actual synthesis of an individual peptide chain may be considered as consisting of two phases, the soluble or enzymatic phase, and the ribosomal phase.

The soluble phase includes those enzymatic steps by which an amino acid is activated and joined through its carboxyl group to the 3' hydroxyl of the ribose at the -CCA terminus of its specific acceptor RNA. These steps require only the activating enzymes (one for each amino acid) the amino acids, the S-RNA's, and adenosine triphosphate (ATP) as an energy source.

The ribosome phase, during which these activated amino acids are linked sequentially into a growing polypeptide, requires the proper spatial juxtaposition of the messenger RNA, at least 20 different amino acyl S-RNA's, GTP, and at least two enzymes. These diverse substances are brought together at the ribosome, which serves as a nonspecific synthesizing apparatus, directed by the information from the messenger RNA. Furthermore, two or more ribosomes may simultaneously utilize a single message, "reading" from the 5' hydroxyl end of the polynucleotide (7), and forming the protein from its N-terminal end. The ribosome itself is a very complex structure, and though one can determine the effect of gross physical alterations on its synthetic capacities, the molecular conformation of the "peptide-bond-forming site" is not known (3).

As a tool for studying protein synthesis, the mammalian reticulocyte has proven to be very useful. All of the steps in protein synthesis, with the exception of the initial transcription which cannot take place in an enucleate cell devoid of DNA, have been recognized and studied in these cells (10). Reticulocytes have many advantages; they are readily available, not easily damaged, and synthesize preponderantly a single well-characterized protein, hemoglobin. However, it must be kept in mind that they are the last stage in a "dying" system. In the production of the erythrocyte, a highly specialized but metabolically moribund cell, the reticulocyte is the last stage in which there is any protein synthesis. These cells have no DNA, and are losing their RNA, without the ability to replace it. Such a cell has little or no capacity for adaptation and develops irrevocably into an erythrocyte unless it is destroyed. It should be pointed out, however, that unlike a logarithmic culture of bacteria, a population of reticulocytes is composed of an entire spectrum of cells, from the very active ones recently released from the bone marrow to senescent reticulocytes which are little different from erythrocytes.

Reticulocytes also offer many advantages as a source of a cell-free synthesizing system. They are easily disrupted by gentle means such as osmotic shock; the ribosomal material is initially free in the cytoplasm rather than bound to membranes and is obtainable in pure form by differential centrifugation alone. The messenger RNA (or at least the capacity to support protein synthesis) is stable, as

one would expect in a system from a cell which, in vivo, synthesizes protein for up to forty hours in the absence of nuclear control. The stability is exemplified by the low levels of endogenous nuclease and by the resistance of the ribosomes toward dissociation by chemical means. The experimental disadvantages of reticulocytes are due mainly to their lack of RNA synthesis. This means that it is not possible to introduce a radioactive label into the RNA or ribosomes in vitro; rather one must provide a radioactive substance to the bone marrow cells of the animal and await the appearance of these cells as reticulocytes in the peripheral blood.

Many investigations of ribonuclease (RNase), both in pure form and in various tissues, have been performed (11). However, most of these have been concerned with the characteristics and specificities of the enzyme itself or with the digestion products resulting from its action. The assays of enzyme activity designed for such studies have necessarily involved large amounts of both enzyme and substrate. Classical measurements of RNase activity have been based upon the release of acid-soluble mono- and oligo-nucleotides, which are then determined as milligrams of phosphorus (12), absorption at 260mμ (13) or counts (from radioactive substrate) (14). The sensitivity of these assays depends upon the size of the substrate RNA. For most RNA's a large number of cleavages must take place before soluble products are released linearly with each additional break, and a plot of soluble products versus either enzyme concentration or time (for a single RNase concentration) is sigmoid. The length of the linear region of

this sigmoidal curve depends upon the homogeneity of the substrate as well as its size. Other determinations have used the spectral shift of the UV absorption of RNA to shorter wavelengths with digestion (15) or the splitting of cyclic 2' - 3' phosphates (16). The limit of detectable RNase for most of these measurements is about 0.1γ RNase/ml.

Recently a number of more sensitive assay systems have been developed, and have been used to measure minute traces of RNase present as contaminants in commercial reagents and enzymes. One such method uses polycytidylic acid for the substrate and is reported to detect 10^{-5} γ RNase/ml (17). Holley et al. (18) have used an assay based on the loss of amino acid acceptor ability of S-RNA. Polatnick and Bachrach (19) described the first system using the loss of infectivity of viral RNA to detect RNase. With Foot and Mouth Disease Virus (FMDV) RNA, they were able to measure RNase at 10^{-4}γ/ml. Philipson and Kaufman (20) have employed a similar assay, using RNA from Poliovirus Type I to obtain a sensitivity of 10^{-5} γ RNase/ml. Using the FMDV determination, it was possible to demonstrate levels of RNase contamination in crystalline desoxyribonuclease of 1:100,000 and in crystalline trypsin of 1:10,000 (21).

In the study of protein synthesis, the integrity of the messenger RNA is of greatest importance; for example, just a few scissions from RNase or from other sources can drastically alter the properties of polyribosomes. Therefore, we wished to develop an operationally useful assay which would measure nuclease activity under conditions favorable for protein synthesis, and which would detect RNase at the

level of a few "hits" per molecule. Since a single endonuclease break destroys the infectivity of the RNA of bacteriophage MS2 toward a bacterial protoplast, MS2-RNA can be used as the substrate for a very sensitive RNase assay.

There has been considerable interest in RNases from various organisms and tissues, especially in ribosomal RNases (whether bound to the ribosome or forming an integral part of it). Since the discovery of the ribosomal RNase of E. coli (22), a number of investigators have examined ribosomes from rat liver (23), rabbit liver (24), and rabbit reticulocytes (24,25,26) for the presence of RNase. Adachi et al. (27) have purified an RNase from rabbit reticulocytes and determined its localization in the cell. However, due to the differences in assay systems, it is not possible to compare our results with theirs.

Our work, with both the cell-free synthesis and with intact reticulocytes, has been mainly concerned with the functioning of the ribosomes and polyribosomes in relation to protein synthesis. In this section we shall consider the presence of RNase in the cell-free system (in various types of cells, in commercial reagents, and in subcellular components from reticulocytes), the dependence of synthesis upon the physical integrity of the monomeric ribosomes, and the question of synthesis-dependent breakdown of polyribosomes during a cell-free incubation.

B. Materials and Methods

The materials and methods in this section refer to the experiments in Sections C. and E.

1. Reticulocytes

Reticulocytes were obtained from anemic rabbits as previously described (28) and washed two times by centrifugation in a saline solution containing 0.154 M NaCl, 0.005 M KCl, 0.005 M $MgCl_2$. After each centrifugation the supernatant solution and the buffy coat were removed by aspiration. All subsequent procedures were carried out at 0°C.

2. Preparation of Fractionated Reticulocytes and Sub-cellular Components

Reticulocytes from an anemic rabbit were separated on a BSA density gradient (Part II, Glowacki and Millette, Materials and Methods) and the cells collected as only three fractions for RNase testing as follows: 1) the pellet and the lower third of the gradient, 2) the middle of the gradient, 3) the top of the gradient, excluding the remaining buffy coat. Cells were washed free of BSA and a small amount removed for staining (See Part II, Glowacki and Millette, Materials and Methods).

Fractionated cells or a total reticulocyte population were lysed using one of two lysis procedures; in Method A, the cells were lysed by the addition of an equal volume of cold water; in Method B they were lysed by the addition of three

volumes of 0.001 M $MgCl_2$ in 0.001 M tris, pH 7.6 at R.T., and the solution restored to isotonicity after 60 sec. by the addition of 0.6 volume of 1.5 M sucrose containing 0.15 M KCl.

After either lysis procedure, the mixture was centrifuged at 500 g for 10 minutes in a refrigerated Servall, giving a pellet ("Cells") and a supernatant solution which was recentrifuged for 10 min. at 12,000 g. The pellet from the 12,000 g centrifugation is referred to as the "Debris" fraction and the supernatant solution as "Lysate". After removing an aliquot of this "Lysate" for RNase testing, the remainder of the solution (usually 0.5 to 6.0 ml for various preparations) was diluted to 7.0 ml with Solution G and layered over 4.0 ml 30% sucrose in Solution G and centrifuged in a Spinco angle rotor at 105,000 g for 3.5 hours. After centrifugation, the upper 7.0 ml were removed from the tube ("Supernatant") and the sucrose layer was discarded. The pelleted ribosomes were rinsed once with Solution G (0.08 M KCl, 0.005 M $MgCl_2$, 0.01 M tris, pH 7.6 at 20°C.) and frozen immediately.

To the pellets referred to above as "Cells" and "Debris" were added volumes of Solution G proportional to the original cell volume, and these pellets were resuspended with a Vortex mixer. The resulting suspension was frozen and thawed two times, with vigorous Vortex mixing after each thawing. They were then centrifuged for 10 min. at 17,000 g. The supernatant solutions

from this centrifugation formed the "Cells" extracts and "Debris" extracts for RNase determinations; the pellets were discarded. Ribosomes were stored as pellets at -70°C. until assayed. All other fractions were stored at -20°C.

Lysates were prepared in a similar fashion from normal rabbit erythrocytes and from bone marrow cells. They were centrifuged once for 10 min. at 12,000 g to remove insoluble material, but no further fractionation was made. Purified bone marrow cells from normal rabbits were provided by Dr. Henry Borsook.

Preparation of subcellular fractions for the cell-free experiments described in Section E was the same as for the RNase experiments, with the further modification that polyribosomes were purified in the following way: Ribosome pellets were gently resuspended in Solution G using a Pasteur pipette. (This sometimes required up to one hour in an ice bath.) At the end of this time the solution was centrifuged at 3000 g for 5 min. and the pellet discarded. Two ml of the ribosome solution was layered onto each of three sucrose gradients (50 ml gradients, 15-30% sucrose in Solution G). These gradients were centrifuged for two hours at 25,000 rpm (75,000 g) in a Spinco SW 25.2 rotor and decelerated without brake. All three gradients were collected simultaneously using a Technicon Proportioning Pump. Fifteen samples (3.5 ml each) were collected from each gradient into centrifuge tubes. Preliminary dilutions were made to determine the position of the optical

density peaks, and 12 samples from each gradient (usually numbers 2 - 13) were diluted to 11 ml with Solution G and centrifuged at 105,000 g for 3.5 to 4 hours. The supernatant solutions were discarded and the pellets stored at -70°C. until used.

3. Assay Procedure for RNase using MS2-RNA and Protoplasts

An aliquot of the sample to be tested was added to an equal volume of the MS2-RNA solution, containing 2 x 10^{12} molecules/ml in 0.05 M tris (pH 7 at 37°C.), in an ice bath. After removal of the zero time aliquot, the solution was incubated at 37°C. and aliquots were removed at ten minute intervals for 60 min. These aliquots (0.1 ml of the incubation mixture) were diluted 100 fold into ice-cold 0.05 M tris. Duplicate 0.4 ml portions of the dilutions were pipetted into tubes for the protoplast assay and frozen immediately in an acetone dry-ice bath. Samples were stored frozen until a few minutes before the infectivity assays. Protoplasts were prepared and the infectivity assays performed according to the method of Guthrie and Sinsheimer (29) using the modification of Strauss (30). Control incubations consisting of the RNA in 0.05 M tris alone were always included, since the efficiency of the infectivity assay depends upon both the protoplast stock and upon the RNA preparation. These controls also demonstrate that in the absence of nuclease the RNA was stable for 60 min. at 37°C.

The infectivity assay for MS2-RNA is linear with the RNA concentration (30). We assume that one break is sufficient to destroy the infectivity of the RNA and that all RNA molecules are equally susceptible to RNase. Strauss (personal communication) has been able to show that the loss of infectivity is directly related to the loss of material from the leading component (27S) of MS2-RNA seen in analytical ultracentrifuge patterns. The fraction of surviving infectivity at any time is given by the zero order term of a Poisson distribution, $P(0) = e^{-kt}$, where k is the mean number of bonds cleaved per molecule per unit time and t is the time. The total number of bonds cleaved per unit time is then $10^{12} k$. In practice, k is evaluated from a semi-logarithmic plot of plaque titer *versus* time. The turnover number, $\S$, (bonds cleaved/min./RNase molecule) for pancreatic RNase has been determined in this system to be 1700. (Note that there are 4.4×10^{13} molecules of RNase/γ of enzyme.) These values were used to convert the value of k found experimentally for each sample into the more familiar units of "equivalent γ of pancreatic RNase/ml." Most samples were diluted to give an average of 3 to 5 hits/molecule/60 min. or 0.5 to 5% survival, and for ease of comparison have been expressed as equivalent γ of pancreatic RNase/ml packed cells for the cell fractions, or per ml solution for the reagents.

4. Other RNase Assays

RNase assays were also performed according to the method of Kunitz (12), measuring the optical density of the soluble products after precipitation of the bulk RNA with MacFadyen's Reagent (31). The substrate was highly polymerized yeast RNA obtained from California Biochemical Company. Details of this assay are given in the legend to Figure I-1.

RNase determinations using C^{14}-RNA as a substrate were also performed for comparison. The labelled RNA used was a heterogeneous preparation of C^{14} MS2-RNA with a sedimentation constant of approximately 4S which had been isolated from MS2 phage grown in C^{14}-uracil. Samples containing RNA and enzyme were incubated for 60 min. at 37°C. At the end of the incubation period samples were chilled, 50 γ of DNA per sample added as carrier, and the samples were precipitated in 7% TCA. After 60 min. on ice, they were filtered onto Millipore filters (HA, 0.45 μ pore size) and the filters counted on planchets in a Nuclear Chicago gas flow counter.

The ribonuclease used as a standard in all of these experiments was Bovine Pancreas RNase Type IA, 5 times crystallized, protease-free, obtained from Sigma Chemical Company. The activity given on the label was 60 KU/mg., where one KU is that amount of enzyme capable of causing the maximum possible decrease in absorption at 300 mμ of a 0.1% solution of yeast RNA in one minute at 25°C. (15).

MS2-RNA for the infectivity assays was provided by James H. Strauss, Jr. and the concentration was determined spectrophotometrically (32).

5. Cell-free Incubation Mixtures and Reagents

Various incubation mixtures, with either the Lysate (Method A) or purified polyribosomes and/or 80S particles plus supernatant, were used for various experiments. The conditions for each incubation are given in the legends to the figures and tables. However, most of these used the "Standard Reagent Mixture". Standard Reagent Mixture contained (per ml) 91 μ moles creatine phosphate, 5 μ moles ATP, 850 γ creatine kinase, 1 μ mole GTP, and 0.3 ml of a mixture of 20 amino acids minus leucine (33). Reagent mixes lacking one or more components were made up to the same volume with Solution G.

Creatine phosphate was obtained from California Biochemical Company, creatine kinase (ATP: creatine phosphotransferase, International Union of Biochemistry number 2.7.3.2) came from either California Biochemical Company or Sigma Chemical Company (see Section C), ATP and GTP were obtained from Pabst Laboratories. Reduced glutathione (GSH) was obtained from Sigma Chemical Company and added to the incubation mixtures as a 0.5 M solution at pH 6.0. Uniformly labelled C^{14}-leucine was obtained from New England Nuclear Corporation.

6. Sucrose Gradient Analyses

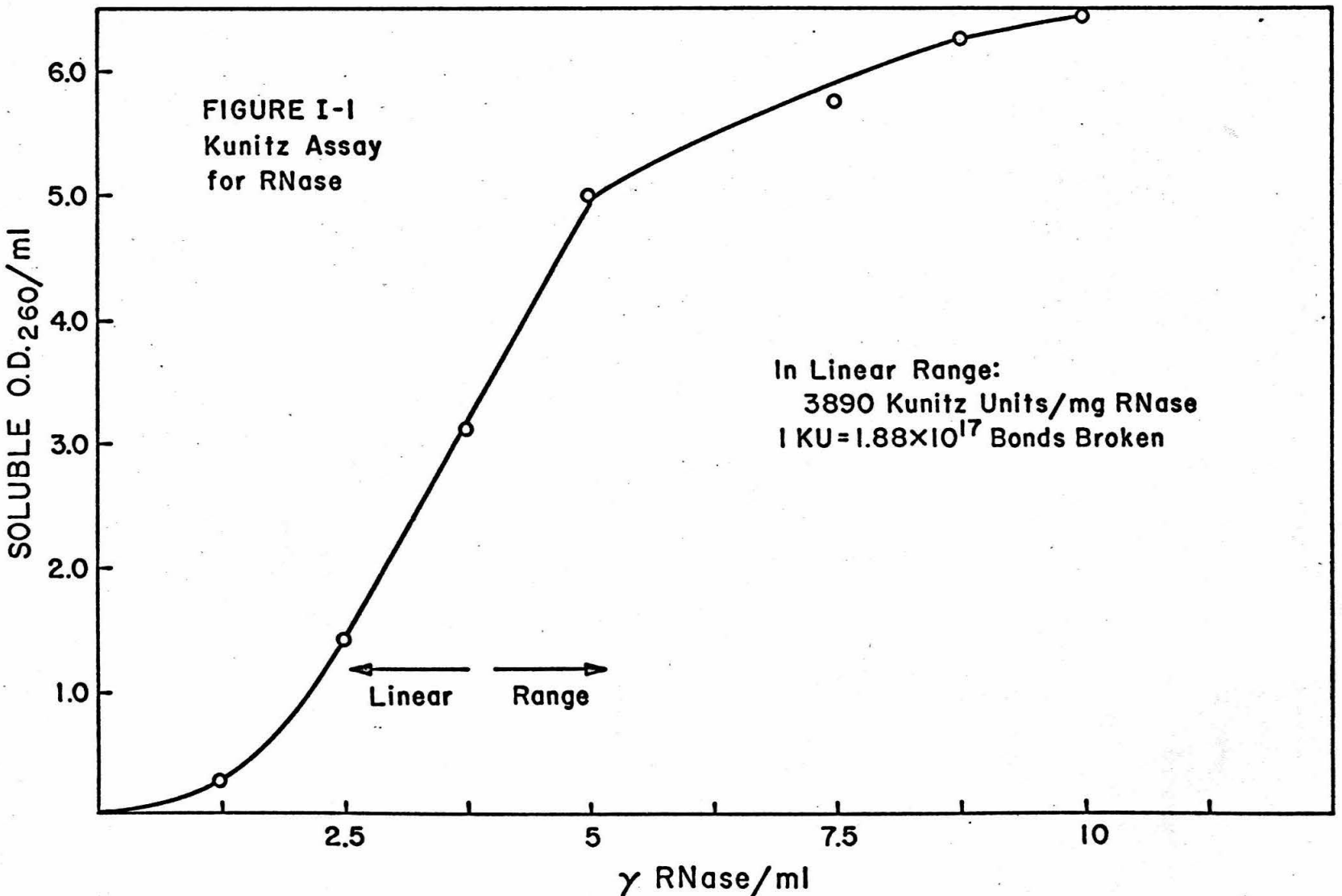
Sucrose gradients of incubations (unless otherwise noted) were 25 ml gradients of 15-30% sucrose in solution G and were spun for 2 hours at 25,000 rpm (63,600 g) in an SW25.1 rotor and decelerated without brake. One ml samples were collected by pumping with a Technicon Proportioning Pump and samples frozen at -20°C. until analyzed. The optical densities were read on a Beckman DK2 Recording Spectrophotometer. Samples were precipitated in 7% tricholoracetic acid (TCA) at room temperature, using unlabelled reticulocyte lysate as carrier, and the precipitates filtered on Millipore HA filters (0.45 μ pore size). The filters were counted on planchets in a Nuclear Chicago Gas Flow Counter.

C. Determination of RNase in Reagents, Various Types of Cells, and Sub-cellular Fractions from Reticulocytes.

Kunitz (12) has defined a Kunitz Unit (not to be confused with the KU described in Materials and Methods) in terms of the soluble phosphorus released by enzyme action as follows: 1 KU = 10^{-3} mg P released/ml of enzyme digestion mixture/10 minutes (where the enzyme digestion mixture contains 0.25 mg total RNA phosphorus /ml). We have calculated that 10^{-3} mg P is equivalent to 0.25 optical density units of nucleotides at 260 mμ. Our standard RNase preparation gave the data shown in Figure I-1, or a value of 3890 KU/mg enzyme using yeast RNA as substrate. The linear range of the assay is very short, and occurs between 5 and 10 γ RNase/ml of digestion mixture or 2.5 and 5.0 γ RNase/ml of total mixture.

The second variety of assay used was the loss of TCA precipitable counts from a radioactive substrate. A calibration curve for this assay using the standard RNase solution is shown in Figure I-2. This assay is roughly 100 times as sensitive as the Kunitz assay and gives a linear response between 0.01 and 0.04 γ RNase/ml of digestion mixture. However, since the linear range is so short, it is not very useful for quantitative measurements.

Figure I-3 shows the type of inactivation curves obtained with the standard RNase and MS2-RNA in the infectivity assay. From the slope of this line one obtains the turnover number of 1660 bonds cleaved/ molecule RNase/min. This value agrees with that found by Strauss in this system (Strauss, personal communication). This corresponds to



Legend on page 17.

Figure I-1 Kunitz Assay for Pancreatic Ribonuclease

The enzyme digestion mixture, containing 0.25 ml of yeast RNA at 4.35 mg/ml in 0.1 M Na acetate at pH 5.0 and 0.25 ml of pancreatic RNase in water, was incubated for 10 min. at 25°C. At this time 0.5 ml of MacFadyen's Reagent was added and the total mixture incubated for an additional 30 min. at 25°C. Samples were filtered through HA Millipore filters and the optical density determined at 260mμ.

The number of bonds broken is determined in the MS2-RNA assay, to show that one KU in the Kunitz assay corresponds to 1.88×10^{17} bonds cleaved in the protoplast assay. No evaluation of the number of bonds cleaved can be made from the Kunitz assay alone.

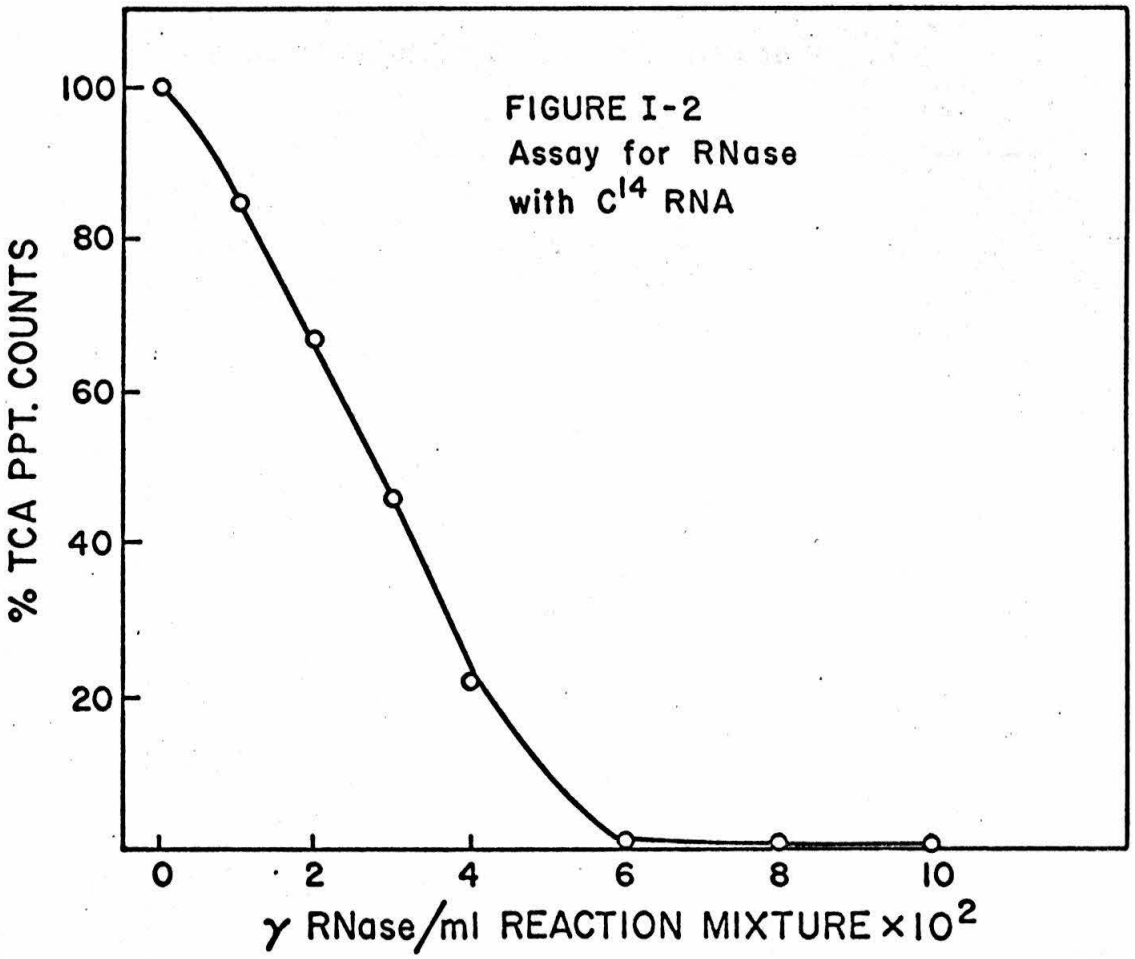
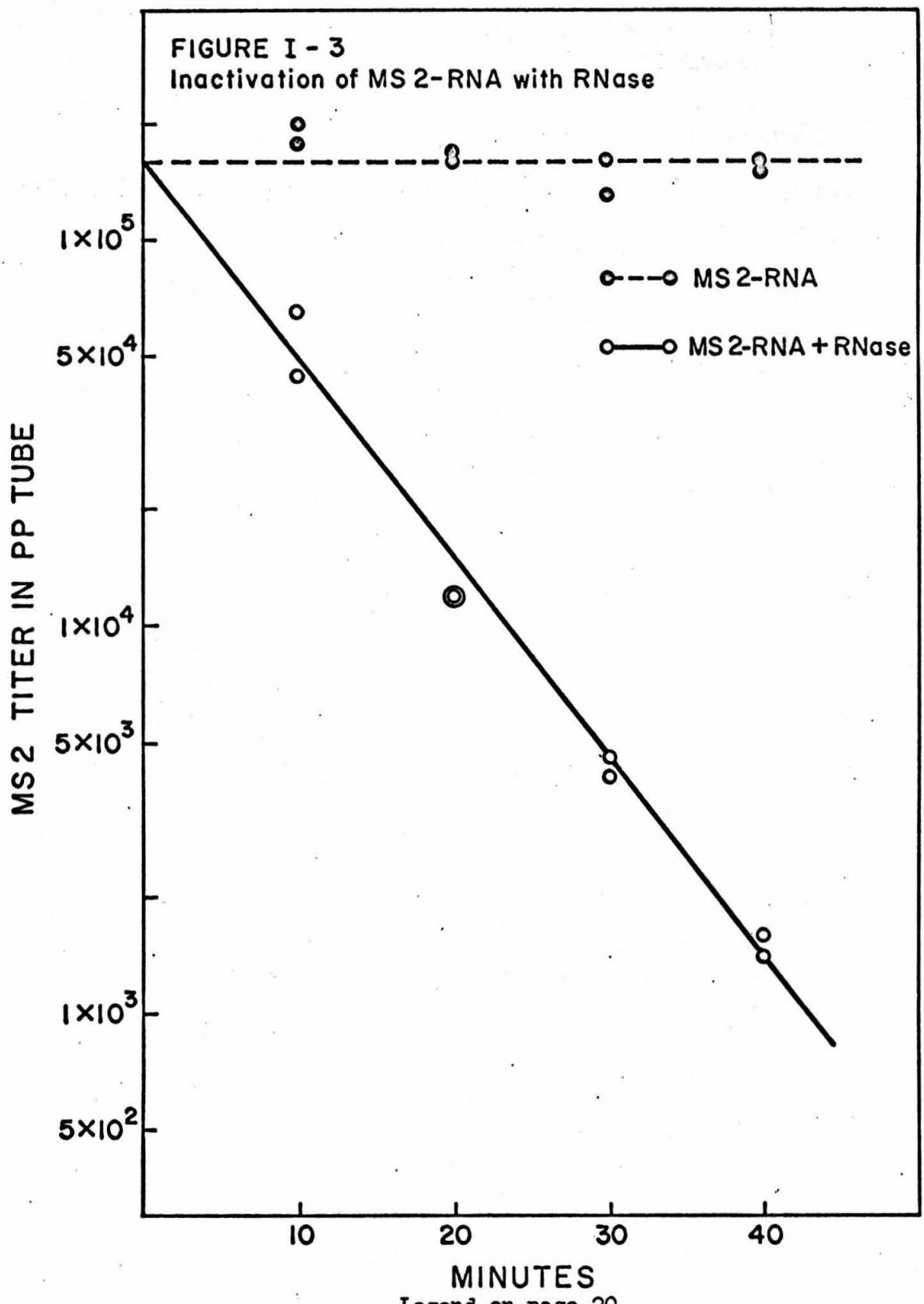


Figure I-2 Assay for RNase Using C^{14} RNA as Substrate

Reaction mixtures containing equal volumes of an RNase solution in H_2O and a solution of C^{14} MS2-RNA in 0.05 M tris, pH 7 at 37°C. were mixed and incubated for 60 min. at 37°C. At this time the samples were chilled and precipitated as described in Methods.

FIGURE I - 3
Inactivation of MS 2-RNA with RNase



Legend on page 20.

Figure I-3 Inactivation of MS2 Infective RNA with Ribonuclease

The original incubation mixture contained MS2-RNA at 2×10^{12} molecules per ml, pancreatic RNase at 1.5×10^{8} molecules per ml, 0.05 M tris and 0.01 M Na phosphate, pH 7.0, and 100 γ BSA per ml. The turnover number (§) is equal to 1660. The dotted line shows the stability of MS2-RNA in 0.05 M tris alone. Protoplast has been abbreviated PP.

7.3×10^{16} bonds cleaved/γ RNase/min. Using RNA at 10^{12} molecules/ml, the assay is linear from about $5 \times 10^{-8}\gamma$ to $2 \times 10^{-6}\gamma$ RNase/ml digestion mixture.

One possible objection to an assay of this type is that one is not sure that the observed inactivation of MS2-RNA infectivity is due to bond cleavage. Other factors to be considered are pH effects (for example, depurination at low pH), or substances in the samples which would interfere with the infectivity assay itself. The pH of many of the incubation mixtures was measured and found to be near neutrality (between 6.5 and 7.0). Due to the low levels of nuclease in most samples, it was not possible to test them in a conventional assay, but for a few of the most active samples, we were able to demonstrate that they would release soluble products from C^{14}-labelled RNA (see Table I-1). The data from the two assays give somewhat different results, but they illustrate that the creatine kinase samples and the bone marrow lysates, which inactivate MS2-RNA, also release soluble nucleotides. Since the linear range of the C^{14} assay is so short, the MS2 assays probably are more reliable.

Some of the samples studied in the protoplast assay gave an initial drop or rise in infectivity after a few seconds at 0°C., followed by a slower exponential decrease in infectivity with time at 37°C. This effect is very sensitive to, and not directly proportional to, the concentration of the sample. The subsequent exponential inactivation is proportional to the concentration of the sample and follows the kinetics expected for nuclease bond cleavage. This initial activation or inactivation is probably due to substances present which interfere

TABLE I - 1

Comparison of Samples Assayed with Both the C^{14} Assay and the Protoplast Assay

Sample	C^{14} RNA Assay		Protoplast Assay
	% TCA Ppt. Counts	Equivalent γ RNase/ml	Equivalent γ RNase/ml
Control	100	none	$< 5 \times 10^{-8}$
Creatine Kinase 5 mg/ml (Sigma 93B-1470)	37.4	$\sim 1 \times 10^{-2}$	5.7×10^{-4}
Creatine Kinase (as above) Heated 10 min. 80°C.	81.7	$\sim 5 \times 10^{-3}$	not determined
Creatine Kinase (as above) Heated 10 min. 80°C., two times	81.7	$\sim 5 \times 10^{-3}$	2.3×10^{-4}
Bone Marrow Cell Lysate	17.1	$\sim 2 \times 10^{-2}$	6.0×10^{-3}
RNase, 0.1 γ/ml	1.3	---	---
RNase (as above) Heated 10 min. 80°C.	5.2	---	---
RNase (as above) Heated 10 min. 80°C., two times	21.1	---	---

Protoplast assays were made on a dilution of the material used for C^{14} RNA assay.

with the assay, perhaps by altering the ionic environment of the protoplast mixture or altering the surface properties of the cells. In such cases the surviving infectivity is calculated from the zero time aliquot rather than from the control RNA sample.

Since pancreatic RNase is stable to heating (11), we also studied the activity of some of the reagent samples before and after heating to 80°C. for 10 minutes. Data on these heated samples are presented in Table I-2. The activity found in the creatine phosphate appears to be completely stable to heating, whereas that found in the creatine kinase, using either the infectivity assay or the C^{14}-RNA assay, is reduced to approximately 40% of the original. This may be due to co-precipitation of RNase with the denatured kinase, for after removing the precipitated protein by centrifugation, a second heating at 80°C. causes no further loss of activity. Moreover, the experimental samples were more stable to heating than the standard RNase.

Our first investigations of RNase with the MS2 infectivity assay were to determine the possible levels of contamination in the reagents added to the cell-free synthesis incubations. These are tabulated in Table I-2. Of the reagents tested, only the mixture of amino acids or a diluted reagent mixture lacking creatine phosphate and creatine kinase showed no inactivation at the concentrations at which they are used in cell-free incubations . Adenosine triphosphate (ATP) had only a very low level of contamination (3×10^{-9}% when expressed as a weight contamination by an equivalent amount of pancreatic RNase), creatine phosphate and phosphoenol pyruvate contained somewhat more (1.4 and

TABLE I - 2

Determination of RNase in Reagents by the Protoplast Assay

Sample	Bonds broken/min/ml	Equivalent γ RNase/ml
Amino acid mixture	no inactivation	$< 5 \times 10^{-7}$
ATP (0.1M, pH 7.0)	1.1×10^{11}	1.5×10^{-6}
Creatine phosphate (Sigma) 5×10^{-4}M	1.6×10^{10}	2.2×10^{-7}
Creatine phosphate (Sigma) 5×10^{-4}M, heated	1.7×10^{10}	2.4×10^{-7}
Creatine phosphate (Cal Biochem) 5×10^{-4}M	1.7×10^{10}	2.3×10^{-7}
Creatine phosphate (Cal Biochem) 5×10^{-4}M, heated	1.7×10^{10}	2.4×10^{-7}
30% Sucrose in Solution G	no inactivation	$< 10^{-7}$
Phosphoenol pyruvate (Sigma) 2×10^{-4}M	2.5×10^{10}	3.4×10^{-7}
Creatine Kinase 500γ/ml		
Sigma 93B-1470	4.1×10^{12}	5.7×10^{-5}
Sigma 93B-1470 heated, 10 min. 80°C.	1.6×10^{12}	2.3×10^{-5}
Sigma 53B-1960	8.5×10^{11}	1.2×10^{-5}
Boehringer 6192104	6.2×10^{11}	8.5×10^{-6}
Boehringer 6103105	5.6×10^{11}	7.7×10^{-6}

Legend to all of Table I - 2 will be found on the following page.

TABLE I - 2 (Continued)

Sample	Bonds broken/min/ml	Equivalent γ RNase/ml
Reagent Mix	1.0×10^{12}	1.6×10^{-5}
+ 0.01 M NaF	9.5×10^{11}	1.4×10^{-5}
+ 0.01 M Na phosphate	1.0×10^{12}	1.5×10^{-5}
- Amino acids, - Creatine phosphate, - Creatine kinase	no inactivation	$< 5 \times 10^{-7}$
- Creatine phosphate, - Creatine kinase	no inactivation	$< 5 \times 10^{-7}$
- Creatine kinase	1.8×10^{11}	2.4×10^{-6}
- Creatine kinase, - Amino acids	2.3×10^{11}	3.2×10^{-6}

Bonds broken/min/ml and equivalent γ RNase/ml refer to the concentration of sample given in the first column although the actual measurements were not all made at this concentration.

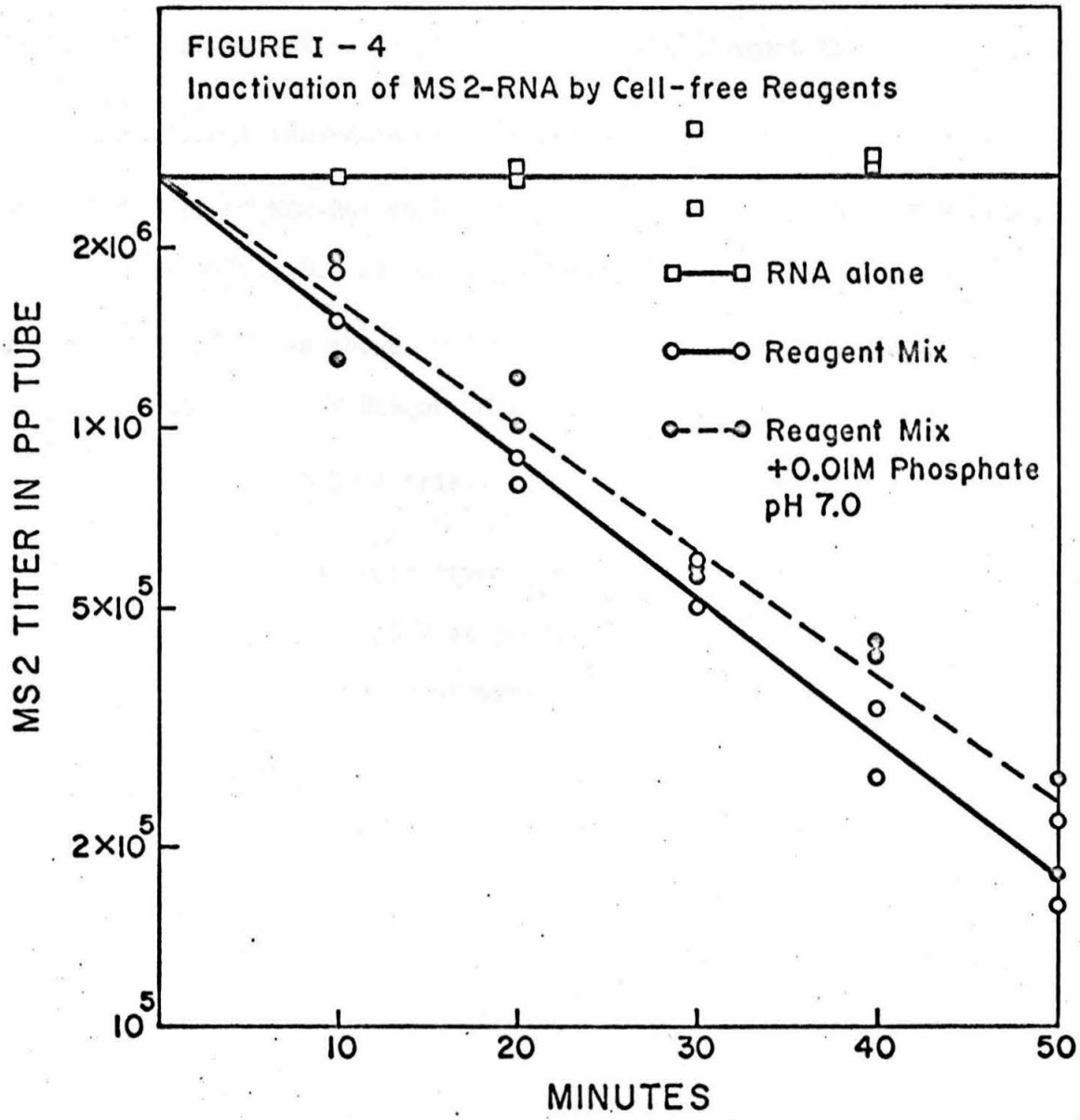
The creatine kinase used in the Reagent Mix was Boehringer Lot 6184310. Reagent Mix has been described in Materials and Methods.

Creatine phosphate samples were heated at 80°C for 10 minutes.

5 x 10^{-7}% respectively), but the greatest amounts were found in the various creatine kinase preparations (0.2 to 1.0 x 10^{-5}%). Different preparations of kinase were tested, including two lots from Sigma Chemical Company, and three from California Biochemical Company (prepared by Boehringer und Soehne, G. m. b. H., Mannheim). The testing of the total reagent mixtures in the presence of 0.01 M NaF and 0.01 M Na phosphate was for another purpose, to be explained in Section E, but is included here to illustrate that there is very little change in activity in the presence of either of these ions. Representative data from which these values were obtained for the reagent mixtures are given in Figure I-4.

A second series of determinations was made to measure the relative amounts of RNase in various types of cells, especially the different classes of reticulocytes. The results obtained from a fractionated cell population using the two varieties of lysis are shown in Table I-3. The amount of RNase in the cells of intermediate age was greater than that found in the youngest cells from the top of the gradient, while the oldest cells and erythrocyte pellets contain much more RNase.

Table I-4 shows the results obtained for various sub-cellular fractions of reticulocytes. We present data using the two different lysis procedures for fractions from two populations of unfractionated reticulocytes and for the youngest cells from the two BSA separations discussed above. The percentage of the total RNase found which is localized in the "Cells" extract is indicated in each case. This was somewhat variable, but for a total cell population approached the levels



Legend on page 28.

Figure I-4 Inactivation of MS2 Infectivity by Reagent Mixture

The original incubation mixtures contained:

O——O 0.5 ml of MS2-RNA at 4×10^{12} molecules per ml in 0.05 M tris, pH 7 at 37°C., 0.4 ml of 0.05 M tris, and 0.1 ml of Reagent Mix.

●- - ● 0.5 ml RNA as above, 0.3 ml tris, 0.1 ml of 0.1 M Na phosphate pH 7 and 0.1 ml of Reagent Mix.

□——□ 0.5 ml RNA, 0.5 ml tris.

Aliquots of 0.1 ml were removed at the times given and diluted 100 fold in 0.05 M tris, pH 7 at 37°C.; 0.4 ml of this dilution was used for the protoplast (PP) assays.

TABLE I - 3

RNase in Cells Fractionated on a BSA Gradient

Cell Fraction	Type of Lysis	Total γ RNase/ml packed cells x 10^3
1 (Pellet and Oldest Cells)	A	44.9
" " " "	B	23.6
2 (Cells of Intermediate Age)	A	4.2
" " " "	B	3.0
3 (Youngest Cells)	A	1.5
" "	B	2.9

Total γ of RNase is obtained from the sum of γ RNase found in the "Cells" extract, "Debris" extract, and Supernatant.

TABLE I - 4a

RNase in Subcellular Fractions from Rabbit Reticulocytes

Preparation	Subcellular Fraction	γ RNase/ml cells	% RNase * in "Cells"
Total cells (1)			
Lysis A	"Cells" extract	1.27×10^{-3}	91
	"Debris" extract	1.41×10^{-6}	
	Lysate	1.75×10^{-4}	
	Supernatant	1.36×10^{-4}	
Lysis B	"Cells" extract	2.47×10^{-3}	92
	"Debris" extract	2.46×10^{-6}	
	Lysate	2.14×10^{-4}	
	Supernatant	7.00×10^{-5}	
Total cells (2)			
Lysis A	"Cells" extract	6.78×10^{-4}	94
	"Debris" extract	3.3×10^{-6}	
	Lysate	2.09×10^{-5}	
	Supernatant	4.59×10^{-5}	
Lysis B	"Cells" extract	4.25×10^{-4}	85
	"Debris" extract	3.84×10^{-7}	
	Lysate	6.5×10^{-6}	
	Supernatant	7.61×10^{-5}	

* % Total RNase in "Cells" Extract

Total RNase is equal to the sum of the Cells Extract, Debris Extract and Supernatant.

Numbers in parentheses are to identify different preparations of anemic blood.

TABLE I - 4b

RNase in Subcellular Fractions from Rabbit Reticulocytes (cont'd)

Preparation	Subcellular Fraction	γ RNase/ml cells	% RNase * in "Cells"
Youngest Cells (3)			
Lysis A	"Cells" extract	1.95×10^{-3}	66.5
	"Debris" extract	5.20×10^{-6}	
	Lysate	7.20×10^{-6}	
	Supernatant	9.9×10^{-4}	
Lysis B	"Cells" extract	9.8×10^{-4}	84
	"Debris" extract	2.67×10^{-6}	
	Lysate	2.80×10^{-5}	
	Supernatant	$1.9. \times 10^{-4}$	
Youngest Cells (4)			
Lysis A	"Cells" extract	1.32×10^{-3}	86
	Lysate	1.18×10^{-4}	
	Supernatant	2.15×10^{-4}	
Lysis B	"Cells" extract	1.24×10^{-3}	60
	Lysate	3.87×10^{-4}	
	Supernatant	8.40×10^{-4}	

* % Total RNase in "Cells" Extract

Total RNase is equal to the sum of the Cells Extract, Debris Extract and Supernatant.

Numbers in parentheses are to identify different preparations of anemic blood.

of 93-99% reported by Adachi (27). This fraction of the RNase may be due either to membrane-bound RNase from reticulocyte ghosts or, more probably, to white cells which are not lysed in the original osmotic lysis and are only disrupted during the later extraction.

In Preparation I, the activity found in the Lysate is greater than that found in the Supernatant after removal of the ribosomes, but in all subsequent preparations the Supernatant shows higher values. From our experiments with ribosomes (see below) we have concluded that this lowered apparent activity in the Lysates is due to the protective effect of the ribosomes.

Table I-5 gives comparative data for lysates from several samples of rabbit bone marrow cells and from a lysate of normal rabbit blood. The erythroblastic cells appear to contain up to 100 times as much RNase per ml of packed cells as the reticulocytes.

No figures for the determinations of RNase in the ribosomes have been given in any of these tables. Ribosomes from all of these cells and a number of separated polyribosome and 80S ribosome preparations have been tested and give no observable inactivation. Suspecting that ribosomes might interfere with the detection of RNase, a series of experiments was performed in which pancreatic RNase was measured in the presence of different amounts of reticulocyte ribosomes. The results of these determinations are shown in Figure I-5. This effect does not appear to be proportional to the number of RNA bonds available from the ribosomal RNA, as the ratio of phosphodiester linkages in ribosomal RNA to those in MS2-RNA was 4 in the first case and 40 in the latter

TABLE I - 5

RNase Determinations on Lysates of Other Types of Rabbit Cells

Type of Cells		Equivalent γ RNase/ml packed cells x 10^3
Normal Rabbit Erythrocytes		1.0
Total Bone Marrow		23.0
Purified Bone Marrow	a)	18.0
	b)	20.0
	c)	8.7
	d)	8.0

Bone marrow preparations a), b), c), and d) were partially purified cell populations, from which most of the erythrocytes and white cells had been removed.

All of these determinations were done on the crude lysates of these cells.

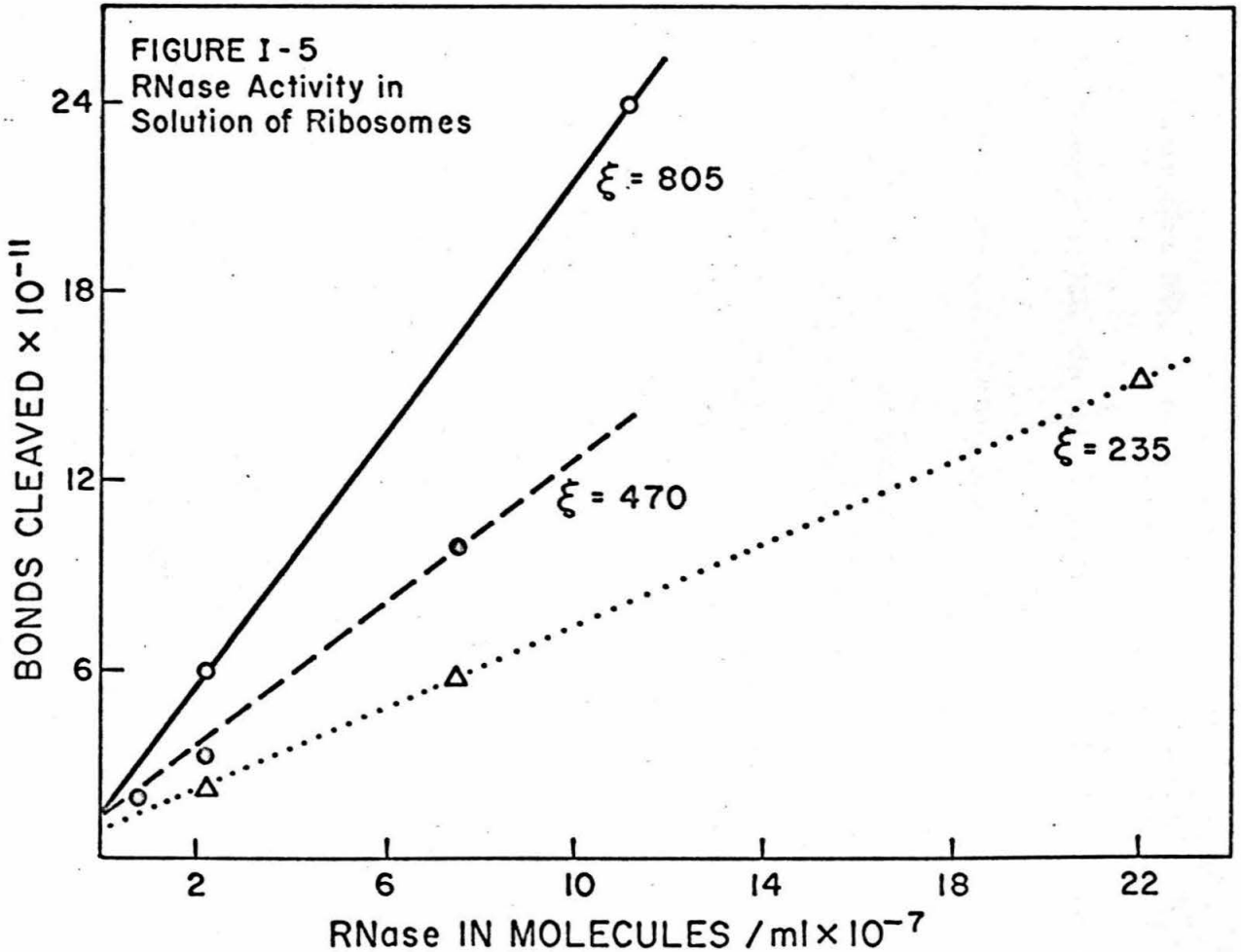


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Figure I-5 Determination of the Activity of Pancreatic RNase in the Presence of Reticulocyte Ribosomes

○——○ RNase plus RNA, no ribosomes

●-----● RNase plus RNA in the presence of 0.169 $O.D._{260}$ ribosomes/ml.

Δ-----Δ RNase plus RNA in the presence of 1.58 $O.D._{260}$ ribosomes/ml.

"Bonds cleaved" is equal to the number of bonds cleaved in 30 min. in a solution of 10^{12} RNA/ml. ξ is equal to the turnover number (bonds cleaved/RNase molecule/min.).

case. The exact nature of the masking effect of ribosomes cannot be ascertained from these experiments, but we can conclude that an accurate determination of RNase in the ribosomes is not possible. A similar masking effect by bacterial ribosomes of RNase has been reported (34). From this experiment, we have determined that the level of endogenous RNase which could be present and undetected in our assays must be less than 5×10^{-7} $\gamma/O.D._{260}$ of ribosomes.

This finding that reticulocyte ribosomes mask the effect of RNase may account in part for the conflicting reports on ribosomal RNase in the literature. Williamson and Mathias could obtain autodegradation of ribosomes only in the presence of 4M urea (25); Stavy et al. (24) found little activity by measuring autodegradation, no increase in activity in the presence of urea and no degradation of an exogenous substrate; while Farkas, et al. (26) found that 0.5% of the ribosomal O.D. was solubilized in 60 min. at 37°C. and that rabbit reticulocyte ribosomes would degrade C^{14}-polyadenylic acid.

These results concerning the amount of RNase in cell fractions and reagents have been used to interpret some of the cell-free incubation results presented in Section E.

D. Controlled Dissociation of Rabbit Reticulocyte Ribosomes and its Effect on Hemoglobin Synthesis.

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Controlled Dissociation of Rabbit Reticulocyte Ribosomes and its Effect on Hemoglobin Synthesis

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The state of aggregation of ribosomes can be ascertained by analytical ultracentrifugation. The extent of aggregation of ribosomes, including those from rabbit reticulocytes, depends on the Mg^{2+} concentration and ionic strength of the medium. In the usual incubation medium in which rabbit reticulocyte ribosomes incorporate amino acids into hemoglobin, the ribosomes exist predominantly as 70 s particles. In solutions without Mg^{2+}, or with low Mg^{2+} concentrations, one 70 s ribosome reversibly dissociates into one 50 s and one 30 s particle.

Various methods are available for the removal of Mg^{2+} from reticulocyte ribosomes in order to achieve dissociation, among them pyrophosphorolysis. By varying the relative concentration of ribosomes and pyrophosphate, any desired extent of dissociation into subunits is feasible.

Ribosomes were tested for their protein synthetic capacity after reversible dissociation. It was observed that once ribosomes had been completely dissociated into subunits they lost the capacity to incorporate amino acids into protein and none of the usual supernatant factors was capable of restoring this property.

The experiments present evidence that in the usual preparation of reticulocyte ribosomes two classes of particles are actively participating in protein synthesis; a small portion—about 5%—of the ribosomes is 10 times more active, and more resistant to dissociation, than the remaining 95% of the ribosomal population.

Introduction

It is now well accepted that ribosomes are intimately involved in protein synthesis and that they are the site of peptide bond condensation in viral, bacterial, fungal, plant and animal cells (Hoagland, 1960).

Ribosomes from a variety of sources have been found to be similar in many respects. Even though they are isolated from tissues in a wide range of particle sizes, the bulk of the ribosomes fall into groups having sedimentation coefficients around 70, 50 and 30 s. Ribosomes isolated from rabbit reticulocytes were shown to contain 82% 78 s, 9% 50 to 60 s and 9% 120 s particles (Dintzis, Borsook & Vinograd, 1958).

The state of aggregation of ribosomes is a function of the ionic strength and the Mg^{2+} concentration of the medium. At optimal concentration of Mg^{2+} the main portion of the ribosomal population has a sedimentation coefficient of about 70 to 80 s, but at lower Mg^{2+} concentrations these particles reversibly dissociate into subunits with S values of 50 and 30 (Hoagland, 1960). Thus Ts'o & Vinograd (1961) dissociated rabbit reticulocyte ribosomes into subunits by dialysis against Mg^{2+}-free media and re-associated them to particles of the original size (70 to 80 s) by dialysis against solutions containing Mg^{2+}. Compared to dissociated *Escherichia coli*

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ribosomes, reticulocyte subunits were inhomogeneous in the analytical ultracentrifuge, and less stable in low ionic strength media.

Susceptibility of ribosomes to disaggregation has been utilized by various investigators (Tissières, Schlessinger & Gros, 1960; Roberts, 1960; Wallace, Squires & Ts'o, 1961) who have attempted to pinpoint the site of protein synthesis on ribosomal particles. In such experiments they dissociated ribosomes which had been prelabeled with radioactive amino acids, and looked for the distribution of radioactivity among the ribosomal fractions. Although this approach allowed Tissières *et al.* (1960) to present convincing evidence concerning the existence of a class of "active 70 s" *E. coli* ribosomes, it does not permit a direct test of the synthetic capacity of the various classes of ribosomal particles.

The main purpose of this paper is to correlate the physical state of ribosomes from rabbit reticulocytes with protein synthesis activity. To make such a study feasible a method was devised for the controlled dissociation of ribosomes. It was desirable that this procedure should yield any extent of dissociation and be sufficiently rapid and mild to permit testing of remaining intact particles for their ability to incorporate amino acids into protein. Since the medium for amino acid incorporation requires the presence of Mg^{2+}, the activity of the dissociated ribosomes was actually measured in the reaggregated state.

Material and Methods

All experiments were carried out at 4°C unless otherwise stated.

Reticulocytes

Reticulocytes were obtained from rabbits (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1952) and ducks (Lamfrom, Hartwell, Stewart & Miller, 1962) and washed as previously described (Lamfrom, 1961).

Preparation of cell fractions

The lysis of reticulocytes and their subsequent fractionation into a ribosome fraction, a pH 5 fraction and a supernatant fraction followed an earlier description (Lamfrom, 1961), except that medium A given in that report has been replaced by a solution C. The composition of solution C is as follows: 0·05 M-KCl, 1×10^{-3} M-tris buffer pH 8·0, $1{\cdot}5 \times 10^{-3}$ M-$MgCl_2$.

A transfer factor was isolated by fractionation of rabbit reticulocyte supernatant on a DEAE cellulose column by a modification of the method of Takanami & Okamoto (1960). A fraction containing the enzyme was eluted at 0·5 mg protein/ml., and was free of hemoglobin and releasing factor (Lamfrom, 1961).

The cell fractions were stored at −80°C and retained full activity for at least 4 months. Ribosomal pellets were preserved under solution C–glycerol (2:1) and all other fractions in the dissolved state. Samples were thawed immediately before use; the ribosomes were dissolved and the ribonucleoprotein (RNP) concentration measured by u.v. absorption (Lamfrom, 1961).

Conditions of incubation

The conditions for incorporation of radioactive amino acids were similar to those reported earlier (Lamfrom, 1961), and are given in the legends to the Tables. In a total volume of 2·7 ml. the incubation mixture contained 0·8 ml. rabbit ribosomes (3·0 mg RNP), 0·4 ml. duck pH 5 fraction (4·0 mg protein), 0·3 ml. rabbit supernatant, 0·35 ml. rabbit transfer factor (1·75 mg protein), 0·45 μmole guanosine triphosphate, 1·8 μmoles adenosine triphosphate, 36 μmoles phospho-creatine, 342 μg creatine kinase, 25·8 μmoles reduced glutathione, 0·18 μmoles glutamine, 0·09 ml. of a complete amino acid mixture minus leucine (Borsook, Fischer & Keighley, 1957), and 0·18 μmoles of [^{14}C]leucine (uniformly labeled L-isomer obtained from Nuclear Chicago) (2310 cts/min/mμmole).

Where necessary, the Mg^{2+} concentration of the ribosomal fraction was brought to $1{\cdot}5 \times 10^{-3}$ M before addition to the incubation mixture.

After incubation for 1 hr at 37°C the samples were cooled in an ice bath. Ribosomes were separated from the soluble proteins by precipitation at pH 5·1, as described (Lamfrom, 1961).

TCA precipitation and counting procedure

The samples were precipitated by TCA, and prepared for measuring radioactivity as reported earlier (Lamfrom, 1961); they were counted on a Nuclear gas-flow counter with micromil end-window. Self-absorption corrections to infinite thinness were made for all samples.

Dissociation of ribosomes into subunits

(a) *Sephadex treatment*

Ribosomal pellets were dissolved in solution C minus Mg^{2+} and passed through two successive columns of G-25 Sephadex (1·3 × 13·0 cm). Solution C minus Mg^{2+} was used for the equilibration of the columns and for the elution of the ribosomes. A 1·5 ml. sample was applied to the first column at 33 mg RNP/ml., eluted and applied to the second column at 16·6 mg RNP/ml., and finally recovered in 3·5 ml. at a concentration of 8·5 mg RNP/ml. Portions for ultracentrifugation were frozen in CO_2– methyl Cellosolve, and stored at −80°C. Samples for measuring protein synthetic activity were used immediately.

(b) *Dialysis*

Ribosomal pellets were dissolved at the desired RNP concentration in solution C minus Mg^{2+}. The solution was then dialysed in EDTA-treated Visking tubing against twice 300 vol. of the same medium for 19 hr. Portions for ultracentrifugation and for incubations were processed as described above for Sephadex-treated samples.

(c) *Pyrophosphate treatment*

The ribosomal pellets were dissolved at the desired RNP concentration in solution C minus Mg^{2+}, containing an appropriate concentration of pyrophosphate (PP) at pH 8·0, and incubated for 10 to 30 min at 0°C. Portions were removed for ultracentrifugation and either analysed immediately or frozen in CO_2– methyl Cellosolve and stored at −80°C for future analysis. The remainder of the sample, to be used for incorporation studies, was treated in the following manner to terminate the PP action. PP was hydrolysed with inorganic pyrophosphatase (PPase) which was kindly supplied by Dr. M. Kunitz, and assayed by his method (Kunitz, 1952). The inorganic P was estimated according to a modification of the procedure by Sumner (1944). Sufficient PPase was added to hydrolyse completely PP in 6 min at 25°C in the presence of $1{\cdot}5 \times 10^{-3}$ M-Mg^{2+}.

Analysis by ultracentrifugation

Portions of ribosome preparations were examined in a model E Spinco ultracentrifuge, equipped with phase plate schlieren optics, at 35,600 rev./min and 4 or 20°C in a 12 mm or 30 mm cell.

Most of the measurements were made at an RNP concentration of 3·7 mg/ml. in media which are specified in the legends. Where necessary a concentration correction was made using a factor found by Sherman & Petermann (1961) for calf liver ribonucleoprotein.

The sedimentation coefficients are given in Svedberg units, normalized to 20°C.

The relative concentrations of the various sedimenting components are expressed in terms of the number of particles in each peak. These were obtained by first determining the relative concentration on a weight basis (c_1) by measurement of the areas under the peaks,† using corrections for radial dilution, but ignoring Johnston–Ogsten effects, and assuming the refractive index for all types of particles to be the same. To calculate the concentration by number of particles the value obtained for c_1 was divided by the molecular weight of the particle under consideration. The molecular weights were estimated from

† The relative areas for the various components were determined by analysis of a single exposure, since the values were the same, within experimental error, as those obtained by analysis of a series of exposures extrapolated to 0 time (= time at speed).

a log-log graph of S versus molecular weight obtained from the combined data of Dintzis *et al.* (1958), Hall & Slayter (1959), and Tissières, Watson, Schlessinger & Hollingworth (1959).

Results

The state of aggregation of ribosomes can be ascertained by analytical ultracentrifugation. The extent of aggregation of ribosomes, including those from rabbit reticulocytes (Ts'o & Vinograd, 1961), depends on the Mg^{2+} concentration and the ionic strength and composition of the medium (Petermann, Hamilton, Balis, Samarth & Pecora, 1958; Ts'o, Bonner & Vinograd, 1958; Roberts, 1960).

The medium which is routinely used for the preparation and incubation of rabbit reticulocyte ribosomes is solution C; in it most of the ribosomes exist as 70 to 80 s particles, while about 6 to 15% of them are present as aggregates of roughly 100, 120, 150 and 180 s, and 5 to 10% are found in the dissociated form.†

A. *Physical state of dissociated ribosomes*

1. *Extent of dissociation of ribosomes into subunits*

In solutions without Mg^{2+} or with low Mg^{2+} concentration (2×10^{-4} M) the ribosomes dissociate into nucleoprotein subunits.‡

Various methods for the dissociation of reticulocyte ribosomes by the removal of Mg^{2+} have already been described in this paper in the section on Materials and Methods. Table 1 shows the maximum extent of dissociation of 70 s particles which

TABLE 1

Maximum dissociation of ribosomes achieved by various procedures

Procedure	% Dissociation
1. Twice through Sephadex	84·4
2. 19 hr dialysis against Mg^{2+}-free medium	97·6
3. 1·3 μmoles pyrophosphate/mg ribosomes	100·00

Conditions for treatment of ribosomes are given under Materials and Methods. RNP concentration during dialysis was 11·7 mg/ml. and during PP treatment 3·7 mg/ml. See text for Sephadex concentrations. Determination of % dissociation is described in text and is based on ultracentrifuge runs at 20°C and 3·7 mg RNP/ml. Prior to ultracentrifugation appropriate dilutions of dialysed and Sephadex-treated samples were made with solution C minus Mg^{2+}.

can be achieved with these three methods. With Sephadex or dialysis, only partial dissociation of ribosomes into subunits can be obtained, and some 70 s particles always remain. With the third method, using PP, complete dissociation of reticulocyte ribosomes into 50 and 30 s subunits is possible.

Figure 1 demonstrates the relationship between the extent of dissociation of the 70 s particles and the PP concentration per mg ribosomes. It is seen that by varying the relative concentration of ribosomes and PP, any desired extent of dissociation

† When reference is made to "undissociated particles" it will include only those with sedimentation coefficient of 70 s and greater; "dissociated particles" will mean those with S value lower than 70, and will almost always be those with 50 s and 30 s; "percent of dissociation" refers to the sum of % of all particles with S value less than 70.

‡ Designation of ribosomal classes as 70, 50 and 30 s has been adopted as a convention and does not represent absolute values (see Fig. 3). These groups correspond to Ts'o & Vinograd's (1961) 80, 60 and 40 s reticulocyte particles.

into subunits is feasible. The degree of dissociation is not strongly temperature dependent; as illustrated by the two curves in Fig. 1 little difference was noted when ultracentrifuge runs were carried out at 4 rather than at 20°C. At lower concentrations of PP/mg ribosomes dissociation is somewhat greater at 20 than at 4°C,

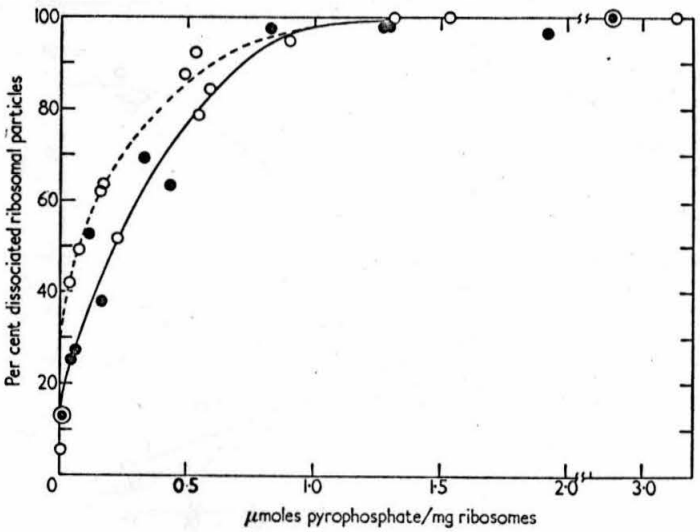

FIG. 1. *Dissociation of ribosomes as a function of pyrophosphate concentration.* Conditions for treatment of ribosomes are given under Materials and Methods. Pyrophosphorolysis was carried out at 0°C, ultracentrifugation at 4°C (●——●) and 20°C (○- - -○).

but in both instances 100% dissociation was noted at a concentration of approximately 1·3 μmoles PP/mg ribosomes resulting in a preparation containing only 50 s and 30 s particles.†

The ribosomal subunits can be partially or completely reassociated to 70 s and higher aggregates by the addition of various amounts of Mg^{2+}. The concentration of $MgCl_2$ in the incorporation medium was sufficient to reaggregate more than 90% of the subunits to particles of 70 s and greater.

2. *Appearance of subunits on dissociation*

In Fig. 2 is shown the relative concentration of 50 and 30 s subunits in ribosomal preparations dissociated to varying extent. Regardless of the method employed for dissociation of the ribosomes, whether the ultracentrifugal analysis was carried out at 4 or 20°C, whether or not the samples were subjected to freezing and thawing, the primary products of dissociation are subunits with *S* values of 50 and 30, which are present in almost equal number at all stages of dissociation. Instability of the 30 s ribosomes probably accounts for the existence of always slightly fewer 30 s than 50 s particles. The instability of 30 s subunits was also described by Ts'o & Vinograd (1961). After dialysis against low ionic strength (1×10^{-3} M-tris pH 7·5) they observed only 70 and 50 s particles and after dialysis against 1×10^{-1} M-KCl and 1×10^{-3} M-tris pH 7·5 they obtained only 50 s particles. The loss of

† Prolonging the incubation of ribosomal preparations in PP from 10 to 30 min at 4°C, or freezing and thawing of PP-treated ribosomes prior to ultracentrifugation, does not increase the degree of dissociation.

30 s subunits appears to be a function of experimental conditions during dissociation. Material with sedimentation coefficient greater than 70 has been omitted from the graph; this constituted 16% of the total ribosomal population at 9% dissociation, decreasing linearly and disappearing entirely by 40% dissociation.

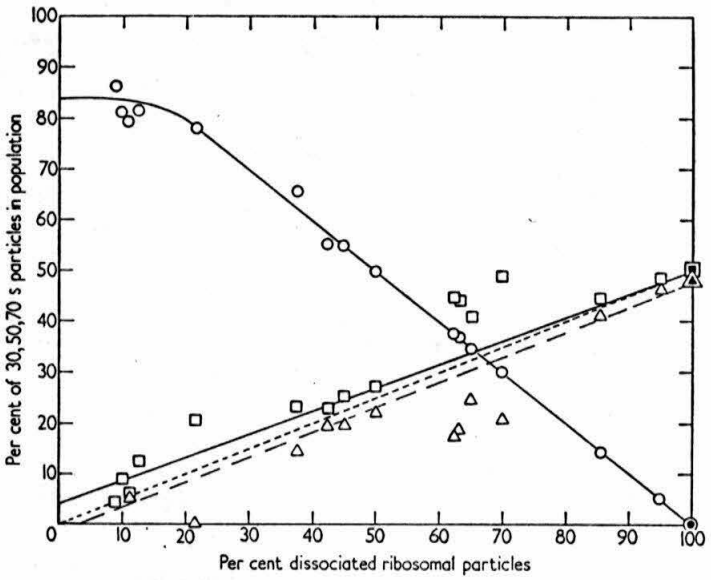

FIG. 2. *Appearance of* 30 s *and* 50 s *particles on dissociation of ribosomal particles.*

○——○ "70 s" particle
□——□ "50 s" particle
△---△ "30 s" particle
- - - - - Theoretical curve common to 30 s and 50 s particles
●, ■, ▲ Identical values from 8 analyses.

When the PP concentration is increased approximately three times above the minimum concentration required for total dissociation, further breakdown occurs and particles of S values lower than 30 begin to appear (S = 5 to 8 and 20 s).

3. *Sedimentation coefficients*

The sedimentation coefficient ascribed to ribosomal particles is dependent on the experimental conditions during ultracentrifugation. As already mentioned in Materials and Methods, the S value is concentration dependent, and increases with dilution. The sedimentation coefficient also depends on the ionic strength of the medium, as previously observed by Ts'o & Vinograd (1961), and it is specifically affected by PP. Figure 3 illustrates that the S value decreases with increasing PP concentration. The total ionic strength of the medium could not be calculated, because the equilibria between ribosomes, divalent cations and PP are not known under the particular conditions of pH and buffering in these experiments. Moreover it should be noted that each type of particle has its own characteristic PP dependence.

B. *Protein synthetic activity of reversibly dissociated ribosomes*

1. *Activity after complete dissociation*

With a method available for quickly and completely dissociating ribosomes into subunits it became possible to determine whether or not ribosomes, after dissociation

and subsequent reassociation, could still function in protein synthesis. To test this idea the following experiment was carried out. Ribosomes were completely dissociated with PP, which was subsequently hydrolysed with PPase. The ribosomal preparation thus obtained was tested for amino acid incorporation in the usual

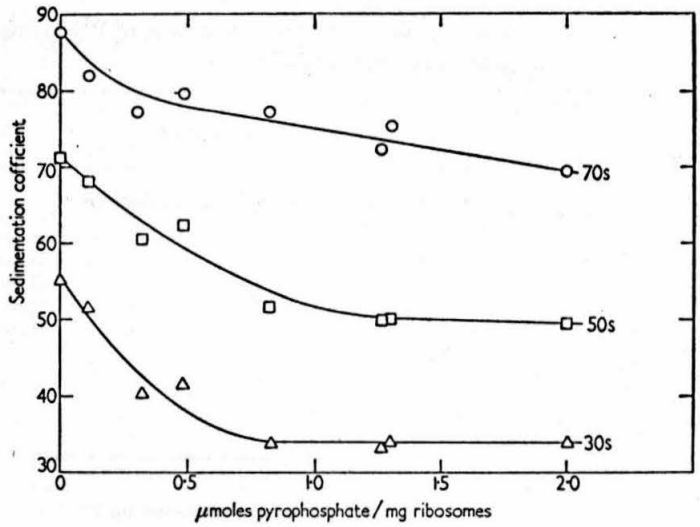

FIG. 3. *S values of ribosomal particles as a function of pyrophosphate concentration.*

Values up to 12% dissociation were obtained from analyses of ribosomes in solution C. All other data represent ribosomes dissociated by PP or dialysed as described in text. Determination of relative concentration of particles is based on ultracentrifugal analysis at 4 or 20°C and is described under Materials and Methods.

The ribosomes were dissolved at 3·71 mg RNP/ml. in a medium containing 0·5 M-KCl, 10^{-3} M-tris pH 8·0 and various amounts PP as indicated. Ribosomal samples containing no PP were dissolved at 3·71 mg RNP/ml. in solution C. Ultracentrifugations were done at 4°C and S values corrected to H_2O at 20°C.

medium containing $1{\cdot}5 \times 10^{-3}$ M-Mg^{2+}, in which the ribosomal subunits reaggregated to the 70 S particles.

In Table 2 the activity of the dissociated ribosomes is compared to that of normal ribosomes which were dissolved in solution C and treated with PPase like the experimental samples. Since orthophosphate, formed by PPase action in experimental samples, was found to inhibit protein synthesis (compare lines 3 and 4 to 1 and 2) it was necessary to add comparable amounts of inorganic phosphate to the ribosomes in control incubations.† The data presented in Table 2 indicate that after reticulocyte ribosomes are completely dissociated into subunits, and subsequently reaggregated, they have lost the capacity for protein synthesis (lines 5 and 6) and incorporation into either the ribosomal fraction or into soluble protein is not significantly greater than for control samples without ribosomes (lines 7 and 8). None of the factors present in the supernatant fraction was capable of restoring the protein synthetic activity of the reassociated subunits (compare line 5 to line 6).

† Attempts to reverse phosphate inhibition by removal of inorganic phosphate with Sephadex were unsuccessful.

2. Correlation between extent of dissociation and activity

Treatment of ribosomes with PP, or by dialysis, or with Sephadex makes it possible to obtain preparations dissociated to any desired extent. It was therefore of interest to correlate the extent of dissociation with the activity for amino acid incorporation.

TABLE 2

Incorporation of [^{14}C]amino acid into ribosome-bound and soluble protein before and after complete dissociation of particles

Ribosomes	Additions		cts/min/mg ribosomes in:			% of control (total)	
	Phosphate	Supernatant	Ribosomes	Soluble protein	Total sample		
1. In solution C		−	312	89	401	100	
2. In solution C		+	1348	1166	2514	100	
3. In solution C	+	−	274	94	368	92‡	100
4. In solution C	+	+	1242	1061	2303	92‡	100
5. In PP		−	17†	5†	22		6†
6. In PP		+	33	20	53		2†
7. —		−	9†	7†	16†		†
8. —		+	9†	7†	16†		†

† Not significant.

‡ Phosphate inhibition varies from one experiment to the next, and may reduce incorporation to as low as 85% without supernatant and 52% with supernatant, compared to control values.

Reaction mixtures were incubated for 60 min at 37°C with final concentrations of reagents as described under Materials and Methods. The following solutions were added as indicated: for experiments 1 to 4 ribosomes were dissolved in solution C; for experiments 5 and 6 ribosomes were dissolved in solution C minus Mg^{2+} containing 1·3 μmoles PP/mg ribosomes. 1·2 μmoles $MgCl_2$ were added to experiments 5 and 6 prior to PPase addition; 7·8 μmoles of orthophosphate were added to experiments 3 and 4; to experiments 7 and 8 solution C was added instead of ribosomes. All reaction mixtures were treated with PPase as described.

Such a comparison is illustrated in Fig. 4(a) and shows that the dissociation of 95% of the ribosomal population is accompanied by the loss of 50% of the protein synthetic capacity. The remaining 50% of the activity is lost on disappearance of the last 5% of intact particles. Assuming all particles to be active this would indicate that those 5% of the particles which dissociate last have approximately 10 times the specific activity of the 95% which disaggregate more readily.

It is of interest to note that the small group of ribosomal particles with high specific activity not only is the last to dissociate, but requires relatively greater concentrations of PP for dissociation than 95% of the population with lower activity. This can readily be seen by comparing Figs. 4(a) and 4(b).

4. Discussion

The observations with rabbit reticulocyte ribosomes presented here give good evidence that one 70 s ribosomal particle dissociates into one 50 s and one 30 s particle. By addition of Mg^{2+} these subunits can be completely reassociated into 70 s ribosomes and higher aggregates.

Our finding that an equal number of 50 and 30 s particles is present at all stages of dissociation at 4 and 20°C indicates that one 70 s reticulocyte particle is composed

of one 50 s and 30 s particle. The data give no indication of other suggested combinations of subunits (Huxley & Zubay, 1960; Petermann & Hamilton, 1960; Roberts, 1960). This is also consistent with a report about reticulocyte ribosomes by Ts'o & Vinograd (1961) that neither 50 nor 30 s particles alone can reaggregate, but that a mixture of these subunits can reform 70 s ribosomes.

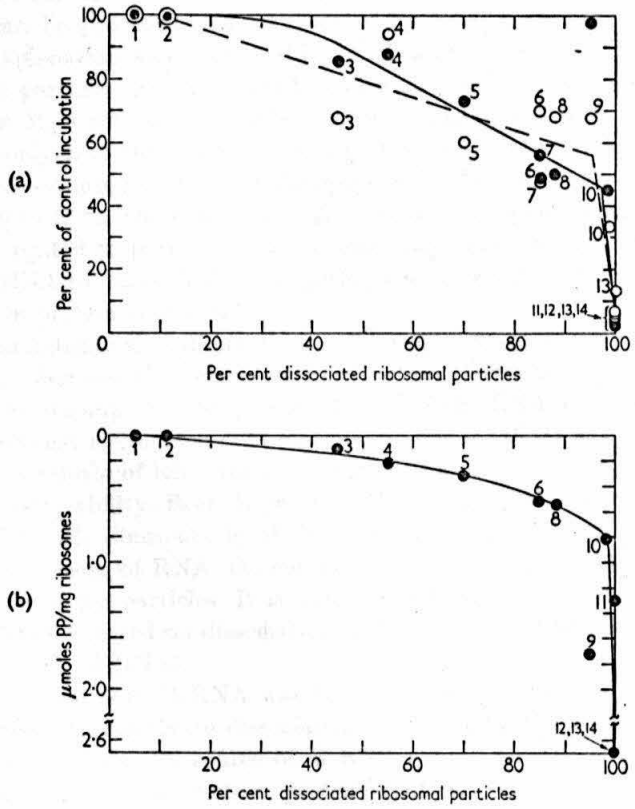

FIG. 4(a). *Protein synthetic activity as a function of extent of dissociation of ribosomes.*

○---○ without supernatant
●——● with supernatant.

Values for this graph were obtained with ribosomes treated as follows:

1 and 2	dissolved in solution C
3, 5, 8, 10, 11, 12, 14	dissociated with PP followed by PPase
4, 6, 9, 13	dissociated with PP followed by PPase followed by Sephadex
7	dissociated by dialysis against Mg^{2+}-free medium.

Each ribosomal preparation was incubated with [^{14}C]leucine in the presence and absence of supernatant and incorporation into protein was followed as described in the text. The protein synthetic activity is expressed as % of control incorporation, following the example given in Table 2. The extent of ribosomal dissociation was estimated by ultracentrifugal analysis of each of the ribosomal preparations, as described in Materials and Methods.

FIG. 4(b). *PP concentration as a function of % of dissociation of ribosomal particles.*
The numbers on Figs. 4(a) and 4(b) refer to identical ribosomal preparations. The data were obtained as described in the legend to Fig. 1.

Ribosomes from reticulocytes are more resistant to dissociation than those from *E. coli* (Tissières *et al.*, 1960) and rat liver (Sachs, 1958) and require more drastic conditions to achieve a comparable extent of disaggregation. See Part III, Section D.

The results described furthermore indicate that under our experimental conditions complete dissociation of ribosomes leads to total loss of protein synthetic activity, and cannot be restored by reaggregation of the particles and addition of a reticulocyte supernatant fraction. Such irreversible loss of incorporating activity on ribosomal dissociation has also been noted in *E. coli* by Tissières *et al.* (1960).

The nature of the irreversible inactivation is not understood, but several possible explanations can be proposed. An observation by Bock (personal communication) may offer an explanation why reassociation of ribosomal subunits with Mg^{2+} did not result in active particles. He dissociated yeast and rabbit reticulocyte ribosomes in a high salt–low Mg^{2+} medium and subsequently reaggregated them with Mg^{2+}. On isolation and analysis of the RNA he noted that after such treatment the ribosomal RNA did not reassociate into units of the original size (30 and 23 s), but remained in smaller units (6 to 12 s). These findings suggest that even though the ribosomal RNP subunits reaggregated to particles of the original size, this was not accompanied by restoration of RNA to its original state; perhaps such disrupted RNA is not capable of functioning in protein synthesis.

Other reports indicate that dissociation of ribosomes involves the release of RNA. Arnstein (1961) observed that complete inactivation of reticulocyte ribosomes with ribonuclease was accompanied by release of 20% of the RNA and disappearance of the higher ribosomal aggregates (133 and 155 s). Sachs (1958) in a study with rat liver had noted a release of RNA from PP-treated ribosomes, with a parallel decrease in the incorporating ability. Beer, Highton & McCarthy (1960) observed that partial dissociation of *E. coli* ribosomes by dialysis against a medium with low Mg^{2+} was accompanied by release of RNA. On complete removal of Mg^{2+} with EDTA all the RNA was lost from the particles. It is difficult to deduce from these data in which instances the RNA released on dissociation is structural RNA and in which it could be messenger RNA (M-RNA).

On the supposition that M-RNA was lost from the particles on dissociation, an attempt was made to reactivate dissociated ribosomes by the addition of a supernatant fraction, as a possible source of M-RNA (Lamfrom, 1961). The inability of this supernatant fraction to restore protein synthetic activity to the reversibly dissociated ribosomes by no means rules out the participation of M-RNA in the reticulocyte system. It may indicate that either the M-RNA of this fraction cannot function under the particular experimental conditions or that the inactivation involved ribosomal alterations of a more structural nature.

It has been shown in the experiments reported here that dissociation of all the ribosomes in a population is accompanied by complete loss of protein synthetic capacity, but that at a time when 95% of the particles are dissociated 50% of the protein synthetic activity remains. When sufficient PP is added to dissociate the final 5% of the 70 s particles the remaining 50% of the incorporating ability is lost. These observations may be explained by assuming that dissociation and reassociation as such does not result in inactivation and that the observed loss of activity is due to our particular experimental conditions which cause damage to the particles of the kind described above. This could account for the gradual loss of incorporating activity with increasing PP concentrations, and the total loss of activity when high concentrations of PP are required to completely dissociate the particles. The fact that under conditions of dissociation inactivation was not time dependent would, however, mitigate against this interpretation.

An alternative interpretation of the data would propose that in the usual preparation of reticulocyte ribosomes two classes of particles are participating in protein synthesis. One type constitutes the bulk of the ribosomal population, has low incorporating ability and is susceptible to dissociation; the other type accounts for approximately 5% of the particles, has 10 times higher specific activity and only dissociates at higher PP concentrations. Experiments are in progress to isolate the 70 s particles at a point where 95% dissociation has occurred and to determine their activity directly. These findings are compatible with the results of Tissières *et al.* (1960) which suggest that in the *E. coli* system a small portion (less than 10%) of the 70 s ribosomes is 15 to 40 times more active, and more resistant to dissociation, than the rest of the ribosomal population.

Evidence is presented that the ribosomes isolated from reticulocytes, and used in the cell free system, represent a heterogeneous population with respect to physical properties and activity. It is unknown whether the range of stability encountered in the ribosomal population is an indication of the true state of the ribosomes in the cell, or whether the 5% active particles represent a random selection by the isolation procedure. Whether the protein synthetic activity endows the particles with resistance to dissociation, or *vice versa*, is as yet unknown.

In spite of the limited significance, an approximate calculation of the protein synthetic capacity of the 5% active 70 s particles was made. Based on the data given in Table 2, and assuming that at the beginning of the incubation the ribosomes are on the average half-filled with non-radioactive peptides, and that the free leucine pool is negligible, one 70 s particle synthesizes 1·15 molecules Hb during 60 minutes. This supports observations by Bishop, Leahy & Schweet (1960) that in the reticulocyte cell-free system even the most active particles only complete protein molecules.

A re-evaluation of the accumulated data on ribosomal particles may be rewarding, considering that observations on the protein synthetic activity of ribosomes reflect the properties of only a small group of ribosomal particles, and that descriptions of physical properties deal with the total ribosomal population, most of which is relatively inactive.

We are indebted to Dr. H. Borsook for his continued support and interest during the course of this work. It is a pleasure to thank Mrs. Christine Ziegler for her capable assistance. This investigation was supported by grant no. H-1624 from the National Institutes of Health, U.S. Public Health Service.

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It was obvious that the interpretation of these results needed to be changed in the light of the discovery of polyribosomes in reticulocytes, which was made some time after this paper appeared (35-38). In the first place, it is clear that the "aggregates" which we mention were probably polyribosomes rather than nonspecific precipitates and our standard method of ribosome preparation, which was designed to maximize the 80S particles, was yielding a synthesizing system which was seriously degraded. Therefore, the conclusions we reached concerning the nature of the protein synthesizing site are in error and the hypothesis that the 80S ribosomes in a reticulocyte exist as two populations, one of which contains 5% of the ribonucleoprotein and 50% of the synthetic capacity, is no longer tenable. Since this population of 80S ribosomes did incorporate protein, we can say in retrospect that the monomers still possessed some messenger RNA and that we had probably broken the polyribosomes by shear during the homogenization steps.

The fact that the original ribosome preparation contained 6 to 15% polyribosomes suggests that these particles were the source of the "active" ribosomes, and it is probable that they were more resistant to dissociation because they possessed messenger RNA and/or growing peptide chains. It is clear today that it is the integrity of the messenger RNA which is important for protein synthesis. In the native state this M-RNA is attached to several 80S monomers to form the active unit, the polyribosome. Our method of dissociating the 80S particles probably removed residual M-RNA from them while separating

the ribosomes into 60 and 40S subunits, leading us to the erroneous conclusion that the act of dissociation was solely responsible for destroying the synthetic capacity. The fact that the addition of supernatant or other factors failed to restore the activity is not surprising, since in order to appear in our assay system the added messenger would have had to initiate chains, and at this time we were unable to demonstrate any chain initiation in our most active preparations. Lamfrom (39) has since been able to show chain initiation in a cell-free system from reticulocytes.

To confirm the reports by others (37,38) that the active protein synthesizing unit in a reticulocyte cell-free system is the polyribosome, this author separated ribosomal material from a cell lysate on a sucrose gradient and tested the fractions in cell-free incubations. The results are shown in Figure I-6. The optical density peak for the 80S monomers is obscured by the hemoglobin present, but an approximate calculated position for this peak has been indicated. It is clear that the most active units are polyribosomes. This is in agreement with our results from studies on intact reticulocytes (see Part II, Section B). In this experiment we did not observe synthesis on the 80S ribosomes such as that reported by Lamfrom and Knopf (39).

In conclusion, the conditions which we used for dissociating ribosomes caused a concomitant loss of synthesizing ability, which we now feel was due to the removal of messenger RNA.

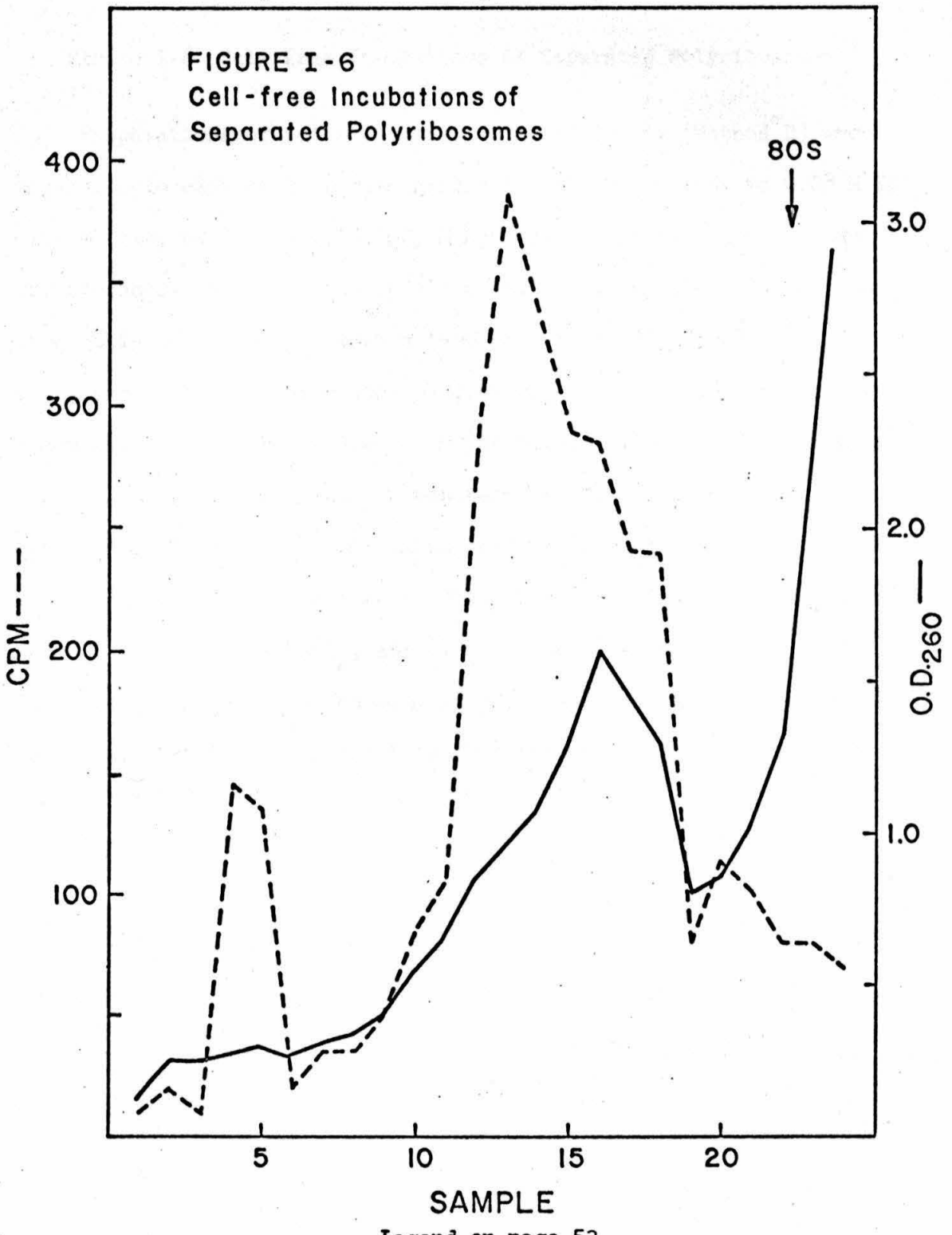

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Figure I-6 Cell Free Incubations of Separated Polyribosomes

Preparations of polyribosomes: 2 ml of lysate (Method B) were layered onto each of 3 sucrose gradients (15-30% sucrose in 0.08 M KCl, 0.02 M tris, pH 7.6 R.T., 0.0025 M $MgCl_2$). These were centrifuged for two hours at 24,000 rpm in an SW 25.1 rotor at 5°C. One ml samples were collected by drop counting into dry vials and stored at -70°C. until used. Samples from one gradient were used for optical density measurements and from another for incubation. To the one ml ribosome fractions were added 0.2 ml Supernatant and 0.3 ml of a reagent mix containing 100 γ S-RNA , 50λ amino acid mixture, 1 μ mole ATP, 0.25 μ mole GTP, 7.6 γ creatine kinase, 20 μ moles creatine phosphate, 10 μ moles GSH, 5 μ moles $MgCl_2$, and 0.05 μ mole C^{14}-leucine at 6.3μC/μM. These were incubated for 45 min. at 37°C. and frozen at -70°C. until analysis. CPM indicates the total TCA precipitable counts in each sample.

E. On the Question of Synthesis-Dependent Breakdown of Polyribosomes

After finding that there were significant amounts of RNase in the reagents we had been adding to the cell-free incubation mixtures (See Section C), we decided to reinvestigate the "energy-dependent" or "synthesis-dependent" breakdown of polyribosomes which has been reported in the literature (39,40,41). Those factors which we found to contain the largest contamination with RNase are the same as those which are usually omitted from experiments designed to show that an energy source is required for breakdown of polyribosomes.

The first step was to re-examine the requirements of the cell-free system in order to see if any of the reagents were unnecessary. The results of such an experiment are shown in Table I-6. In this preparation the cell lysate (prepared by the Method A lysis) had no requirement for added co-factors, and indeed, was strongly inhibited by the presence of Reagent Mixture containing creatine kinase.

A time course of incorporation was performed with unfractionated lysate in the presence of all reagents and in the same mixture lacking creatine kinase. This is shown in Figure I-7. This also indicates that the lowered activity of the preparation with the kinase added is due to the presence of nuclease, as the initial rate of synthesis is almost the same in both cases but continues for a longer time in the kinase-free incubation.

However, when separated ribosomes and a supernatant solution were used, (see Table I-6), there was a definite dependence on the added

TABLE I - 6

Cell-free Incubations with Various Reagent Mixtures

Reagents Used	Total Counts Incorporated in 20 Min. at 37°C Type of Incubation		
	Lysate*	80 S‡	Polyribosomes‡
Complete	283	1409	1239
Complete, - kinase	1878	739	1068
Complete, - kinase, - creatine phosphate	1365	128	472
Complete, - kinase, - amino acids	1700	876	948
Complete, - kinase, - amino acids, - creatine phosphate	1700	122	528
No reagents added	1750	67	432

*Lysate incubations contained 0.8 ml cell lysate (Method A), 12 μ moles GSH, 0.08 ml C^{14} leucine at 5×10^{-4} M and 0.13 ml Reagent Mix.

‡Incubations with purified 80S ribosomes and polyribosomes contained 0.7 ml Supernatant, 0.1 ml C^{14} leucine at 5×10^{-4} M, 15 μ moles GSH, 0.2 ml RNP at 11 mg/ml in Soln. G, and 0.25 ml Reagent Mix.

Reagent Mix has been described in Materials and Methods.

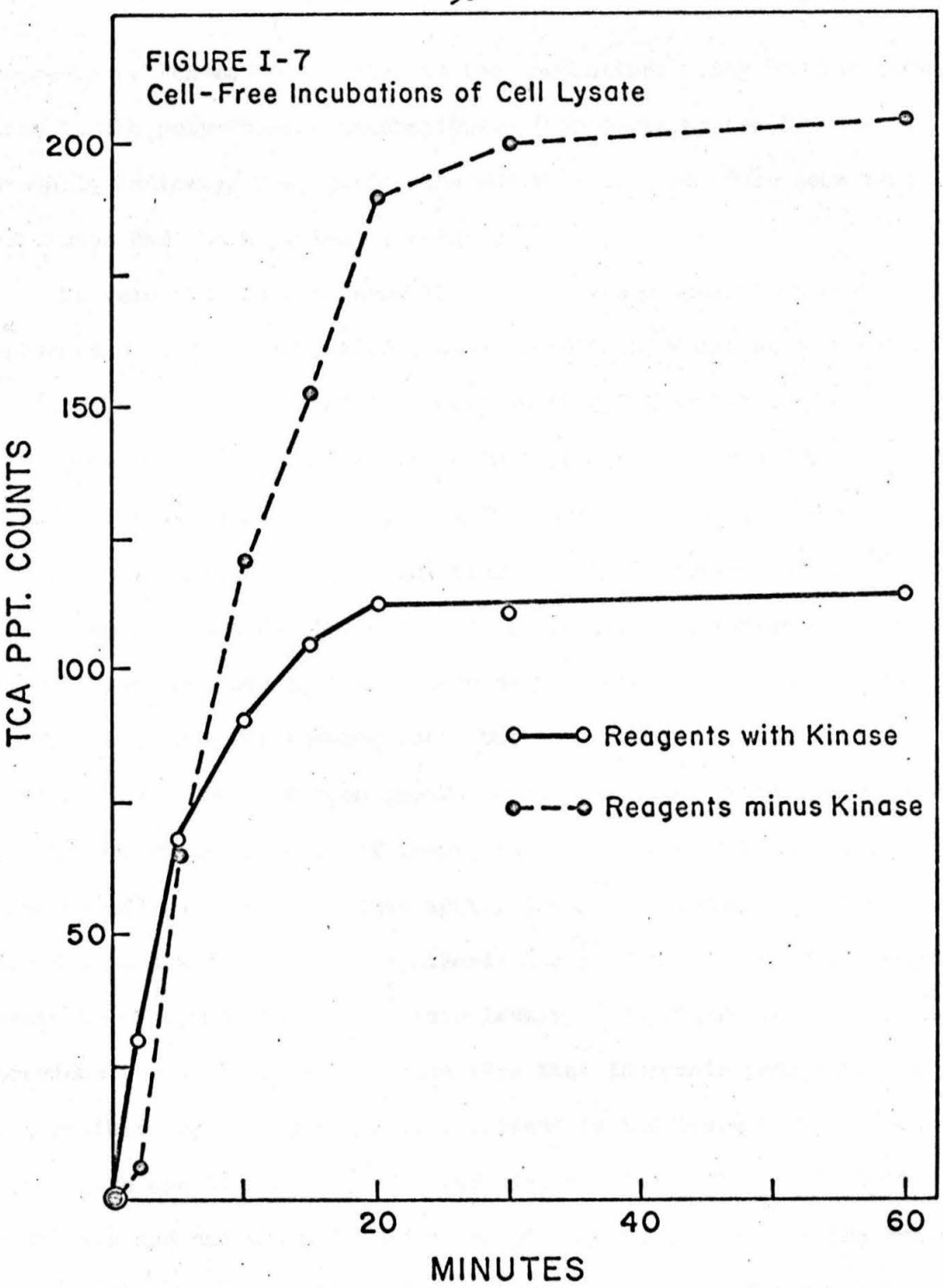

Figure I-7 Cell Lysate Incubated in the Presence of Reagent Mix with and without Kinase.

Incubation mixtures are the same as for the Lysate incubations in Table I-6.

reagents which was more marked in the incubations using 80S ribosomes than in the polyribosome incubations. (Synthesis by the 80S fraction probably indicates that during the purification procedure some polyribosomes had been broken by shear.)

We were also able to show that incubation of polyribosomes in the presence of the reagents alone, under conditions where no synthesis could take place, resulted in breakdown (see Figure I-8).

Since we had shown that the effect on synthesis due to added creatine kinase was very small and the nuclease level was relatively high, it was decided to omit the kinase from all subsequent incubations.

However, in order to separate the processes of nuclease action on polyribosomes and synthesis-dependent breakdown, it was necessary to find a method for slowing down but not stopping synthesis so that samples which had been incubated for different lengths of time but had the same amount of incorporation could be compared for polysome breakdown. We found that adding inorganic phosphate to the system would slow down the rate of synthesis and performed time course experiments to determine the appropriate levels (See Figure I-9.). We had previously done a control to make sure that inorganic phosphate did not inhibit the nuclease activity present in the Reagent Mix. (See Table I-2b and Figure I-4). We had also considered NaF to inhibit synthesis and had shown it had no effect on the nuclease of the Reagent Mix. This procedure was abandoned, however, because of reports by Marks et al. (42) that incubation of intact reticulocytes with NaF caused breakdown of polysomes.

FIGURE I – 8
Degradation of Polyribosomes by Reagent Mix

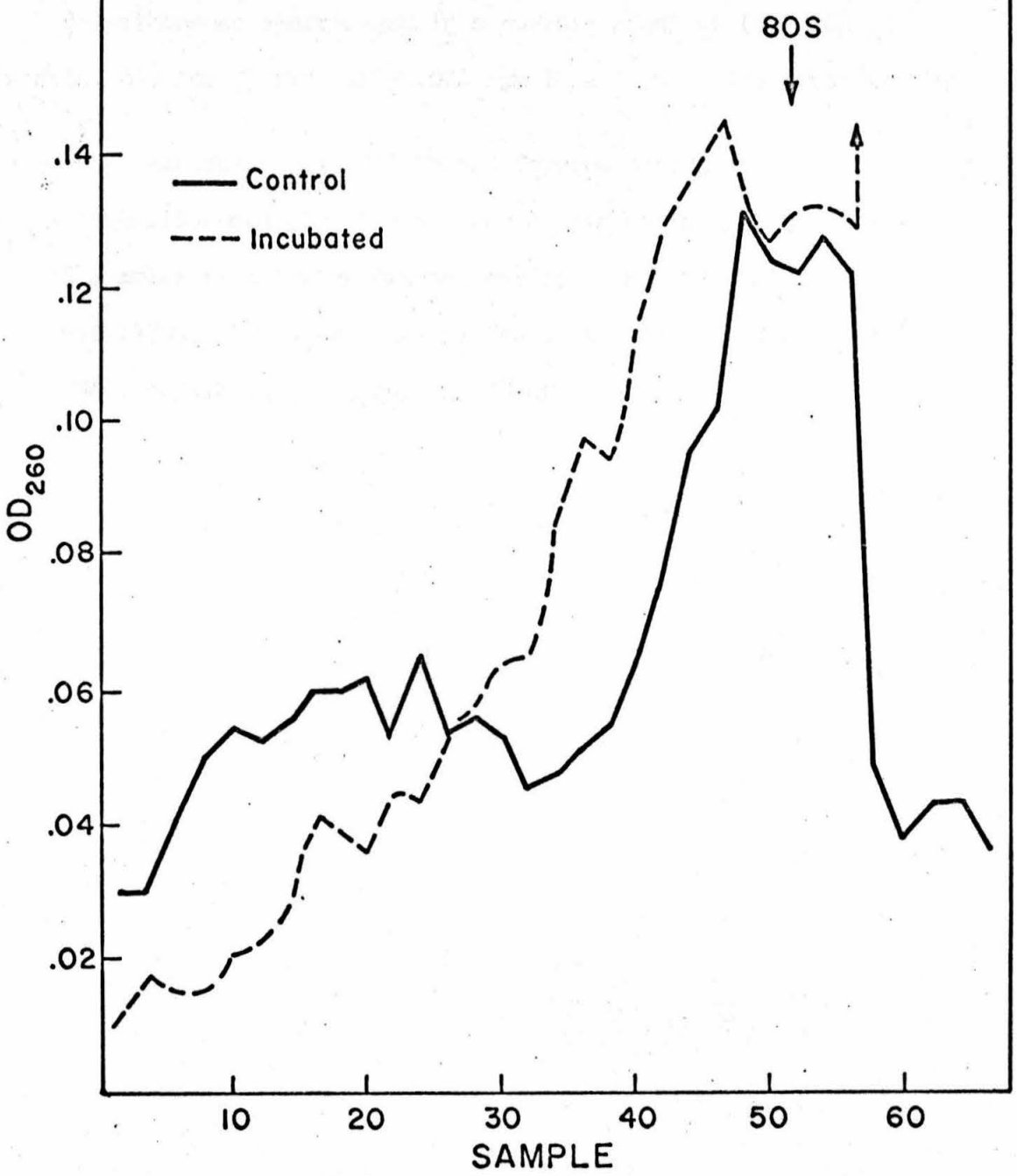

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Figure I-8 Degradation of Polyribosomes Incubated with Reagent Mix

Polyribosomes centrifuged in a sucrose gradient (5 - 20% sucrose in Soln. G) for 73 min. at 30,000 rpm in a Spinco SW39 rotor at 5°C.

——— 50λ ribosomes plus 50λ Soln. G layered onto gradient.

- - - 50λ ribosomes plus 50λ of reagent mix containing 1 μ mole ATP, 5 μ moles creatine phosphate, and 15γ creatine kinase (Sigma 93B-1470). Ribosomes plus reagents were incubated for 5 min. at 37°C. before layering onto gradient.

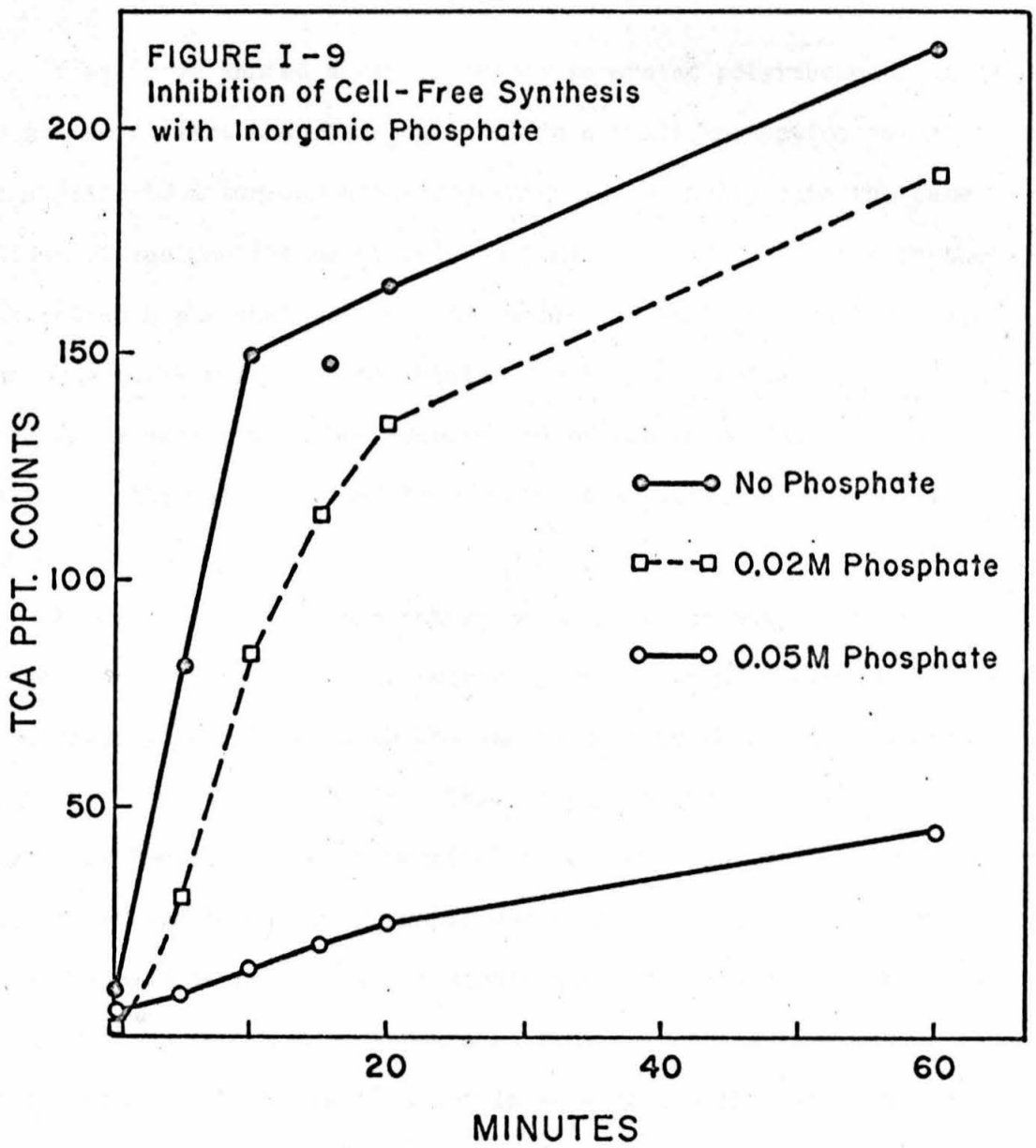

Figure I-9 Inhibition of Cell-free Protein Synthesis by Inorganic Phosphate

Incubations contained 0.21 ml of ribosomes at 7.2 mg/ml in Soln. G, 0.26 ml Supernatant, 0.07 ml of Reagent Mix minus kinase, 8 μ moles GSH, 0.05 ml C^{14}-leucine (5×10^{-4}M), and 0.08 ml of additive. Additives were Soln. G (●——●), 0.2 M Na phosphate in Soln. G (□----□), or 0.5 M Na phosphate in Soln. G (○——○). Samples were incubated at 37°C; 25 λ aliquots were precipitated with 14% TCA at the times given.

Ideally we wanted a system (using separated polyribosomes in order to get sufficient absorbing material in a small incubation volume to be applied to a sucrose gradient) which would incorporate the same amount of radioactive amino acid in 5 minutes and 10 minutes in the control as a phosphate-containing sample did in 15 and 40 minutes. This was quite difficult to obtain, for the exact time course of the incubation varied with the preparation of ribosomes and supernatant factors. The data obtained from one of these experiments are given in Table I-7.

Though the results are not as unambiguous as one might wish, these data indicate that the degree of breakdown of polyribosomes is more closely correlated with the length of time of incubation than with the amount of synthesis. This would indicate that while there may be synthesis-dependent destruction of polyribosomes, the major portion of the total breakdown is due to RNase present in our system. This RNase is largely in the reagents added to provide the "energy-generating system" and so appears as "energy-dependent" breakdown of polysomes. This result is not in agreement with other results obtained for the reticulocyte system (39,40), but may explain why our cell-free synthesizing systems stop making protein after only a few minutes. This facet of our cell-free incorporation mixtures precludes any possibility of investigating the rate of attachment and release of monomers from the messenger RNA during cell-free synthesis. Noll *et al.* (41) have carefully considered the problem of synthesis-dependent *versus* RNase-dependent breakdown in the rat liver system

TABLE I - 7

Degradation of Polyribosomes during Cell-free Synthesis

Incubation	% Synthesis	% of Total Optical Density at 260mμ		
		Polyribosomes		80S
		Σ 1 - 8	Σ 9 - 16	Σ 17 - 24
Control				
0 minutes	3.0	35.6	28.3	36.1
5 minutes	29.1	19.8	32.0	48.0
10 minutes	71.1	19.9	27.8	52.2
Phosphate				
0 minutes	3.0	39.4	28.0	32.5
15 minutes	55.7	16.1	26.0	57.9
40 minutes	100.0	8.9	47.6	64.2

Total Synthesis was determined from an aliquot of the incubation which was TCA precipitated before the sucrose gradient separation. The 40 min. phosphate sample is considered 100% synthesis.

Numbers refer to the samples on the sucrose gradients. Σ 1 - 8 also includes the pellet.

Incubations contained 0.3 ml RNP at 12.4 mg/ml, 0.11 ml Reagent Mix (minus creatine kinase), 13 μ moles GSH, 0.09 μ mole C^{14}-leucine (20 μC/μM), 0.5 ml Supernatant and 0.11 ml of Soln. G for the Controls or 0.11 ml 0.2 M Na phosphate, pH 7.0, for Phosphate samples. The entire incubation was layered onto a sucrose gradient and analyzed as described in Materials and Methods.

and have been able to separate these two effects, to show that monomers can attach to polyribosomes, move down the messenger RNA, and be released when the peptide chain is completed. Though the mechanism is quite certainly the same for the reticulocyte system, comparable data do not exist. This may be due to the problem of contamination of the reagents with low levels of RNase which we have considered here.

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PART II

Polyribosomes and Hemoglobin Synthesis in the Maturing Reticulocyte

A. Introduction

The experiments in this section were conducted with Dr. Robert Millette and are the only research with whole reticulocytes that the present author has done. Although Dr. Millette presented these studies in greater detail in his thesis, I felt it wise to limit this presentation to the two joint publications, three explanatory appendices, and a discussion of the literature on this subject which has appeared subsequent to these articles (i.e. since October 1964). Both papers are presented in their entirety, with the exception of the analytical ultracentrifugation section from the Journal of Molecular Biology paper, which has been included in Part III.

B.

Polyribosomes and the Loss of Hemoglobin Synthesis in the Maturing Reticulocyte

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The fate of the protein-synthesizing system in rabbit reticulocytes as they mature to erythrocytes has been examined. Reticulocytes were fractionated by buoyant density centrifugation on a 21 to 30% bovine serum albumin gradient. The least dense cells at the top of the gradient are larger, stain as very immature reticulocytes, and are most active in protein biosynthesis. The cells banding in denser regions of the albumin are less active in protein synthesis, are smaller, and have very little stained reticulum. Most erythrocytes present in the population are pelleted at the bottom of the tube. Therefore, from these cytological and biochemical criteria, the albumin gradient separates the cells according to their degree of maturation.

Cells from six fractions of the bovine serum albumin gradient were incubated for 5 to 20 minutes with radioactive leucine, lysed, and the ribosomal material purified by centrifugation through 30% sucrose. Ribosomes from each fraction were examined by zone centrifugation on sucrose gradients. Samples from the gradients were analyzed for ribonucleoprotein and acid-precipitable radioactive material.

While the most immature reticulocytes contained up to 40 times as much ribosomal material as the most mature ones, the percentage of this material present as polyribosomes decreased only from 68 to 34%. The pentamer persisted as the major ribosomal aggregate throughout maturation. This evidence indicates that the process of maturation involves primarily the loss of total ribosomal material rather than a sequential breakdown of polyribosomes to 80 s particles. The specific activity of the polyribosomes was highest in the youngest reticulocytes and decreased with increasing cell age. However, in all fractions the amount of newly synthesized soluble protein was directly proportional to the amount of growing peptide chain associated with the polyribosomes, rather than the total amount of polyribosomal material. This indicates that there is an increasing fraction of inactive polyribosomes with progressive cell maturation.

Polyribosomes were also studied in the analytical ultracentrifuge, with band centrifugation over 90% D_2O. Five discrete peaks were seen, with $S_{20,w}$ values of 80, 130, 153, 190 and 220, corresponding to the single ribosomal particle (monomer) and the dimer, trimer, tetramer and pentamer respectively.

1. Introduction

Reticulocytes undergo dramatic biochemical and cytological changes in their conversion to mature erythrocytes. During this process their RNA is lost and hemoglobin synthesis stops (Holloway & Ripley, 1952; Lowenstein, 1959; London, 1961;

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Schweiger, 1962). Although it has been shown that the RNA is ultimately degraded to purines and pyrimidines (Bertles & Beck, 1962), the precise sequence of events at the ribosomal level has remained obscure.

It has been well established that the subcellular site of hemoglobin synthesis is the polyribosome (Warner, Rich & Hall, 1962; Marks, Burka & Schlessinger, 1962; Gierer, 1963). Therefore a study of the activity and fate of these structures in relation to the progressive loss of hemoglobin synthesis in the maturing reticulocyte was undertaken.

Previous studies in this laboratory have shown that rabbit reticulocytes may be fractionated by buoyant density centrifugation in a bovine serum albumin gradient (Borsook, Lingrel, Scaro & Millette, 1962). Cell fractions were obtained which showed a gradient of protein-synthesizing capacity from the most active cells at the top to the least active cells at the bottom of the gradient. Such a fractionation provides a means of studying the exact nature of the loss of the protein-synthesizing apparatus within a given cell population.

In the present experiments we have first demonstrated that the position of the reticulocytes in the albumin gradient is a function of their degree of maturation. We have then correlated their state of maturation and protein-synthesizing capacity with the concentration, activity and state of aggregation of their ribosomes. Our results indicate that the progressive loss of hemoglobin synthesis observed in these reticulocyte fractions is directly associated with the loss of total ribosomal material, both 80 S ribosomes and polyribosomes, and a decrease in the ability of polyribosomes to incorporate amino acids. This second effect appears to be the result of an increasing percentage of inactive polyribosomes during the course of cell maturation.

2. Materials and Methods

(a) *Materials*

Blood containing 80 to 90% reticulocytes was obtained by cardiac puncture from rabbits which had been made anemic by injections of phenylhydrazine (Lingrel & Borsook, 1963). [1-^{14}C]leucine, 40 μc/μmole, was purchased from Isotopes Specialties Co. and diluted before use. Mallinkrodt Analytical Grade sucrose was used for gradients. Bovine albumin, fraction V powder, was obtained from Armour Pharmaceutical Company. The membrane filters were Schleicher & Schuell, type B-6.

(b) *Cell separation on albumin gradients*

Blood cells were centrifuged out of the plasma and washed once with NKM (0·153 M-NaCl, 0·005 M-KCl, 0·005 M-$MgCl_2$). Linear bovine serum albumin (BSA†) gradients of 21·7 to 30·1% (w/w) in NKM containing an equimolar amount of $NaHCO_3$ in place of NaCl were used to separate cells by buoyant density centrifugation. Four ml. of a suspension of cells diluted 1:1 with NKM were layered onto each of three 25-ml. gradients and centrifuged in a Spinco 25·1 rotor at 19,000 *g* for 90 min at 5°C. Approximately 20 preliminary fractions were collected from the gradients, using a Technicon proportional pump, through a glass capillary inserted to a point just above the erythrocyte-rich pellet (Leif & Vinograd, 1964). All fractions, including the pellets, were diluted fourfold with NKM and centrifuged to free them from albumin. Adjacent fractions were pooled to give six final cell fractions of roughly equal volume, resuspended in NKM; samples were removed for staining and cell counting, and the cells recentrifuged. These six fractions of packed cells were used for the whole-cell incubations.

† Abbreviations used: BSA, bovine serum albumin; TCA, trichloroacetic acid.

(c) *Whole cell incubation and ribosome preparation*

The reagent mixture for the incubations was made up as follows: amino acid mixture without leucine (Lingrel & Borsook, 1963), 5·0 ml.; 0·14 M-$MgCl_2$ in 0·4 M-glucose, 0·25 ml.; 0·164 M-tris, pH 7·8, at 20°C, 2·5 ml.; 0·01 M-sodium citrate in NKM, 2·0 ml.; 0·01 M-$NaHCO_3$ in NKM, 3·0 ml.; fresh anemic plasma, 0·75 ml.; 0·27 mM-$Fe(NH_4)_2(SO_4)_2$ in NKM, 0·85 ml. (Borsook, personal communication). For 1·0 ml. of packed cells, 3·1 ml. of the above mixture was used.

After preincubation at 38°C for 20 min in the absence of leucine, [1-^{14}C]leucine was added (1 μmole/ml. of cells at 15 μc/μmole) and incubation continued, usually for 10 min. The incubation was terminated by chilling the reaction mixture and washing the cells in ice-cold NKM. All subsequent operations were carried out at 5°C. The cells were lysed osmotically by a modification of the method of Schweet, Lamfrom & Allen (1958). Two volumes of 0·0015 M-$MgCl_2$ in 0·001 M-tris, pH 7·5, at 20°C, were added. After 30 sec the lysate was returned to isotonicity by the addition of 0·6 vol. of 1·5 M-sucrose containing 0·15 M-KCl. Lysates were spun for 10 min at 17,300 *g* to remove cell debris and unlysed cells. The clarified lysates were layered over 2·5 ml. of 30% sucrose (w/v) in solution P (0·08 M-KCl, 0·0015 M-$MgCl_2$, 0·01 M-tris, pH 7·5 at 20°C) and centrifuged 4 hr in a Spinco SW39 rotor at 39,000 rev./min to obtain ribosomal material relatively free of contaminating hemoglobin. The pellets and the last 0·5 ml. of supernatant liquid were gently resuspended in 1·5 ml. solution P. See Appendix 1, Section C.

(d) *Sucrose gradients*

Approximately 10^7 plaque-forming units of bacteriophage ϕX174† were added to each ribosomal suspension as a sedimentation marker and these suspensions were layered onto linear sucrose gradients (15 to 30% w/v in solution P). Gradients were centrifuged 130 min at 24,800 rev./min in a Spinco 25·1 rotor at 5°C and decelerated without brake. 1-ml. fractions were collected from three gradients simultaneously using a Technicon proportional pump and stored at −70°C until analysis.

(e) *Analysis of sucrose gradients and other measurements*

Samples were analysed for ribonucleoprotein by absorbance at 260 mμ, using either a Beckman DK2 recording spectrophotometer or a Beckman DU spectrophotometer. Samples were assayed for incorporated [^{14}C]leucine by precipitation in hot 5% TCA using 0·5 mg casein per sample as carrier and collection of precipitate on membrane filters for gas-flow counting. Standard plaque-assay procedures were used for determining ϕX174. Hemoglobin content was determined by absorbance at 522 mμ on samples diluted into no. 1 developer saturated with CO (Allen, Schroeder & Balog, 1958) using an extinction coefficient of 0·564/mg/ml. (Hutchinson, 1960). Cells were stained with new methylene blue (Brecher, 1949) and counterstained with Wright's stain. Cell counts and size distributions were determined on a Coulter electronic particle counter, model A, after dilution into a modified Eagle's saline described by Brecher, Jakobek, Schneiderman, Williams & Schmidt (1962). Volume distributions were compared to those of normal human and normal rabbit erythrocytes in the same medium.

(f) *Analytical ultracentrifugation*

Analytical ultracentrifugation of reticulocyte polyribosomes was carried out in the Spinco model E analytical ultracentrifuge using band centrifugation (Vinograd, Bruner, Kent & Weigle, 1963). The supporting medium was solution P in 90% D_2O, and photographs were taken with monochromatic light at 265 mμ.

3. Results

Reticulocyte separation on albumin gradients

After centrifugation on the BSA gradients, the reticulocytes were collected as six cell fractions, numbered 1 to 6 from the bottom to the top of the gradients. The

† Bacteriophage ϕX174 and its host bacterium *Escherichia coli* C were kindly supplied by Michael J. Yarus.

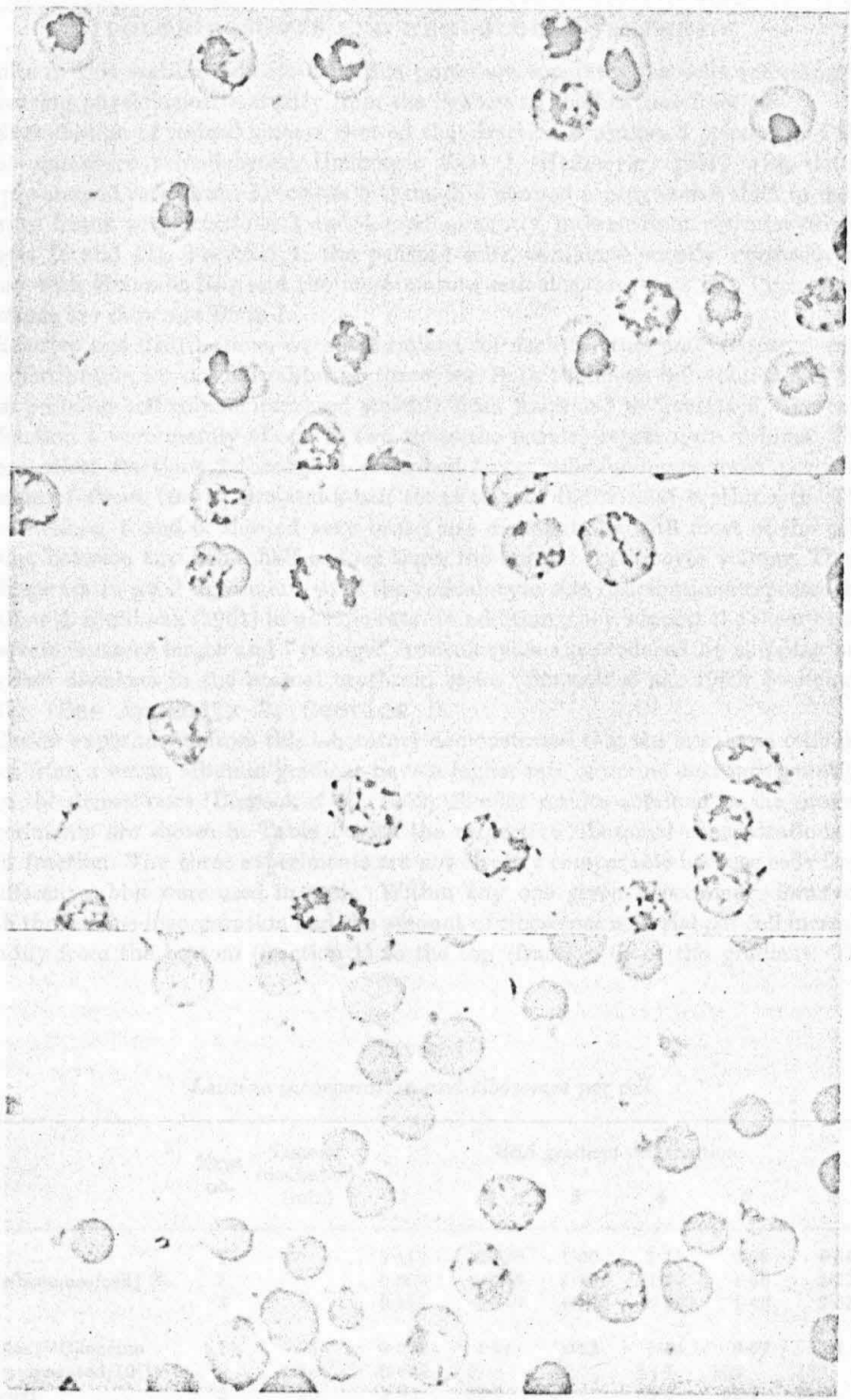

PLATE I. Photomicrographs of reticulocyte fractions from the BSA gradients, stained as described in Materials and Methods. Bottom: fraction 1; middle: fraction 3; top: fraction 6.

[*facing p. 119*

results in this section indicate that this procedure separates the cells according to increasing physiological maturity from the lightest to the heaviest fraction.

Examination of stained smears showed that fraction 6 consisted primarily of the most immature reticulocytes, Heilmeyer class I (Heilmeyer, 1931) with dense, deeply-stained reticulum. Fractions 5 through 2 showed a progressive shift to more mature forms with fractions 3 and 4 predominantly intermediate reticulocytes of classes II and III. Fraction 1, the pelleted cells, contained mostly erythrocytes, many with Heinz bodies, and the most mature reticulocytes, class IV. Typical cell fractions are shown in Plate I.

Relative size distributions were determined for each fraction and compared with the distribution for normal rabbit erythrocytes. Both the mean cell volume and the most probable cell volume increased steadily from fraction 1 to fraction 6. The cells of fraction 1 were mainly of one to two times the normal erythrocyte volume. The intermediate fractions 2 through 4 contained larger cells having a most probable volume of about two to two-and-a-half times that of the normal erythrocyte. The top fractions, 5 and 6, showed very broad size distributions with most of the cells having between two-and-a-half to four times the normal erythrocyte volume. These findings are in good agreement with the reticulocyte size distributions reported by Brecher & Stohlman (1961) in anemic rats. In addition, they support the theory that in severe anemias larger and "younger" reticulocytes are produced by skipping one or more divisions in the normal erythroid series (Borsook *et al.*, 1962; Stohlman, 1961). See Appendix 2, Section D.

Earlier experiments from this laboratory demonstrated that the less dense reticulocytes from a serum albumin gradient have a higher rate of amino acid incorporation than the denser ones (Borsook *et al.*, 1962). Similar results obtained in the present experiments are shown in Table 1 with the respective ribosomal concentrations in each fraction. The three experiments are not directly comparable because cells from a different rabbit were used in each. Within any one given experiment, however, both the leucine incorporation and the amount of ribosomal material per cell increase steadily from the bottom (fraction 1) to the top (fraction 6) of the gradient. The

TABLE 1

Leucine incorporation and ribosomes per cell

	Expt no.	Time of incubation (min)	BSA gradient cell fraction 1	2	3	4	5	6
	1		0·112	0·620	1·03	1·71	2·59	4·64
mg ribosomes/cell† *	2		0·089	0·753	0·899	1·10	1·56	1·67
	3		0·117	0·540	0·759	0·953	1·45	2·63
μmoles [^{14}C]leucine	1	5	0·071	1·44	2·96	5·44	8·57	15·1
incorporated/10^{12}	2	10	0·850	27·8	41·3	68·7	104	136
cells‡	3	20	3·60	23·2	37·4	56·0	99·7	216

† Calculated from total ribosomal material from sucrose gradient analyses and total number of cells in each fraction. *This should read mg ribosomes/10^{12} cells.

‡ Based on total TCA-precipitable radioactive material in the cell lysates.

cells in the lightest fractions are up to 200 times as active in protein synthesis and contain up to 40 times as many ribosomes as those in the heaviest fractions.

It is well documented that the amino acid incorporation as well as the amount of RNA is greatest in the youngest reticulocytes and decreases with maturation (Holloway & Ripley, 1952; Lowenstein, 1959; Schweiger, 1962; Gavosto & Rechenman, 1954; Bertles & Beck, 1962). These results, therefore, provide further evidence for an increasing degree of cell maturation from the top to the bottom of the gradient.

Thus several cytological and biochemical criteria are in accord that buoyant density centrifugation on an albumin gradient fractionates the reticulocyte population according to its degree of physiological maturity; the position of a reticulocyte on the gradient is a function of its age. (This result has been further verified by following the *in vivo* maturation of a narrow fraction of immature reticulocytes transfused into a normal rabbit (Millette & Glowacki, manuscript in preparation).) This fractionation provides an opportunity to investigate in detail the macromolecular changes occurring during reticulocyte maturation.

Sucrose gradient analysis and amino acid incorporation

Sucrose gradients of the ribosomal material from each of the six cell fractions are shown in Fig. 1. The optical density profile shows two major peaks, a narrow one of 80 s single ribosomes and a broader peak of polyribosomes. Most of the TCA-precipitable radioactive material is found in the polyribosomal region. The very low levels of radioactivity in the 80 s region indicate that negligible polysome degradation to single ribosomes occurs during the isolation procedure. Most of these counts are probably due to contaminating radioactive hemoglobin from the supernatant solution. The maximum leucine incorporation appears in the same position on each gradient, indicating that ribosomal aggregates of similar size must be responsible for the bulk of protein synthesis in all classes of reticulocytes.

The specific activities of the ribosomal material from the sucrose gradients are plotted in Fig. 2. This illustrates again the negligible incorporation into 80 s ribosomes. It is interesting to note that the polyribosomes from the most active reticulocytes have a higher specific activity than those from the least active. This difference in specific activity is not due to failure of the polysomes from the less active cells to reach saturation labeling during the incubation period, for the same labeling differences are observed for both five- and twenty-minute incubations. As will be shown later, the apparent decrease in the ability of polyribosomes to incorporate amino acids probably results from an increasing number of inactive polyribosomes in the more mature cells. Evidence for this in *in vitro* maturation has been reported by Marks, Rifkind & Danon (1963).

A summary of results from the six sucrose gradients and their respective ribosomal supernatant fractions is presented in Table 2. The protein synthesizing activity of the cells, represented by the total counts in hemoglobin (line 1), shows a large decrease from the youngest cells (fraction 6) to the oldest (fraction 1). A corresponding but less severe decrease is seen in the total ribosomal and polysomal material (lines 3 and 4). As a result the amount of protein synthesized per polyribosome as measured by absorbancy at 260 mμ (A_{260}) (line 5) is greatest in the most immature cells. On the other hand, the polysomal incorporation (line 7) very closely parallels the incorporation into hemoglobin (line 1). The percentage of ribosomes found as polyribosomes varies comparatively little with increasing state of maturation of the reticulocytes,

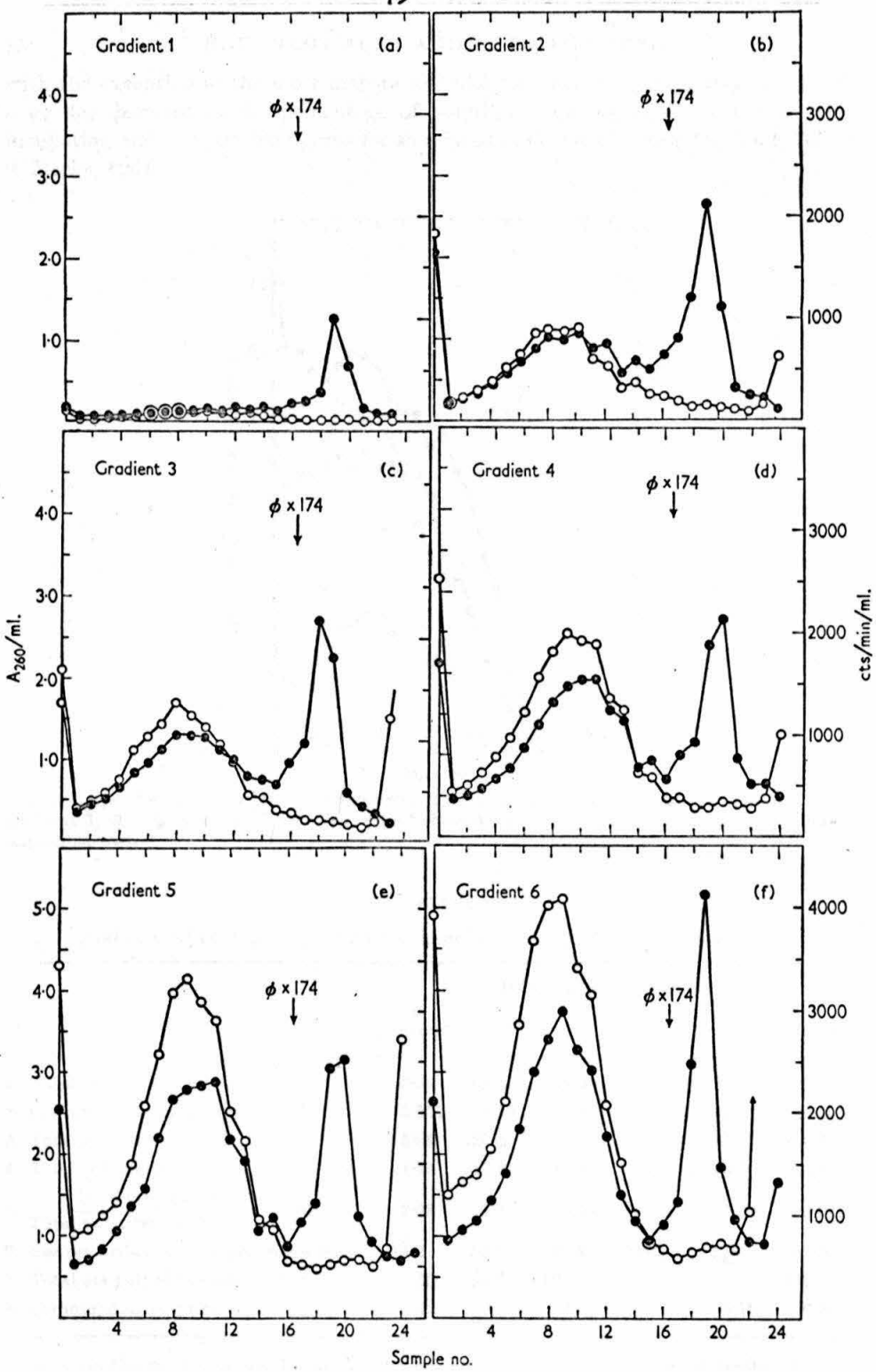

FIG. 1(a) to (f). Sucrose gradients of ribosomal material from the six cell fractions of the BSA gradients. Each fraction was incubated 10 min with [^{14}C]leucine under conditions described under Materials and Methods. Gradients are numbered from the densest to the lightest BSA fraction. All results are normalized to 2·0 ml. of cell lysate (corresponding to approx. 0·7 ml. packed cells). ϕX174 is included for sedimentation marker. (●) A_{260}/ml.; (○) cts/min/ml.

with the exception of the most mature reticulocytes. Marks *et al.* (1963) have found a similar decrease in the percentage of polyribosomes (75 to 47%) with *in vitro* incubation and comparable figures for simulated *in vivo* maturation (Rifkind, Danon & Marks, 1964).

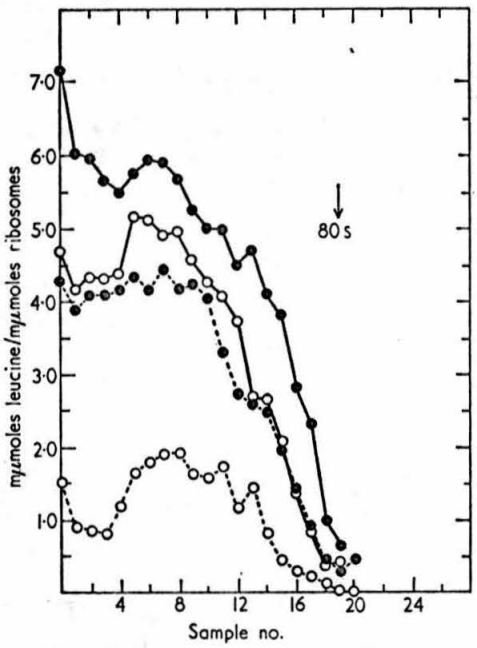

FIG. 2. Specific activity of the polyribosomes. ○--○, gradient 1; ●--●, gradient 2; ○—○, gradient 3; ●—●, gradient 6. Gradient 4 (not shown) falls between 3 and 6; gradient 5 is superimposable with 6.

TABLE 2

Amino acid incorporation and ribosomal content of reticulocyte fractions

	BSA gradient cell fraction 1 (bottom)	2	3	4	5	6 (top)
1. Total cts hemoglobin $\times 10^{-4}$	3·98	63·4	88·0	153	227	325
2. Cts/min/mg hemoglobin $\times 10^{-3}$	0·377	9·19	10·8	18·5	36·8	46·5
3. Total ribosomes (A_{260})	5·09	20·6	23·3	30·0	41·1	48·5
4. Total polyribosomes(A_{260})	1·74	11·8	13·4	19·8	28·5	32·8
5. $\frac{\text{Total cts protein}}{\text{Total polyribosomes } (A_{260})} \times 10^{-4}$	2·28	5·36	6·59	7·71	7·94	9·91
6. Per cent ribosomes as polyribosomes	34·2	56·6	57·3	66·1	69·4	67·5
7. Total cts polyribosomes $\times 10^{-2}$	5·20	94·7	124	200	315	374
8. Cts/min/A_{260} polyribosomes	1·42	3·77	4·41	4·78	5·19	5·34

Based on the results of the 10-min amino acid incorporation and 2 ml. of lysate (Fig. 1). Total cts hemoglobin are the total TCA-precipitable material in the ribosomal supernatant solutions. Total ribosomes represent total A_{260} found on the sucrose gradients. Total polyribosomes include sucrose gradient samples 1 through 14 plus pelleted polyribosomes.

When one plots the amount of incorporation into protein *versus* the amount of ribosomal material or the amount of polyribosomal material (Fig. 3(a)), one finds a linear relationship extrapolating to finite values of the abscissa. This is again indicative of some inactive ribosomes and polyribosomes. However, the most precise measure of protein-synthesizing ability is the amount of radioactivity associated with the polyribosomes (Fig. 3(b)). In all of our experimen we have found that the total amino

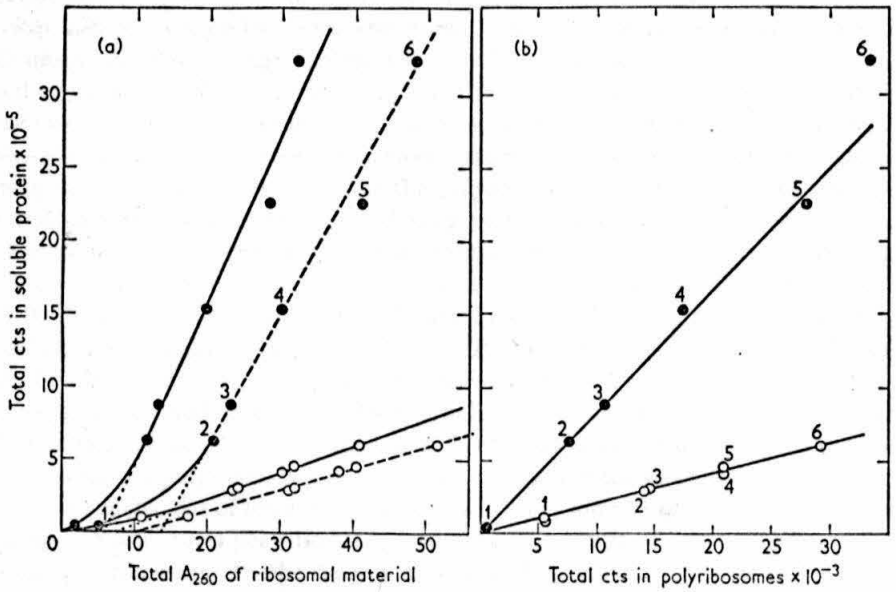

FIG. 3(a) and (b). Incorporation of [^{14}C]leucine into soluble protein. (a) As a function of the total amount of ribosomal material (dashed lines) and polyribosomal material (solid lines); (b) as a function of total counts on polyribosomes. (○) 5 min incubation with [^{14}C]leucine; (●) 10 min incubation with [^{14}C]leucine. The numbers correspond to the BSA cell fraction.

acid incorporation is directly proportional to the total counts on polyribosomes. Assuming the functionally active ribosomes have the same degree of labeling in all stages of cell maturation, this means that the rate of protein synthesis is proportional to the number of active ribosomes in polyribosomes.

4. Discussion

Rabbit reticulocytes can be fractionated according to their degree of physiological maturity by means of buoyant density centrifugation in an albumin gradient. This was verified by concordant data from cytological characteristics, size distributions, ribosomal contents and amino acid incorporation. The progressively less dense fractions not only stained as more immature reticulocytes but were shown to be larger, contain more ribosomes and exhibit a higher level of amino acid incorporation. We have then used these reticulocyte fractions to investigate the role of the polysome in hemoglobin synthesis in relation to the process of reticulocyte maturation. Although others have examined the fate of reticulocyte polysomes, following both *in vitro* maturation (Marks *et al.*, 1963) and *in vivo* maturation (Rifkind *et al.*, 1964), this system provides a means of studying the differences in the protein-synthesizing apparatus in a cell population of varying stages of maturation without the use of prolonged incubations.

In our studies we have found a small but progressive decrease in the percentage of ribosomal material present as polyribosomes, but no evidence for an orderly shift to smaller aggregates with maturation. Although the more mature cells contained fewer ribosomes, the main polyribosome peak as measured by absorbance and radioactivity appears in approximately the same position in the sucrose gradients as it does in gradients of material from the youngest cells. This is in contrast to electron microscope studies by Marks *et al.* (1963) and by Rifkind *et al.* (1964) for *in vitro* and *in vivo* maturation, and agree well with the observation of Mathias, Williamson, Huxley & Page (1964), who found mainly tetramer and pentamer ribosomal aggregates in electron microscope sections of reticulocytes containing very few ribosomes, i.e. the most mature cells. However, it seems likely that a balance is maintained within the cell between the concentration of polysomes and single ribosomes, thereby maintaining the integrity of the polysomes for hemoglobin synthesis up to the very last stages of maturation. It may very well be that the increase in single ribosomes that is observed in the more mature fractions is largely a result of the dynamic interaction between ribosomes and messenger RNA which would be expected from the scheme of ribosomal attachment and release from the polysomes observed in the cell-free system (Goodman & Rich, 1963; Hardesty, Miller & Schweet, 1963; Hardesty, Hutton, Arlinghaus & Schweet, 1963). We have evidence from cell-free experiments and indications from whole-cell studies that the rate of protein synthesis depends on the concentration of free ribosomes as well as polyribosomes.

The maximum polysomal labeling we have observed in the most active reticulocytes is about six molecules of leucine per ribosome. This is somewhat below the theoretical labeling of 8·5 leucines per ribosome, assuming an average of one-half a hemoglobin peptide per ribosome. In the least active cells this labeling is further reduced to approximately two leucines per ribosome at the pentamer peak. This submaximum labeling may be due to several causes. In the initial work on this problem the cells were first incubated with radioactive amino acid and subsequently separated on the albumin gradient. The incorporation into soluble protein was comparable to that reported here, but only very low counts were detected in either the polysomes or 80 s ribosomes. This is thought to be due to a chase effect caused by the release of cold leucine from the breakdown of non-heme proteins during the prolonged handling of the cells in the albumin and washings. It is known that the non-heme proteins, but not the hemoglobin, turn over in reticulocytes (Borsook, 1964), and that stroma proteins are degraded during reticulocyte maturation and re-utilized for hemoglobin synthesis (Schweiger, 1962). This same sort of breakdown may occur during these incubations and be expressed in the figure of 6 rather than 8·5 leucines per ribosome. However, the differences in the specific activities of the polysomes from the various cell fractions cannot be attributed to this. We have determined, by isotope dilution studies, that the internal leucine pool not only contributes negligibly to the observed incorporation but is essentially the same in the bottom as well as in the top fractions.

A more likely explanation for the lower than theoretical labeling and the decreased polysomal counts in the more mature cells is the presence of an increasing percentage of inactive ribosomes or polysomes with increasing maturation. The evidence supporting this is (1) the decreased specific activity of the polysomes in the more mature cells, (2) the extent of protein synthesis is directly proportional to the total counts on the polysomes and not to the total amount of polysomes in each fraction,

(3) the amount of protein synthesized per polysome (A_{260}) decreases with increasing maturation, and (4) the plot of total hemoglobin incorporation against total ribosomal or polyribosomal A_{260} extrapolates to a finite concentration of ribosomes at zero hemoglobin synthesis. A similar decrease in specific activity of polysomes has also been noted in *in vitro* maturation studies (Marks *et al.*, 1963) and in reticulocytes from patients with thalassemia major (Marks & Burka, 1964). Since the reticulocytes are in the process of losing their ribosomes, it seems likely that the lowered polysomal incorporation in the older cells can be best explained by the presence of damaged or inactive ribosomes in the polysomal aggregates, damaged S-RNA and/or damaged messenger RNA blocking the normal transcription mechanism.

The results we have presented show that the loss of protein-synthesizing activity in the maturing reticulocyte involves primarily the loss of total ribosomal and polyribosomal material. However, a concomitant decrease in the percentage of ribonucleoprotein present as polyribosomes occurs, but there is no shift to smaller polyribosomes. At the same time there is a progressive decrease in the capacity of the polyribosomes for protein synthesis, which is probably due to an increasing proportion of inactive or damaged pentamers. All three of these factors enter into the process of the maturation of the reticulocytes.

See Discussion Part II - D

The authors are grateful to Professor Henry Borsook for his interest and support of this project.

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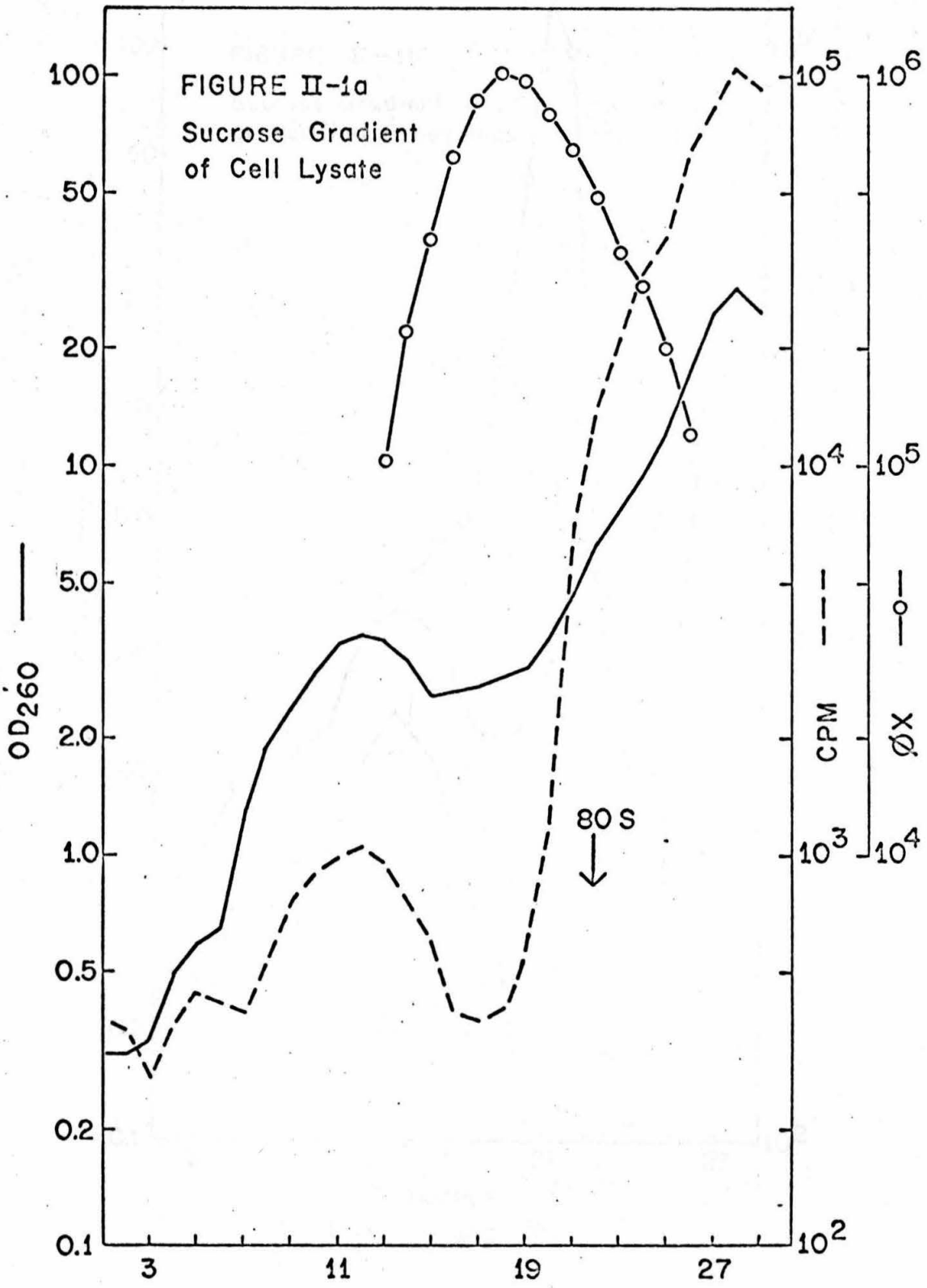
C. Appendix 1. - Comparison of Sucrose Gradients of Lysates and of Purified Ribosome Preparations

In our preliminary experiments we had hoped to be able to layer the entire lysate from an incubated cell fraction onto a sucrose gradient and avoid possible artifacts introduced by the additional centrifugation of the ribosomes through sucrose. However, it is clear from Figure II-1a that the presence of large amounts of hemoglobin in these lysates obscured both the radioactivity and optical density profiles in the region of the 80S ribosomes. At the same time the peaks were definitely spread out, as can be seen by comparison of the marker, ØX174, on Figures II-1 a and b. Though there should be no interaction between a bacteriophage of 114S and hemoglobin at 4S, it is apparent that the hydrodynamic properties and/or density stabilization of the sucrose gradient are altered by the presence of large amounts of protein. Since the profile in the polyribosome region appears unaltered by the purification, this procedure was adopted for all subsequent experiments.



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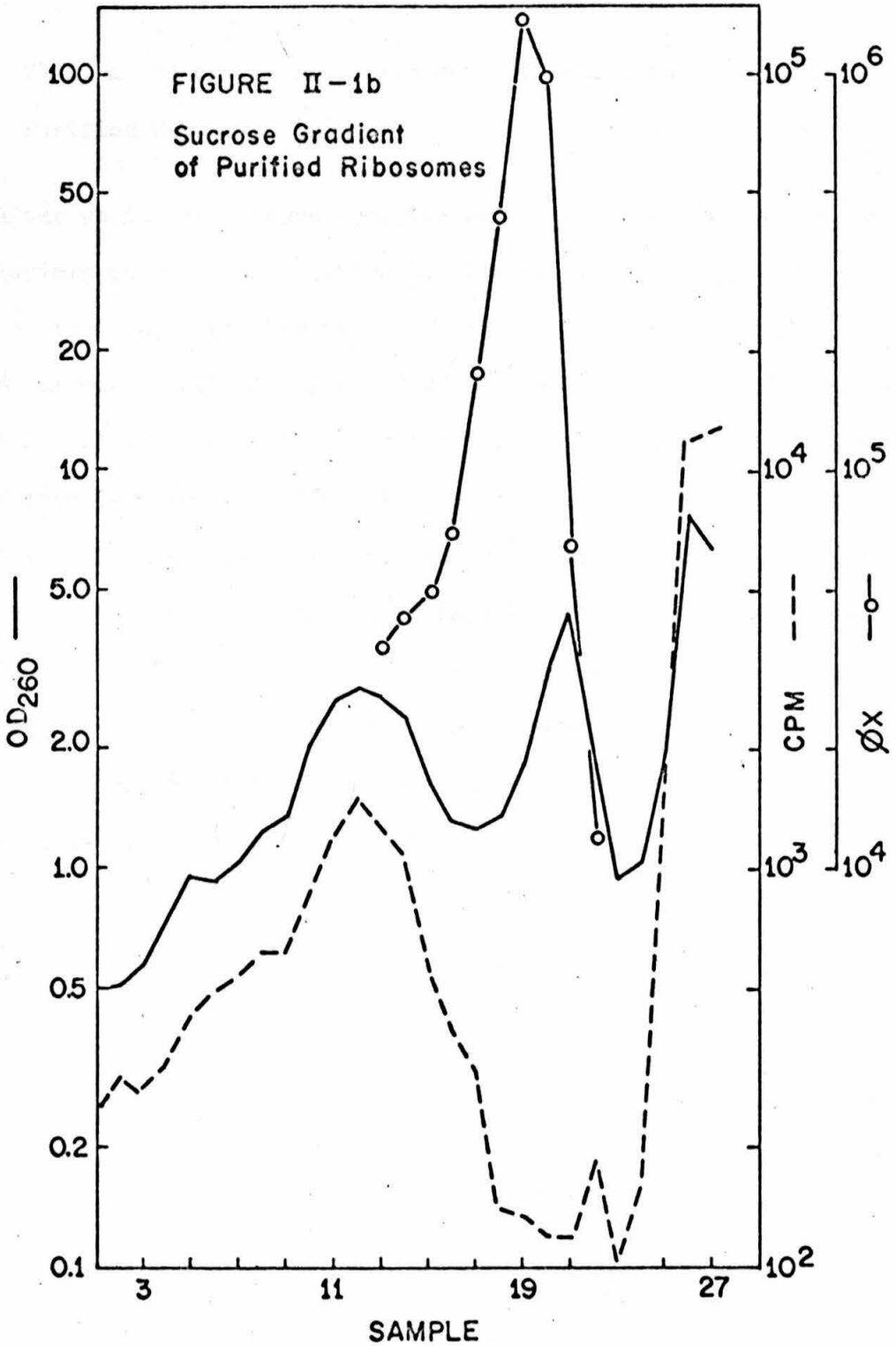

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Figure II-1, a and b: Sucrose Gradients of Cell Lysate (a) and Purified Ribosomes (b).

After whole cell incubation, the cells were lysed as described in Materials and Methods, Section B. Two ml of lysate were set aside on ice, while another two ml aliquot was layered over 2.5 ml of 15% sucrose in Solution G (0.08 M KCl, 0.005 M $MgCl_2$, 0.01 M tris, pH 7.6 at 20°C.), and centrifuged for 3 hours at 37,000 rpm (114,000g) in a Spinco SW39 rotor at 5°C. The pelleted ribosomes were resuspended in Solution G. Bacteriophage were added to the lysate and the ribosome suspension and these solutions were layered onto 25 ml sucrose gradients (15-30% sucrose in Solution G) and centrifuged at 24,000 rpm (58,500 g) for two hours at 5°C. in a Spinco SW 25.1 rotor. Fractions were obtained by drop collecting and the samples were analyzed as described in Materials and Methods, Section B.

D. Appendix 2. - Size Distribution of Reticulocytes from Various Regions of the BSA Gradient

Although not presented in the published article, Figure II-2 has been included here to illustrate the size distributions of the cells from the various fractions of the BSA gradient. The frequency distribution in three fractions has been plotted as a function of threshold voltage on the Coulter counter, and with respect to the location of normal rabbit erythrocytes on a comparable graph. The linearity of threshold voltage with cell volume was determined using rabbit and human erythrocytes, whose average cell volumes are known to be 61 and 87 μ^3 respectively (1).

Figure II-3 depicts the distribution of cells throughout the BSA gradient from which the fractions in Figure II-2 were taken. Seventeen samples were collected by pumping and pooled into six fractions containing nearly equal volumes of cells. This profile is typical, though minor variations occurred in different experiments, due to individual characteristics of the rabbit and differing degrees of reticulocytosis.

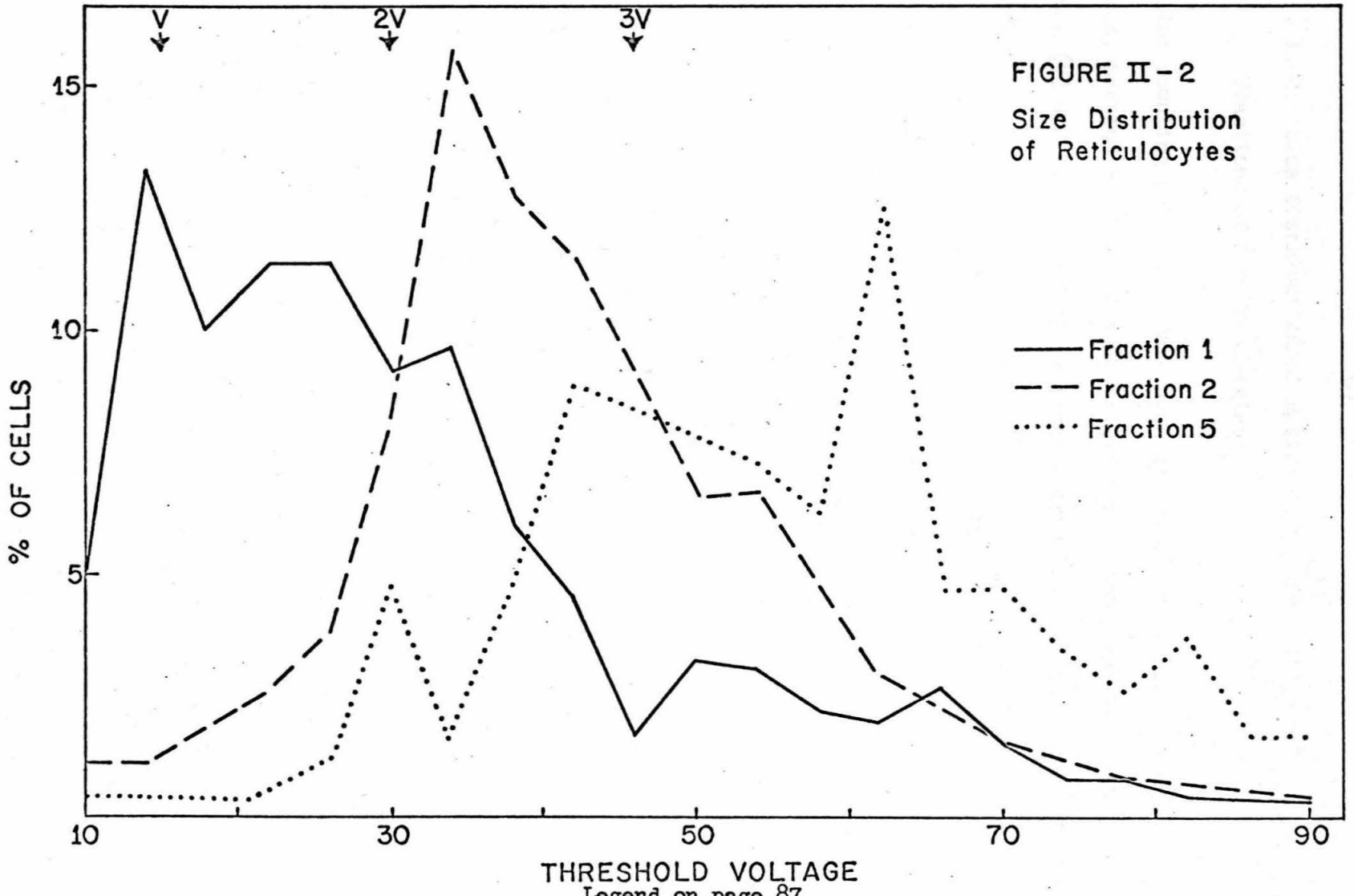

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Figure II-2: Size Distribution of Reticulocytes from Different Fractions of the BSA Gradient

The counting procedures have been described in Materials and Methods, Section B. The threshold voltage for normal rabbit erythrocytes (V) and for two and three times this volume (2V and 3V) are marked.

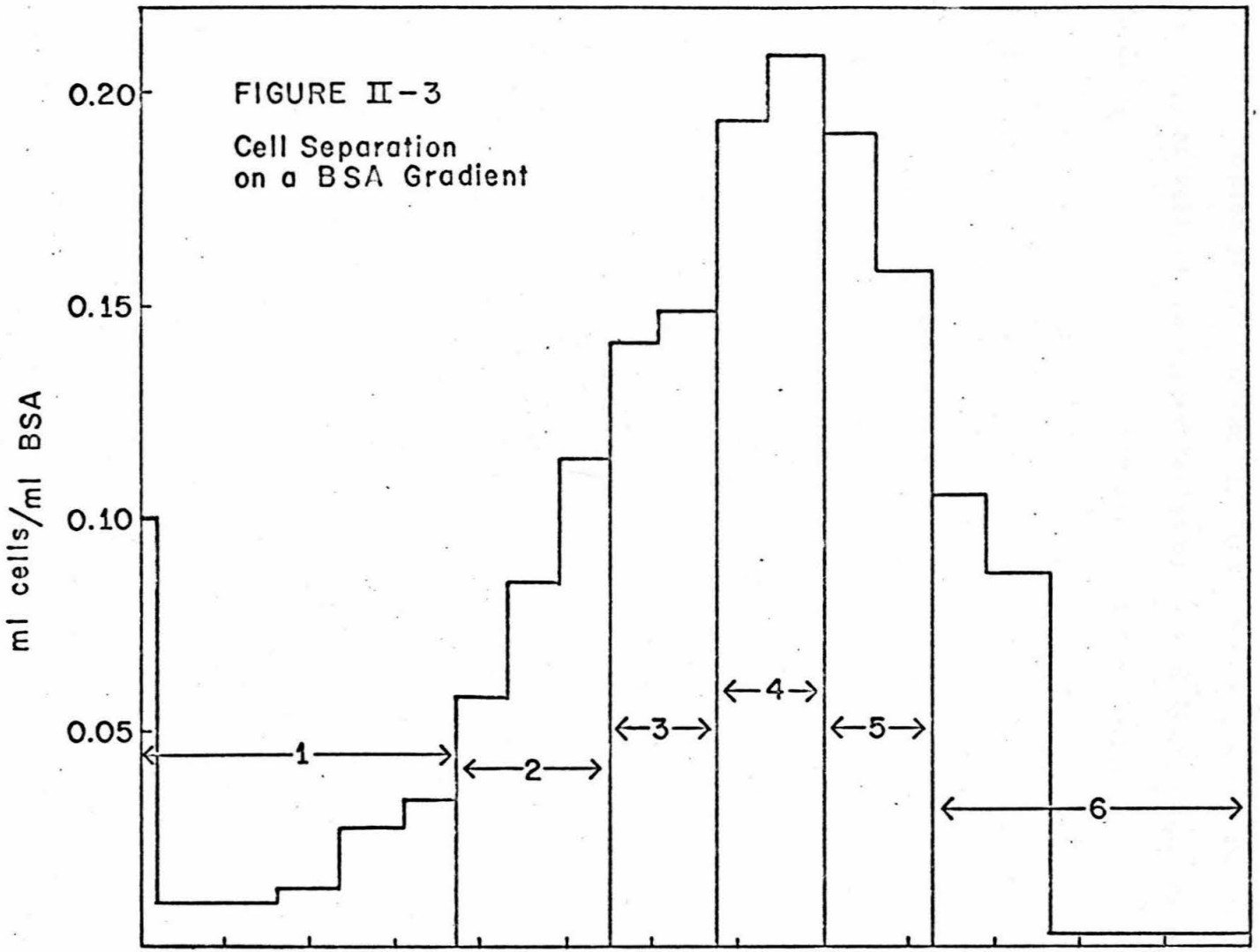

FIGURE II-3

Cell Separation
on a BSA Gradient

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Figure II-3: Cell Separation on a BSA Gradient

The samples pooled for the six final fractions are indicated (1 - 6), as well as the concentration of cells in each sample, as a function of the distance from the bottom of the gradient.

E.

(*Reprinted from Nature, Vol. 204, No. 4964, pp. 1207–1209, December 19, 1964*)

In vivo Maturation of Immature Reticulocytes transfused into a Normal Rabbit

WE have recently published the results of an investigation of polyribosomes and the loss of synthesis of hæmoglobin in maturing rabbit reticulocytes fractionated by buoyant density centrifugation in an albumin gradient[1]. Several cytological and biochemical criteria were applied to show that this procedure fractionates the reticulocyte population according to their degree of physiological maturity. This communication presents additional proof, using *in vivo* maturation of a fraction of the youngest reticulocytes, that the position of the cells in the albumin gradient is a function of their age. In addition, these investigations provide an estimate of the life-span of the reticulocytes produced in phenylhydrazine-induced anæmia.

Reticulocyte fractionation by buoyant density centrifugation in bovine serum albumin (BSA) gradients and *in vitro* incubations were performed as previously described[1]: 1·75 ml. of the most immature cells were isolated from 9·7 ml. of reticulocytes from a phenylhydrazine anæmic rabbit and incubated in the complete reaction mixture with 2 mc. of L-leucine-4,5-^{3}H, 3·37 mc./μmole (Nuclear Chicago), for 1 h at 37° C. These tritium-labelled cells were washed, suspended in 2 vol. of rabbit serum containing 5 mg/ml. sodium citrate and injected into the marginal ear vein of a normal rabbit. Blood samples of 10 ml. each were taken from the ear of the recipient after 1 h and at various intervals thereafter for 15 days (Fig. 1). After measuring blood and cell volumes, the cells were washed in NKM (0·153 M NaCl, 0·005 M $MgCl_2$, 0·005 M KCl) and suspended in 0·5 vol. of NKM. Five to six ml. of this cell suspension were fractionated on a 25-ml. BSA gradient, and collected in 1-ml. fractions. Cells from each fraction were washed with NKM and lysed with 0·8–1·6 ml. of lysing solution[1]. Stroma pellets were washed with 0·2–0·4 ml. of 0·1 M KCl, 0·1 M *tris*, *p*H 7·5, and the washings added to the stroma-free lysate.

Radioactive protein was determined by treating 0·1–0·5 ml. of lysate with 5 ml. of 1 per cent HCl in acetone to remove the hæm. The protein precipitate was centrifuged, washed with acetone, dried, and dissolved in 3 ml. water. One-tenth-ml. aliquots of these final protein solutions were added to 10 ml. of Bray's solution[2] for liquid scintillation counting.

The distribution of radioactivity in the blood samples analysed on BSA gradients is shown in Fig. 1. The time of sampling, given in hours after the initial injection of labelled cells, is indicated for each curve. In the first

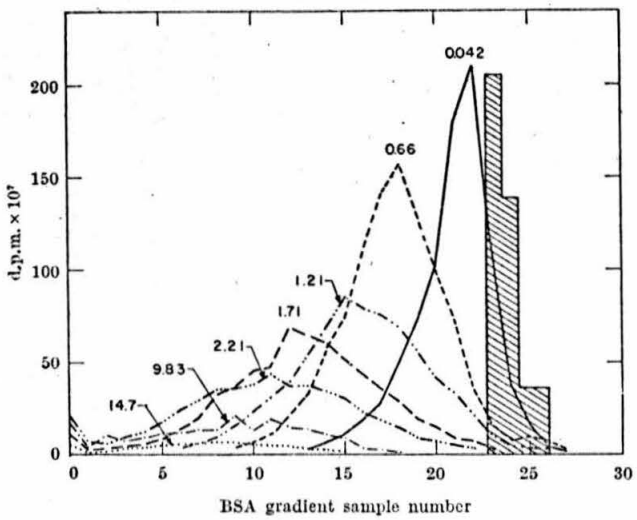

Fig. 1. Distribution of radioactivity in the blood cell samples as analysed on BSA gradients. Time, in days, after injection of tritium-labelled reticulocytes is indicated for each curve. Gradient samples are numbered from the densest (1·090 g cm^{-3}, 24° C) to the lightest (1·065 g cm^{-3}, 24° C). Cross-hatched area represents the position of the initial band of young reticulocytes as isolated from a phenylhydrazine anæmic rabbit

sample, taken 1 h after injection, the labelled cells re-banded at almost the same position as on the original gradient from which they were isolated. In the blood samples taken during the first two days following injection, the tritiated cells banded in successively lower (denser) positions in the gradient as they matured *in vivo*. During this time the position of the peak of radioactivity on the gradients moved linearly with time toward more dense fractions (Fig. 2). A similar but slower increase in density with age has been observed in normal rabbit erythrocytes by Leif and Vinograd[3]. The results presented here confirm our earlier report based on cytological and biochemical evidence that the position of the cells in the gradient is a function of their age.

After the first two days, the banding position of the tritiated cells rapidly levels off two-thirds of the way down the gradient at fraction 9. This final banding position corresponds in density to that of the lightest normal erythrocytes. We have previously shown that the least dense cell fractions consist of the most immature, macrocytic reticulocytes[1]. The present findings show that such cells mature to erythrocytes which are less dense than the majority of normal erythrocytes. This maturation, in terms of buoyant density changes, occurs in about 48 h. Since the injected reticulocytes band well above the normal erythrocytes during the first day, this method offers the possibility of studying the biochemical events of maturation *in vivo*. Such studies have been precluded in

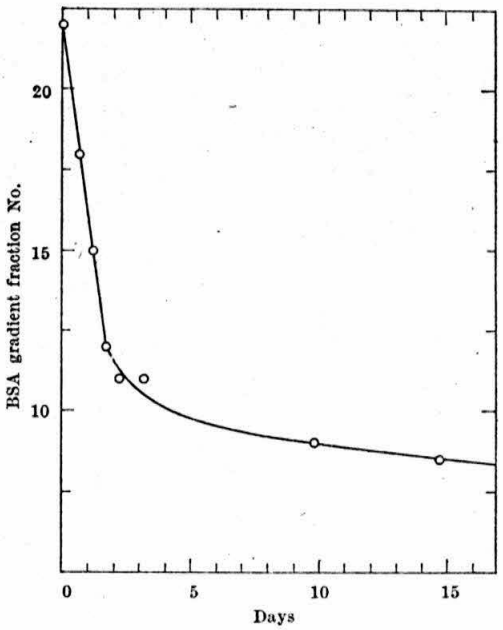

Fig. 2. Position of peak of radioactive cells as analysed on BSA gradients, as a function of time after transfusion

anæmic animals due to the constant influx of new reticulocytes from the bone marrow.

A graph of the per cent survival of the labelled cells versus time (Fig. 3) shows two distinct populations. One population, approximately 55 per cent of the injected cells, shows a life-span of about two days. The remaining 45 per cent of the cells disappear from the circulation after 17 days. A similar survival curve of sickle cells transfused into normal human beings has been reported by Singer and Fisher[4]. This is evidence for the extremely short life-time of the red blood cells which arise in response to a hæmolytic crisis. Previous reports, based on *in vivo* incorporation of ^{14}C-glycine[5] and incorporation of iron-59 (ref. 6), have indicated considerably long half-lives, of about 19 days, for such reticulocytes. However, the present findings are not only consistent with the results of *in vivo* ^{14}C-valine labelling of reticulocytes reported by Borsook *et al.*[7], but are in excellent agreement with the survival times observed by Mazur and Carleton for ^{14}C-glycine labelled reticulocytes transfused into normal recipients[8]. Such extremely short life-spans are not unexpected since survival times as short as four days have also been observed in several hæmolytic anæmias of human beings[9–11].

We thank Prof. Henry Borsook for his advice.

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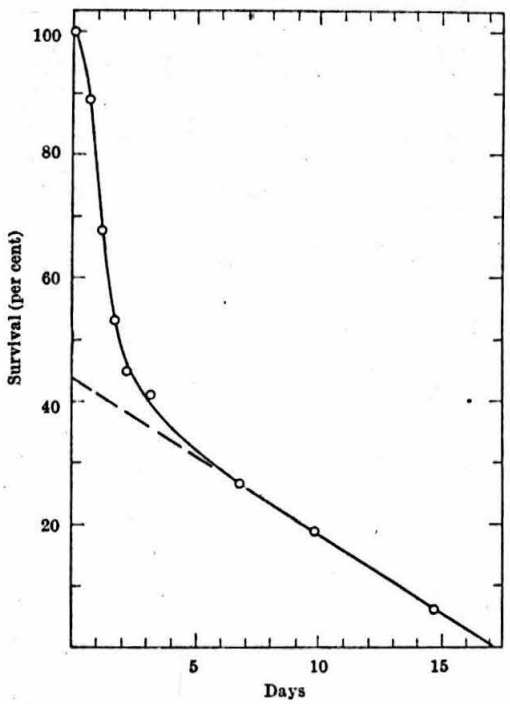

Fig. 3. Per cent survival of tritium-labelled reticulocytes as function of time after transfusion. Values represent per cent of initial radioactivity injected, corrected for cell samples removed

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F. Discussion

In a recent paper by Danon and his co-workers (2) our conclusions concerning the retention of polyribosomes up to and including the final stages of reticulocyte maturation have been seriously questioned. These authors argue that contamination of Fraction 1 with cells belonging in Fraction 4 or 6 could account for the polysomes we have found.

There are several objections to this explanation. Firstly, it is our opinion that Danon et al. have indulged in circular reasoning, for they categorically consider all cells having clusters of three or more ribosomes (by electron microscopy) to be "young" cells, and contaminants of their system. From this premise they conclude that "old" cells have only single ribosomes. On the other hand, Mathias et al. (3) observed reticulocytes by electron microscopy, and classified them according to the total number of ribosomes present, assuming that more mature cells had fewer ribosomes. Using this criterion they have observed that very mature cells contain clusters of up to 4 or 5 ribosomes. This supports our conclusion that polyribosomes are present in the most mature cells. Rowley (4) correlates the maturation of reticulocytes with the loss of total ribosomal material and with the loss of polyribosomes, but makes no statements regarding the relative amounts of single particles and clusters.

A second objection to the view of Danon et al. is that if the polyribosomes in our Fraction 1 are from "young" cells, they must be selectively from damaged cells, since the specific activity of these ribosomes is so much lower than that found in Fractions 4 or 6. It

is not possible to distinguish at the present time between polyribosomes from mature cells which have a lessened activity and polyribosomes from contaminating "young" cells which are damaged, and therefore inactive.

We cannot exclude the possibility that the last stage in the maturation of a reticulocyte to an erythrocyte contains only single ribosomes and due to increased osmotic fragility is lost from our preparations. However, we have found no evidence for the massive conversion of polyribosomes to 80S particles which has been claimed by other groups and which has already been considered in the Discussion of the article reprinted in B.

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PART III

Analytical Ultracentrifugation of Ribosomes and Ribosomal RNA from Reticulocytes

A. Introduction

Almost ten years after the discovery of ribonucleoprotein (RNP) particles and their role in protein synthesis in living cells (1), a great deal of confusion and contradictory evidence exist concerning the physical characteristics of ribosomes and ribosomal RNA. An excellent review of this field up to 1963 is given by Petermann (2). The problems involved with physico-chemical studies are due partly to the complexity of the particles, which contain two major RNP subunits, each of which is composed of RNA and as many as 15 different basic proteins. Moreover, the diversity of techniques employed to isolate and purify ribosomes from many different types of tissues makes comparison of results uncertain. It is relatively difficult to obtain particles which are at the same time pure and functionally intact, and in contrast to other types of biological material, it appears that the observed physical characteristics depend not only upon the ionic strength and the balance of divalent and monovalent cations in the ribosomal suspension medium, but also upon the particular monovalent cation used (3). Furthermore, marked differences exist between the more thoroughly studied bacterial ribosomes (4) and RNP from higher organisms, including yeast (5), fungi (6), higher plants (7), and mammals (8,9,10,11). Reticulocyte ribosomes, in particular, are

larger, contain a lower percentage of RNA, are more stable in solutions of low ionic strength and low magnesium ion concentration, are able to bind soluble RNA (S-RNA) and messenger RNA (M-RNA) more tightly, and are more difficult to dissociate into RNP subunits than bacterial ribosomes. This section deals with sedimentation studies in the analytical ultracentrifuge, first of polyribosomes, then of the 80S monomer and its subunits, and finally of ribosomal RNA.

Polyribosomes (polysomes, ergosomes) were first discovered in rabbit reticulocytes (12,13), and they have since been observed in many other types of cells (14).

The sedimentation of polyribosomes has been studied largely in sucrose gradients in the preparative ultracentrifuge, or with Schlieren optical systems in the analytical ultracentrifuge, using large amounts of material. Boundary centrifugation using ultraviolet optics uses small enough amounts of material to eliminate concentration effects, but interpretation of boundaries in multi-component systems is uncertain at best. Good analytical data on polyribosomes can be obtained, however, using the band centrifugation method of Vinograd et al. (15).

The basic monomeric ribosome from higher organisms is known to be an RNP particle with an S-value of approximately 80 and a molecular weight of 3.5 to 4.5 million (9,16,17), roughly half protein and half RNA by weight (9), with a buoyant density of 1.45 to 1.59 (18,9), depending upon the organism from which it is derived. Bacterial ribosomes, on the contrary, have a sedimentation coefficient of 70S, a molecular weight of 3 million (4,19) and contain up to 63% RNA (4).

In 1960 it was reported that by reducing the magnesium ion concentration below 10^{-3} molar one could separate bacterial ribosomes into well-defined RNP subunits with S-values of 30 and 50S (4). There were also reports that by manipulation of the relative concentrations of divalent and monovalent cations or by using chelating agents, one could obtain partial dissociation into comparable subunits (40 and 60S) of ribosomes from pea seedlings (20), rat liver (8), and reticulocytes (21,22). In 1961 H. Lamfrom and I conducted an extensive study of the dissociation of reticulocyte ribosomes and were able to obtain complete dissociation into 40 and 60S subunits. Our published results have been presented in Part I, Section D. Since this publication, various other investigators have studied dissociation of 80S ribosomes (24-27), and a discussion of their findings in relation to ours is presented in Section D of this part.

Although the chemistry of nucleic acids in general and the physical characteristics of DNA in particular have been thoroughly investigated, the study of high molecular weight RNA's has been continually hampered by the difficulty in obtaining homogeneous undegraded material for examination. This is due largely to the stability and wide distribution of RNase and the lack of a convenient and effective enzyme inhibitor. In addition, "model" compounds, polyribonucleotides of defined size and composition, have not been readily available. In the physical studies of viral RNA's, the criterion of infectivity may be used to show that a preparation is undegraded during treatment; but such a test does not exist for ribosomal RNA.

Several different aspects of R-RNA which have been studied are the following: 1) the amount of RNA in a single 70 or 80S monomer, 2) the molecular weight of the RNA or RNA's found, 3) the distinctions between the RNA isolated from the large (50 or 60S) subunit and that from the small (30 or 40S) subunit, 4) the possibility that the high molecular weight RNA's are composed of smaller polynucleotide subunits joined together by hydrogen bonds or other non-phosphodiester linkages. Since all of these questions have been asked most frequently for bacterial R-RNA, a summary of the current knowledge will be useful for comparison with data for the 80S ribosome, although this author has been chiefly concerned with points 2) and 4) for rabbit reticulocyte ribosomes.

It is now well established that a 70S ribosome has an RNA content of 1.5×10^6 daltons and that this RNA is isolated as two large pieces, one (23S), of molecular weight approximately 1×10^6, and the other (16S), molecular weight 0.5×10^6 (29). However, the controversy over whether or not each of these centrifugal components is composed of smaller RNA subunits held together with hydrogen bonds as suggested by Kurland (30) and substantiated by other groups (29) was not resolved until 1964, when Stanley and Bock were able to prepare a nuclease-free sample with which to do physical measurements and could show conclusively that each of these RNA molecules is a single integral chain (31). Preliminary evidence for this result had also been obtained by Bogdanova et al. (32). Concerning point 3), Aronson (33) has been able to demonstrate differences in base composition and sequence between the RNA's isolated from

the 50 and 30S RNP subunits. This is further supported by evidence that the RNA from the two subunits hybridizes with different segments of the bacterial DNA (34). Kurland (30) and Aronson and McCarthy (35) have found that while the 30S subunit contained only 16S RNA the 50S could contain either 23 or 16S, but this result is dubious, since a single RNase nick in a 23S molecule could produce two 16S pieces.

Comparable data on R-RNA from higher organisms do not exist, and the facts available are often contradictory. Spirin's review (29) gives information obtained before 1964 and points out the difficulties in comparing information about RNA which has been prepared from different types of tissues in many different ways. In general, RNA is obtained from 80S ribosomes as two main components, one roughly three times the size of the other, with S values variously reported to be 25 to 30S for the larger and 14 to 18S for the smaller. The molecular weights for these components are roughly 1.5×10^6 and 0.5×10^6 (calculated by a variety of means, some more dependable than others); however, some authors find that a preparation containing RNA's of 16 and 28S gives only a single value of 0.55×10^6 for the molecular weight by light scattering (9,36,37).

The two components appear to be different, as do the 23 and 16S of E. coli. The 18 and 30 S R-RNA's from rabbit liver have been shown to have differing electrophoretic behavior on agar gels (38). Moreover, Montagnier and Bellamy (39) have shown base analysis differences between 18 and 30S RNA's from ascites tumor cells, and Monro (40) has shown similar results with 19 and 28S RNA's from rat liver ribosomes.

Most of the analytical ultracentrifuge studies of R-RNA have been done with material from liver (rat, calf or chicken) or reticulocytes (sheep or rabbits), and have been concerned in one way or another with the secondary structure of RNA in solution, the existence of smaller RNA's, or with interconversion of the 28 and 16S RNA's by aggregation and dissociation (41). We have examined R-RNA from reticulocytes under a variety of denaturing conditions. These experiments are reported in Section E.

B. Materials and Methods

1. Preparation of Ribosomes

Polyribosomes (Section C) and ribosomes for RNA preparations were obtained by the method of Glowacki and Millette (Part II, Section B). All preparations were stored as pellets at -70°C.

2. Preparations of Ribosomal RNA

R-RNA for most of the experiments was prepared by the LiCl method of Barlow et al. (42). After the initial separation the material was precipitated with 0.2% potassium acetate and 2.5 volumes of 95% ethanol. After three precipitations, the final pellet was dissolved in 0.001 M EDTA, pH 7.1, and centrifuged at 10,000 g to remove insoluble material. The clarified solution was dialyzed for 3 hrs. against the same buffer. Ribosomal RNA was also prepared by the phenol method of Kirby (43). Since the yield from the phenol preparation is very low and the material contains S-RNA, RNA prepared by the LiCl method was preferred for the ultracentrifugation studies. All RNA preparations were stored at -70°C.

3. Analytical Ultracentrifugation of Ribosomes

All analytical ultracentrifugation was performed with a Spinco Model E analytical ultracentrifuge.

Polyribosomes were examined by band centrifugation (15) using a single sector Kel-F bandforming centerpiece of the gap-transfer type (44). The sedimentation solvent was 90% D_2O containing

Solution P (0.08 M KCl, 0.0015 M $MgCl_2$, 0.01 M tris, pH 7.6 at 20°C.). Photographs were taken with monochromatic light at 265 mμ and the films traced with a Joyce-Loebel Mark III Microdensitometer. S values were corrected for the density and viscosity of the D_2O solution and reported as $S_{20,w}$.

4. Formaldehyde Treatment of RNA

The stock formaldehyde (HCHO) solution was Mallinckrodt Analytical Reagent Grade Formaldehyde containing 36-38% formaldehyde, and 10-15% methanol as a preservative. Solutions of RNA were diluted into a buffer containing 0.02 M Na phosphate pH 7.0 and 1/20 volume of the stock HCHO solution. Reaction curves were performed by heating aliquots of a stock solution for various lengths of time to 80 or 100°C. and reading the optical densities on a Beckman DK-2 Recording Spectrophotometer after cooling the samples to room temperature. Ultracentrifugation was performed with D_2O sedimentation solvents containing 1.8% formaldehyde.

5. Ultracentrifugation of R-RNA

Reticulocyte R-RNA was analyzed by band centrifugation. The sedimentation solvent was either D_2O containing various buffers (listed in the figures and tables) or 96-99% DMSO. The solution in the sample well (containing the RNA) was an aqueous solution with the same buffer as the sedimentation solvent for the D_2O runs.

For DMSO runs, the sample was in 50% DMSO, 0.001 M EDTA, and 50% dimethyl formamide. For some centrifuge runs a single sector band-forming centerpiece, double channel, Type III (44) was used and photography and densitometry were the same as for polysome determinations. All runs used ultraviolet optics and monochromatic light, 265 mμ for D_2O and 275 mμ for DMSO. Later runs used a Type III double-sector, double channel, band-forming centerpiece (44), and traces were obtained directly using the Spinco Photoelectric Scanning System. S-values given have been calculated as center-of-mass S-values. These were obtained by dividing each peak on each trace into two equal areas with a vertical line and using these lines as the center of mass position for the material. Relative amounts of components were calculated from the areas under the peaks. Since the relative correction for radial dilution in a band centrifugation is only 5% ($R_{peak}1$ / $R_{peak}2$), this correction was omitted. Peak positions were corrected for time required to scan the cell when the photoelectric system was used.

Data on the density of D_2O solutions (45), viscosity of D_2O (46), and density and viscosity of aqueous salt solutions (47) were used to correct values to $S_{20,w}$. Although the data on the density and viscosity of DMSO were available (48) no correction to $S_{20,w}$ was made, for the value of $\bar{v}$ for this material is not known in DMSO. S-values are not corrected for the density

or viscosity of 1.8% HCHO because no information was available on either of these properties for dilute HCHO solutions.

6. Abbreviations Used

EDTA - ethylenediaminetetraacetic acid

DMSO - dimethyl sulfoxide

D_2O - deuterium oxide (heavy water)

DMF - dimethyl formamide

C. Analytical Band Centrifugation of Reticulocyte Polysomes

The analytical ultracentrifugation portion of the article by E.R. Glowacki and R.L. Millette, "Polyribosomes and the Loss of Hemoglobin Synthesis in the Maturing Reticulocyte", which appeared in the Journal of Molecular Biology, 11, 119 (1964), is included here. Written permission has been obtained from the copyright owner, Academic Press. The references in the body of the reprint have been numbered and included in the bibliography for this section. The results shown here have been since reproduced several times, using a double-sector band-forming cell and the photoelectric scanning system.

Analytical ultracentrifugation

To determine the sedimentation coefficients of the various polyribosome components, band sedimentation (Vinograd *et al.*, 1963) was used because the method offered ease of resolution of many molecular species and required a low enough concentration of material (7 μg of ribosomes per run) so that the correction for extrapolation to zero concentration is negligible. No attempt was made to determine relative amounts of the different peaks, since the material is subjected to an unknown amount of shear when it passes between the centerpiece and the window of the centrifuge cell as it layers onto the D_2O solution.

Average figures from several runs of $S_{20,w}$ values for the five clearly separable components were 80, 130, 153, 190 and 220 s. These values are lower than the approximate figures obtained from the sucrose gradients (86, 123, 181, 205 and 238) by calculation according to the method of Martin & Ames (49). The analytical values are very close to those found by Gierer (50) in a similar ionic strength. Using his equation for S-value against number of ribosomes in the polyribosome, our values correspond to a regular series from the monomer (80 s) to the pentamer (220 s). The pentamer is the principle polyribosome peak appearing in the sucrose gradient analyses.

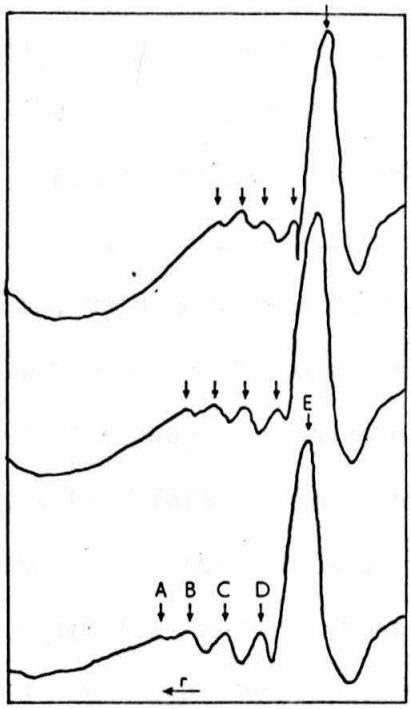


FIG. 4. Analytical band centrifugation of polyribosomes. Densitometer tracings of films taken after 12, 14 and 16 min at 20,410 rev./min. Sample was 10 μl. of 3·0 A_{260}/ml. layered over 0·55 ml. solution P in 90% D_2O. $S_{20,w}$ for the peaks are: A = 216, B = 197, C = 160, D = 126, E = 75 s.

D. Analytical Ultracentrifugation of 80S Ribosomes and Their Subunits

Data on the analytical ultracentrifugation of 80S ribosomes and their subunits have already been presented in Part I, Section D. Since the time at which this work was published, a large amount of work on ribosomal dissociation has been done (3, 24-27). Although a complete description of the physical dissociation of ribosomes is not possible with the data available at this time, one can divide ribosomes into various types in terms of the ease with which they may be dissociated. The 70S particle from bacteria is the most labile, and will separate easily into two well-defined subunits whenever the Mg^{++} concentration is reduced below 10^{-3} M. The 80S ribosome from reticulocytes appears to differ from the 80S particles observed in other tissues.

We had found that reticulocyte ribosomes dissociated completely into one 60 and one 40S subunit in the presence of pyrophosphate, and others have reported this result using EDTA as the chelating agent (3). We were unable to achieve complete dissociation in solutions containing 0.05 M KCl and 0.001 M tris, with no magnesium present. Recently, Philipps has shown that reticulocyte ribosomes dissociate completely with concomitant release of the nascent peptide chain in solutions of 10^{-5}M $MgCl_2$ containing 0.04 M NaCl, but give heterogeneous material from 50 to 80S in the same Mg^{++} concentration in the presence of 0.05 M KCl. Ts'o and Vinograd (22) reported that after dialysis against solutions of KCl in low magnesium, reticulocyte ribosomes gave a broad 50S peak in the ultracentrifuge patterns. They hypothesized

that the 30S material was being degraded, but they may have been observing an intermediate form.

The third type of ribosome is the 80S monomer of the "liver" type, which is incompletely dissociated even in the presence of low concentrations of chelating agents. On increasing the level of EDTA, the 50-60S intermediate breaks down to give 60 and 40S subunits (25). Wettstein has reported that this alteration from the "first dissociation product" to the subunits is associated with the release of the nascent peptide (51). Similar 50-60S ribosomes have been found in yeast (24) and Jensen Sarcoma (28).

We feel on the basis of our dissociation studies that reticulocyte ribosomes are more easily separated into subunits than ribosomes from other types of mammalian tissues. In the light of the extensive studies of liver ribosomes by Siekevitz, we believe that the conclusion we stated in the publication, that reticulocyte ribosomes are more difficult to dissociate than liver ribosomes, is in error. We had based this conclusion on the work of Sachs (52).

E. Analytical Ultracentrifugation of Rabbit Reticulocyte Ribosomal RNA

This study of the sedimentation properties of reticulocyte ribosomal RNA was originally undertaken because we felt that improved techniques for RNA purification (the LiCl method) and improved centrifugation techniques (band centrifugation with and without the photoelectric scanning system) warranted a new study of ribosomal RNA in this laboratory. In addition we wished to look for a 22S component, such as that reported for rat liver microsomal RNA (41).

Several RNA preparations from purified reticulocyte ribosomes, two prepared by the LiCl method ("A" and "C"), one by the phenol method ("B"), and one of the larger component after separation on a sucrose gradient ("D"), were compared in the same buffer, 0.02 M Na phosphate, pH 7.0. These results are given in Table III-1. In all of these preparations there are two major components, the "28S" and the "16S" and a variable amount of the minor component, "22S". The 41S component observed by Barlow (42), was not seen in any of these preparations and may be an aggregate which does not appear when the centrifugations are carried out with a very low RNA concentration. S-RNA was also present in the phenol extracted RNA, but not in the LiCl preparations.

Any of these preparations (and especially "C," which was used for the later studies) compare well with the starting material used by Petermann, which contained a 28S component (49% of the total), 22S (9%), 16S (27%) and 8S (15%) (41). Though no smaller components of defined S-value could be found in our material, a variable amount of heterogeneous trailing material (up to 15% of the total) was present.

TABLE III - 1

Ultracentrifugation of Various RNA Preparations in 0.02 M Na Phosphate, pH 7.0

RNA Preparation	S Values			% Material			Ratios of S	
	"28"	"22"	"16"	"28"	"22"	"16"	"28"/"16"	"28"/"22"
(1) LiCl RNA "A"	25.4	19.6	14.7	55.0	12.0	33.0	1.72	1.30
(2) Phenol RNA "B"	24.4	----	14.6	38.4	----	61.6	1.67	----
(3) LiCl RNA "C"	28.3	----	15.1	62.5	6.2	31.3	1.87	----
(4) "28S" isolated from a sucrose gradient	28.2	21.3	----	56.8	43.2	----	----	1.32

On Table III-1 (as well as Tables III-2 and III-3) the relative amounts of the various components have been calculated ignoring this trailing RNA. In none of the preparations were we able to achieve the theoretical percentages of 28 and 16S (75% and 25%). (This theoretical figure assumes that each 80S ribosome contains one molecule of each type, and that the two types have a ratio of 3:1 in molecular weight.) However, other evidence indicates that there is some slight degradation even in our best preparations.

Due to the minor differences in RNA samples, it was decided to perform all further physical studies on one preparation and "C" was chosen. This RNA gave stable patterns after several days at -20°C. and after several months at -70°C. and showed no alteration after repeated freezing and thawing.

Table III-2 illustrates comparative data for reticulocyte R-RNA centrifuged in a variety of different buffers. Though the S-values appear to be quite dependent upon the ionic strength and the nature of the buffer used, the ratios of the S-values fall into two groups; one set of values is approximately 1.76 and the other approximately 1.88. Using S-values determined by band centrifugation for the 16 and 23S components of E. coli R-RNA, 17.8 and 25.5 respectively, (Strauss, personal communication) and their reported molecular weights of 0.55×10^6 and 1.12×10^6 (30), one obtains the formula:

$$S = k M^{0.52}$$ (See Figure III-1)

This value for the S on M dependence falls between that given by

TABLE III - 2

LiCl RNA "C" Analyzed in Various Buffer Systems

Buffer	S Values			% Material			Ratios of S	
	"28"	"22"	"16"	"28"	"22"	"16"	"28"/"16"	"22"/"16"
(1) 0.20 M NaCl + 0.001 M EDTA	34.5	26.0	19.5	57.3	8.9	33.8	1.77	1.33
(2) 0.02 NaCl, + 0.001 tris, + 0.001 EDTA	25.6	20.4	15.3	49.8	12.8	37.4	1.74	1.33
(3) 020 M NaCl + 0.001 M tris	30.0	----	15.5	62.7	8.5	28.7	1.93	----
(4) 0.01 M Na phosphate	26.5	18.8	14.4	60.0	8.2	31.0	1.88	1.30
(5) 0.01 M Na phosphate + 0.01 M Na citrate	29.4	----	15.6	62.0	8.5	29.6	1.88	----
(6) 0.02 M Na phosphate + 0.003 M EDTA	27.2	20.9	14.8	56.3	12.6	36.5	1.84	1.41

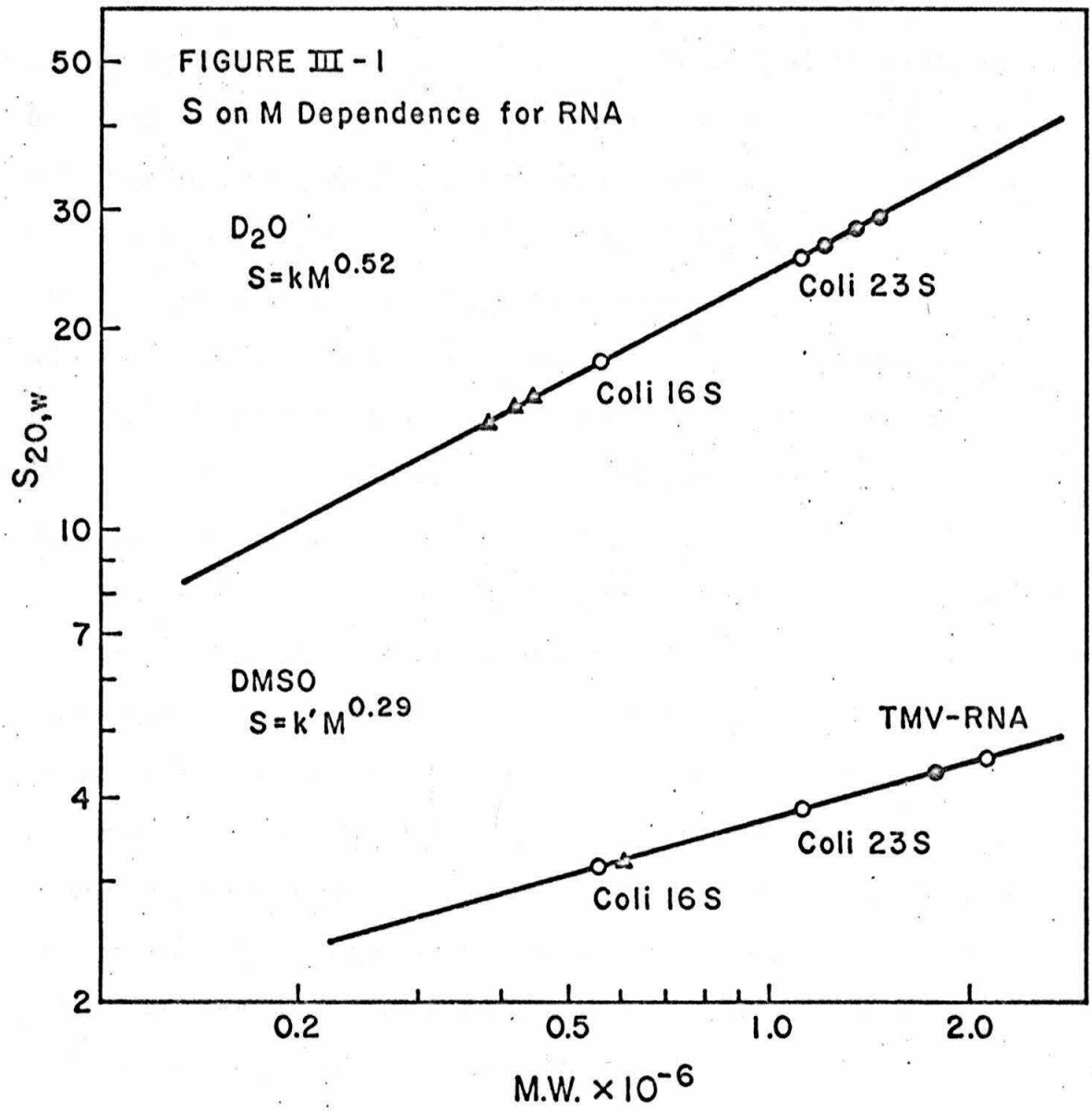


Figure III-1 S on M Dependence for RNA

The slopes on this graph were derived from the points with open circles (Coli and TMV-RNA). The solid circles and solid triangles represent the S-values found for reticulocyte RNA ("28S" and "16S" respectively) which have been plotted on the line. The molecular weights of the reticulocyte components were not determined independently. Coli ribosomal RNA was centrifuged in D_2O solution containing 0.2 M NaCl, 0.001 M EDTA, pH 7.1. Reticulocyte ribosomal RNA was centrifuged in D_2O solutions containing various salt concentrations.

Kurland (30) of 0.56 and that reported by Spirin (53) of 0.476 for bacterial R-RNA. Using S values from Tables III-1 and III-2 one can calculate molecular weights for the major reticulocyte components of 0.4 to 0.45 x 10^6 (16S) and 1.2 to 1.5 x 10^6 (28S). These values are in agreement with those found by Petermann et al. (41) who found molecular weights of 0.55 x 10^6 (16S), 0.96 x 10^6 (22S) and 1.47 x 10^6 (28S), for R-RNA from rat liver microsomes. Thus one 28S molecule and one 16S molecule can completely account for the known RNA content of the reticulocyte ribosome of 2 x 10^6 daltons (9).

Two problems concerning reticulocyte ribosomal RNA still remained: 1) the nature of the 22S component and 2) the possibility that these large molecules were made up of smaller polynucleotide chains. The 22S material had not been observed in rabbit reticulocyte R-RNA, either by Barlow et al. (42) or by Cox and Arnstein (54), though it is unlikely that it would have been detected under their conditions of centrifugation. With respect to point 2) above, both Petermann et al. (41) and others (9) had found that the fundamental unit of ribosomal RNA had a molecular weight of 0.5 x 10^6, which is the size of the 16S material. This would mean that the 28S and 22S species were aggregates, probably held together by hydrogen bonds or magnesium ion "bridges".

Since various organic solvents are known to destroy hydrogen bonds in nucleic acids at room temperature, as evidenced by the loss of hypochromicity and the disappearance of optical rotation (55), we decided to examine reticulocyte R-RNA in such a solvent. Dimethyl

sulfoxide (DMSO) was chosen because it is transparent to light of wavelength 275mμ, was dense enough to serve as the sedimentation solvent for band centrifugation (ρ = 1.10) (48), and because data on the sedimentation of other RNA's in this solvent were available (Strauss, personal communication). Lines 1 and 2 of Table III-3 show the results obtained by centrifugation in DMSO before and after treating the RNA with HCHO (see below). The S-values are much lower in DMSO than in aqueous solvents, partly because of the more extended configuration of the RNA and partly because the data have not been corrected for the density and viscosity of the solvent. The dependence of the S-value on concentration is greater in DMSO than in aqueous solution; this can be seen in the front-spreading of the peaks in the tracing (Figure III-3g) and in the fact that the S-values determined from the peak position and from the center of mass are more disparate in DMSO than in D_2O solvents.

Using the S-values obtained for E. coli 16 and 23S R-RNA and for TMV-RNA for sedimentation in DMSO (3.16, 3.86, and 4.60 respectively) (Strauss, personal communication), the molecular weight for TMV-RNA of 2.1×10^6 (56), and the molecular weights used above for E. coli R-RNA, one obtains the formula:

$$S = k' M^{0.29} \quad \text{(See Figure III-1)}$$

From this one can calculate molecular weights for the 16 and 28S components of 0.6×10^6 and 1.8×10^6. Though there is an increase in the relative amounts of 22S and 16S material in this solvent, a

TABLE III - 3

LiCl RNA "C" Under Denaturing Conditions

Treatment	S Values			% Material			Ratios of S	
	"28"	"22"	"16"	"28"	"22"	"16"	"28"/"16"	"22"/"16"
(1) 99% DMSO + 0.001 M EDTA	4.29	3.71	3.10	52.2	13.1	34.7	1.38	1.20
(2) 96% DMSO + 0.001 M EDTA + 1.8% HCHO reacted in aqueous soln. + 1.8% HCHO, 24 hrs, 40°C.	4.50	3.57	2.94	56.9	12.2	30.7	1.53	1.21
(3) 1.8% HCHO 24 hrs. 40°C.	16.2	12.4	9.4	50.3	15.1	34.6	1.73	1.32
(4) 1.8% HCHO 5 min. 80°C.	15.4	12.0	8.9	49.2	13.1	37.6	1.73	1.34
(5) 0.01 Na phosphate + 0.01 Na citrate 1 min. 90°C.	27.8	20.7	15.5	43.2	14.6	42.3	1.79	1.34

All reactions with formaldehyde were in 1.8% HCHO, 0.02 M Na phosphate. Centrifugations for lines (3) and (4) were in D_2O containing 1.8% HCHO and 0.02 M Na phosphate, pH 7.0.

sizeable fraction of the 28S material is unchanged, even under conditions which destroy hydrogen bonds.

Reaction of the RNA with formaldehyde was used as an alternative method of disrupting secondary structure. Frankel-Conrat (57) had shown that formaldehyde reacted reversibly with the amino groups of cytidylic, adenylic, and guanylic acids through the formation of a Schiff's base. Doty et al. (58) used the reaction rate of RNA with formaldehyde to estimate the degree of intramolecular hydrogen bonding of the macromolecule in solution and had shown that the fully reacted material possessed optical properties identical with those obtained after thermal "melting". Furthermore, HCHO had been shown to inactivate RNase (59), so that an RNA preparation should be stable at elevated temperatures even if contaminated with the enzyme. Conditions for complete reaction of reticulocyte R-RNA were established by performing melting curves in formaldehyde at 80° and 100°C. Another sample was incubated at 40°C. overnight. The data obtained are shown in Figure III-2. Initially there was a rapid increase in optical density at 260mμ and a concomitant shift in the absorption maximum from 257 to 259mμ. This was followed by a slow increase in absorption with time, with no further shift in the spectrum. This second phase is probably due to hydrolysis or other factors rather than to additional reaction with HCHO. On the basis of these results, two conditions for complete melting were chosen: 24 hrs. at 40°C. or 5 min. at 80°C. Analytical ultracentrifugation patterns for the RNA are very similar for these two procedures (lines 3 and 4 of Table III-3). The

FIGURE III-2 Melting of RNA in 1.8% HCHO

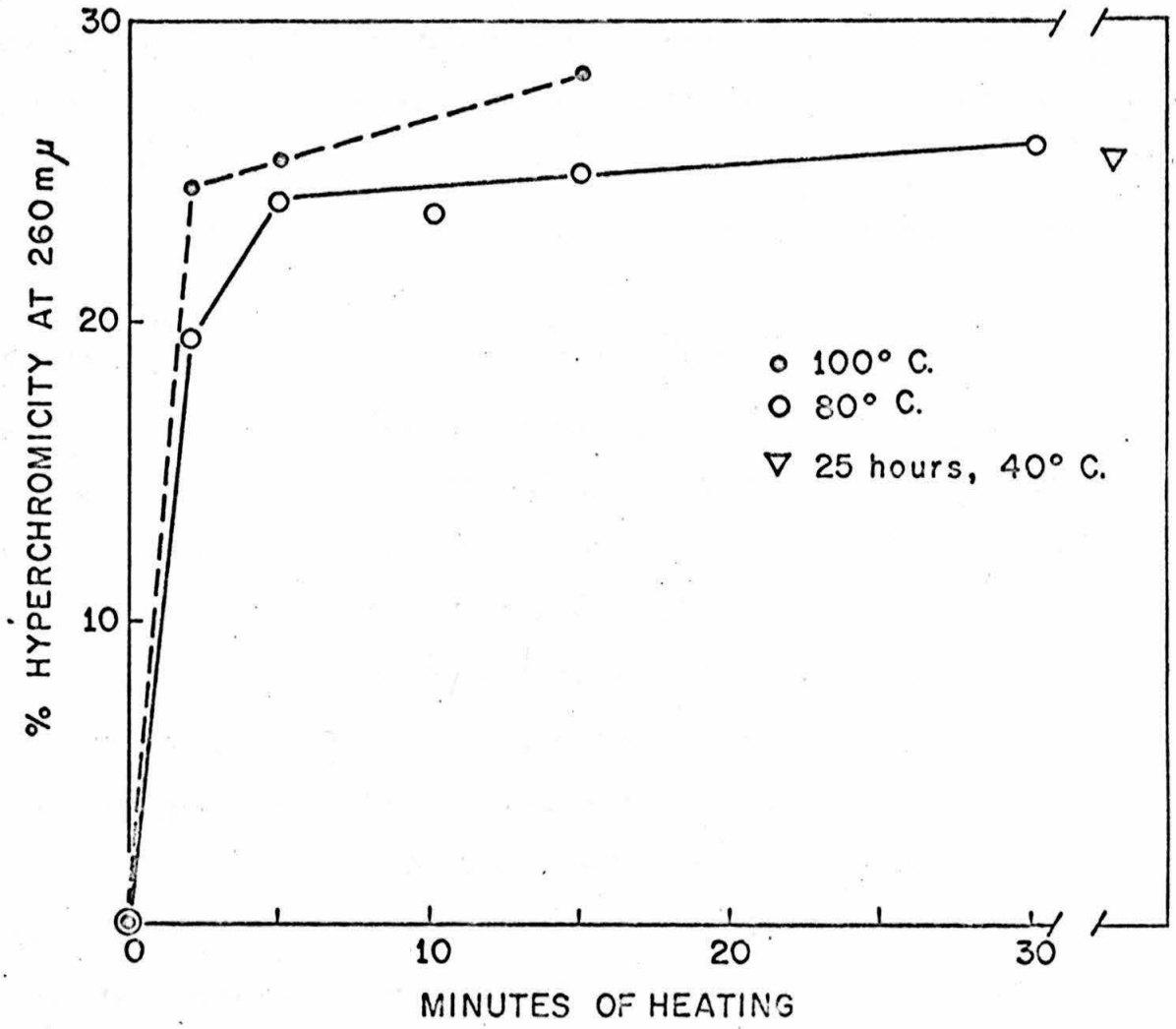


Figure III-2 Melting of Reticulocyte R-RNA in 1.8% Formaldehyde

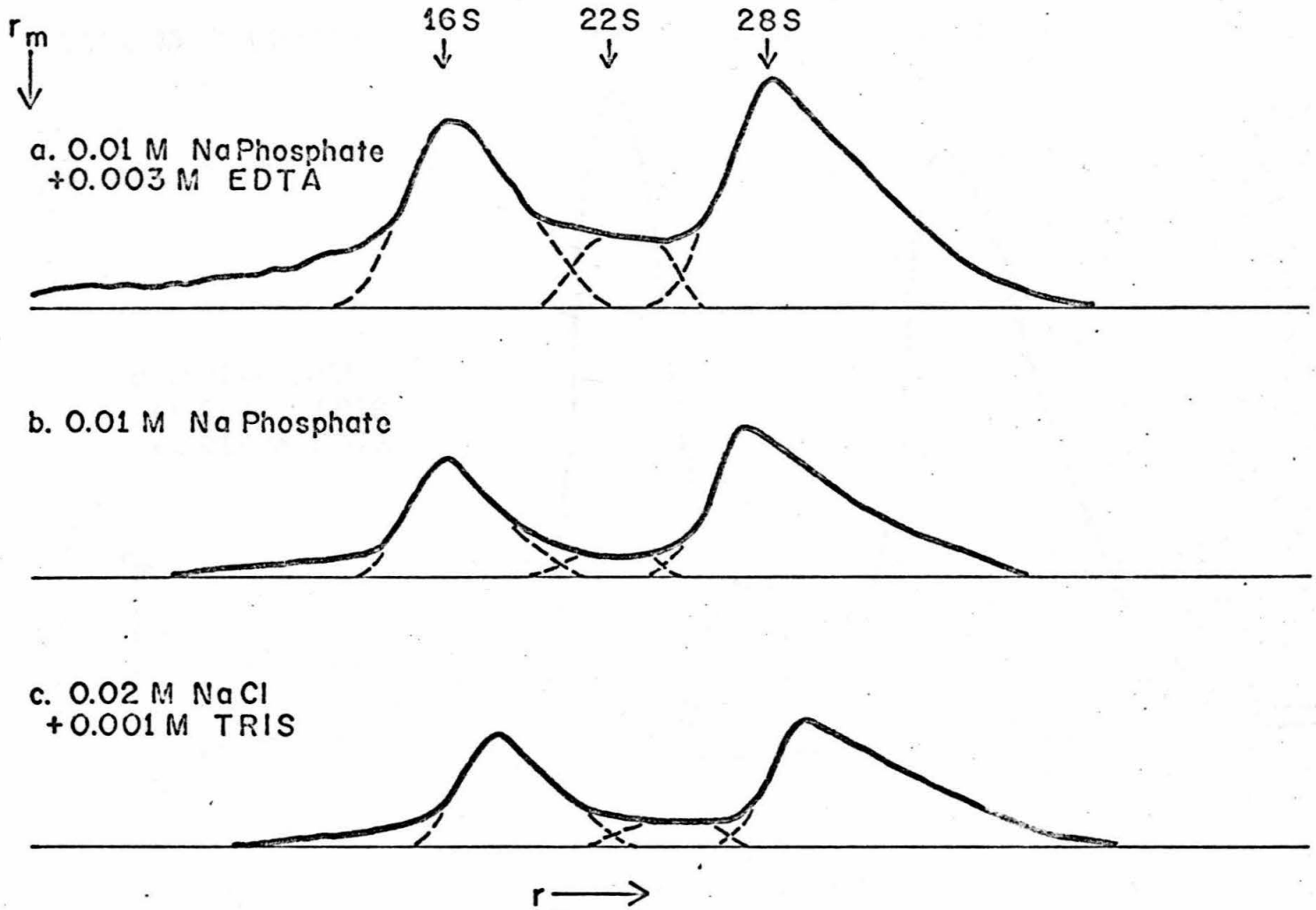
Samples were heated for various lengths of time in a buffer containing 0.02 M Na phosphate, pH 7.0 and 1.8% HCHO, at either 80°C. or 100°C. and cooled to 25°C. before determining the optical density at 260mμ. An additional sample was incubated at 40°C. for 25 hours. The zero time sample was RNA in 0.02 M Na phosphate without HCHO.

formaldehyde treated material was also run in the DMSO system (Line 2 of the same table).

The last method used for denaturation of the RNA was thermal melting in 0.01 M Na phosphate, 0.01 M Na citrate, pH 7.0. This buffer and the conditions of heating were used in order to make our results directly comparable to those of Petermann et al. (41). Initially, we found that the RNA was very unstable in this buffer, giving a large amount of 16S material and smaller products, and even showing progressive deterioration at 25°C. during the course of the centrifugation. After treating both the aqueous and D_2O solutions with bentonite (60), we obtained the results shown in Table III-2, line 5 (before heating) and in Table III-3, line 5 and Figure III-3f (after heating). The relative amounts of the 16S, 22S, and 28S RNA are very close to the results obtained after DMSO or formaldehyde treatment.

All of these results are at variance with those reported by Helmkamp and Ts'o for pea seedling microsomal RNA (55) and with Petermann et al. (41) for rat liver microsomal RNA, but are in agreement with the recent findings of Stanley and Bock on the 16 and 23S RNA components from bacterial ribosomes (31). Helmkamp and Ts'o (55) found that denaturation with formamide, DMSO, or heat destroyed the secondary structure of the R-RNA as measured by optical rotation, and caused a simultaneous decrease in the sedimentation constants. Before denaturation the RNA was present as two well-defined components of 27-28S and 17-18S, while after treatment it formed a single heterogeneous boundary of 8-11S. Petermann et al. found similar results (41),

FIGURE III-3 Ultracentrifugation of R-RNA



Legend on page 125.

FIGURE III-3 (cont'd)

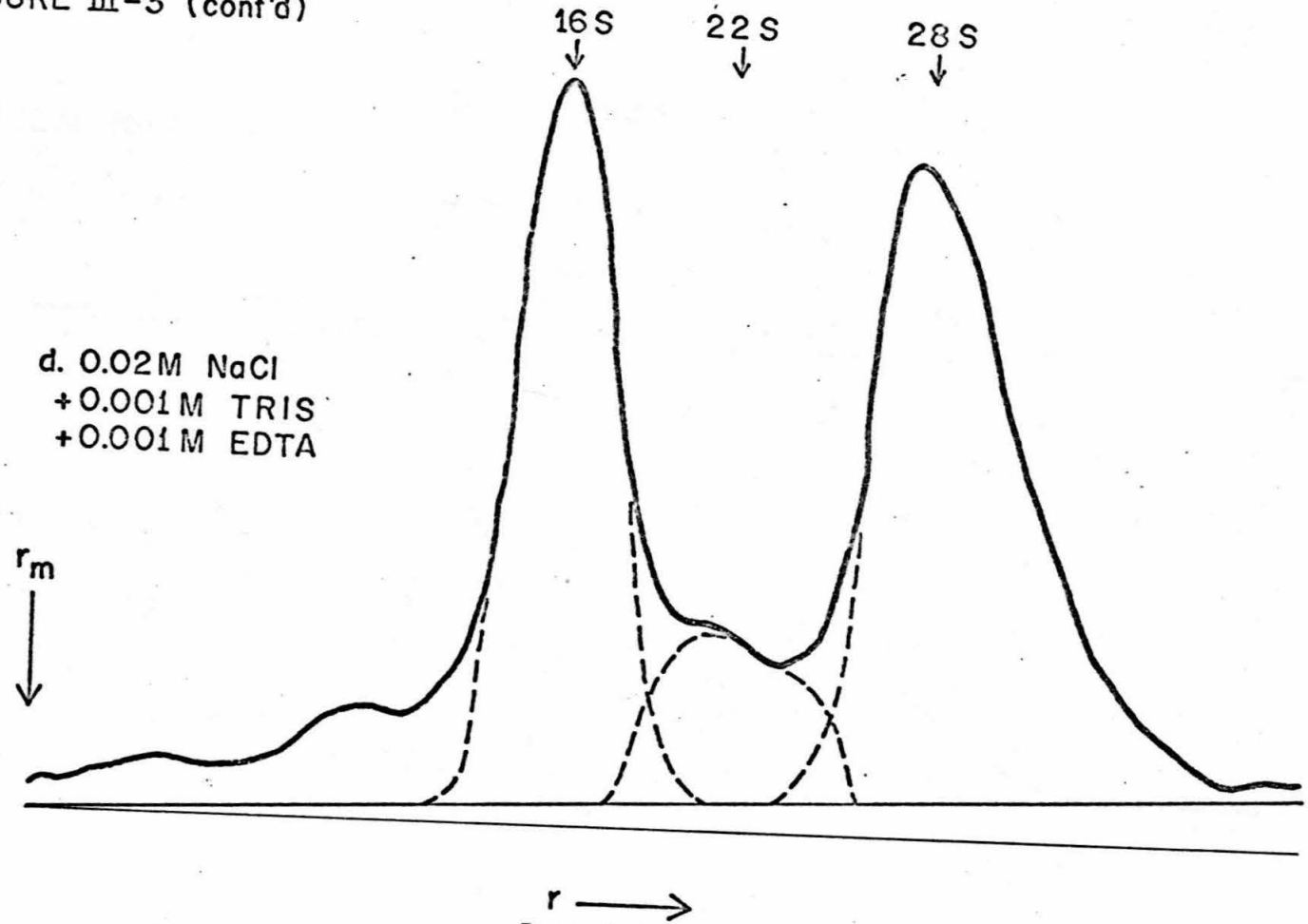


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FIGURE III-3 (cont'd)

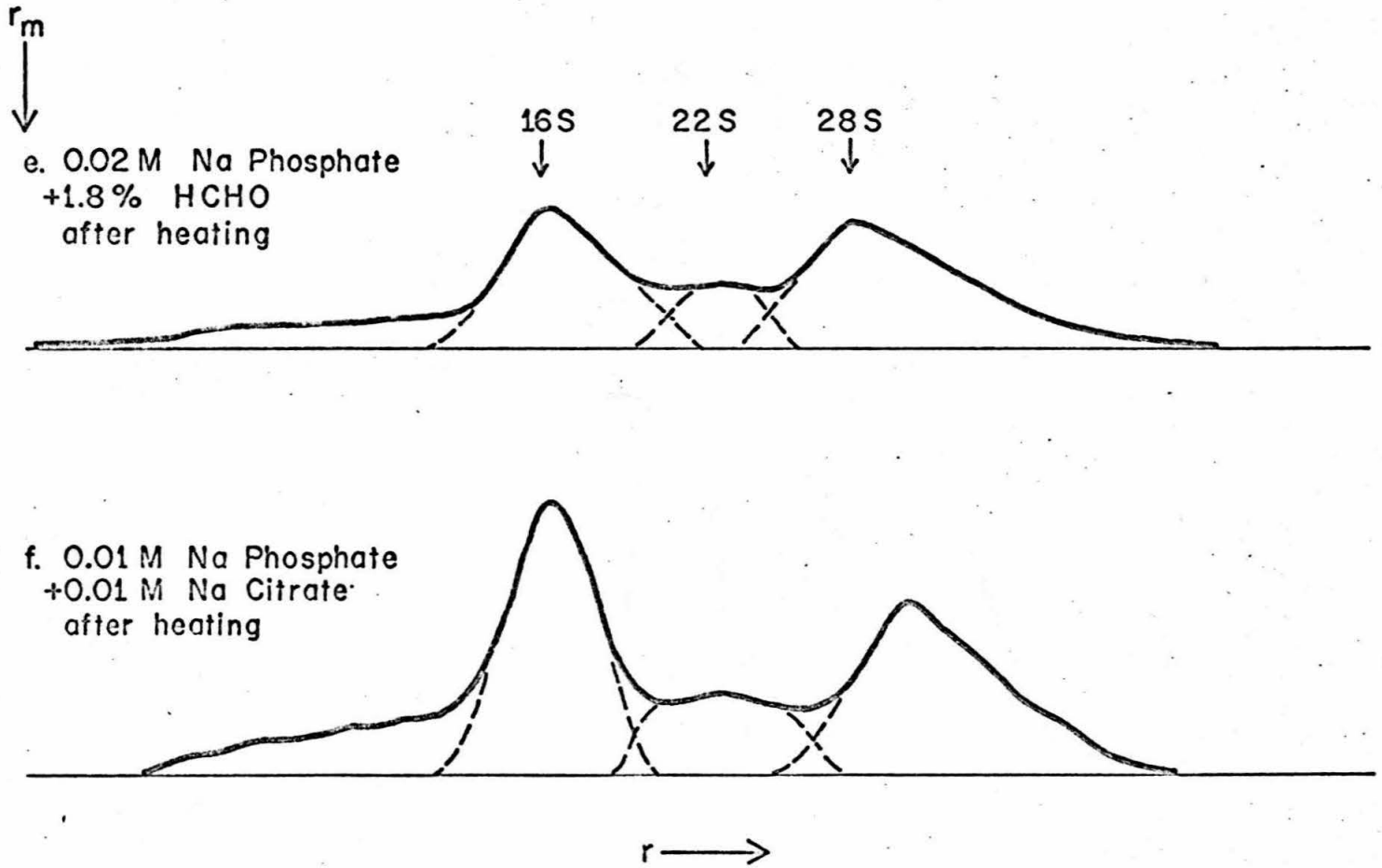

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FIGURE III-3 (cont'd)

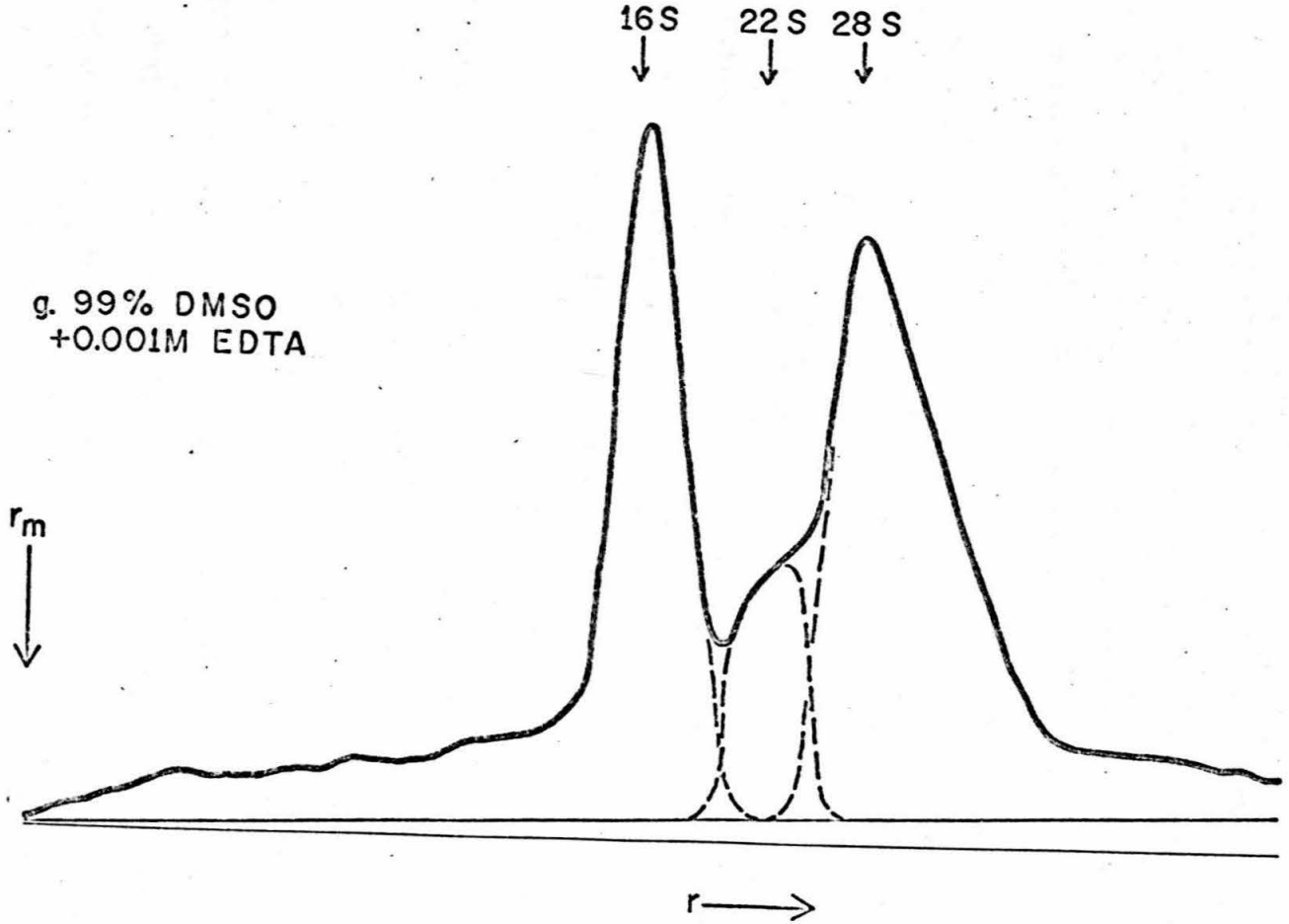

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Figure III-3 Analytical Ultracentrifugation Traces of Ribosomal RNA

The same preparation of LiCl Ribosomal RNA ("C") in different solvents and after various treatments, was used for these runs. The solid lines are the scan or tracing, while the dotted lines show the division into components. R_m indicates the position of the meniscus. All runs were done at 25°C. Both the aqueous sample and the D_2O sedimentation solvent contained the buffers given on the traces, except for g).

a), b), and c) Photoelectric scanner tracings with 265 mμ light, after 24 min. at 50,740 rpm.

d) Densitometer trace of picture taken with 265 mμ light after 36 min. at 50,740 rpm.

e) Photoelectric scanner trace with 265 mμ light after 48 min. at 52,640 rpm. Material heated 5 min. 80°C. in 0.02 M Na phosphate, 1.8% HCHO before run.

f) Photoelectric scanner trace with 265 mμ light after 28 min. at 50,740 rpm. Material heated for one min. at 90°C. before run.

g) Densitometer trace of picture taken with 275 mμ light after 112 min. at 56,100 rpm. Sample was 50% DMF and 50% DMSO containing 0.001 M EDTA pH 7.1. Sedimentation solvent was 99% DMSO containing 0.001 M EDTA, pH 7.1.

showing that removal of magnesium, formamide treatment, or heating caused the 28S and 22S material to "dissociate" into 16S RNA and a slower heterogeneous peak. They conclude from these results that the 16S RNA is the fundamental unit of ribosomal RNA. (However, in the Appendix in The Physical and Chemical Properties of Ribosomes (2), a reference is made to unpublished results of Petermann and Pavlovec that they were able to obtain 28S RNA which was stable to heating at 90°C.) From comparison with our data, it appears that both of these groups were studying RNA preparations which contained a large number of cleavages (probably due to nuclease action) which were only revealed when the secondary structure of the molecules was destroyed. Stanley and Bock (31), on the other hand, were unable to demonstrate comparable "subunits" in E. coli ribosomal RNA using formaldehyde treatment, DMSO, low ionic strength, or thermal denaturation; they concluded that both the 16 and 23S RNA's were naturally occurring integral polynucleotide chains.

From our experiments we can conclude that the native reticulocyte ribosomal RNA exists as two forms, one of S value 28 and molecular weight approximately 1.5×10^6 and the other of 16S and molecular weight 0.5×10^6. In high salt and in the absence of EDTA our preparation contains little intermediate material. However, after thermal melting, in the presence of organic solvents, in salt solutions containing EDTA, and after formaldehyde treatment, there is an increased proportion of 16S and 22S RNA. These molecules probably come from damaged 28S RNA which contained "nicks" in the ribose-phosphate

backbone which are only revealed when the secondary structure of the molecule has been destroyed.

That the fragments produced by a small number of such cleavages should correspond closely to 2/3 and 1/3 of the 28S RNA may mean that ribosomal RNA contains preferential breaking points. Huppert and Pelmont (61) have found defined intermediates of 24, 21, 15, 12, and 8S in R-RNA from Erlich Ascites tumor cells after RNase digestion of a preparation originally containing only 28 and 18S material. Midgley (62) observes that the 23S material from bacterial ribosomes is labile and forms two 16S molecules. He feels, however, that this preferential breakage is due to some special type of bond, and not to nuclease action.

Since some of the methods we have used for denaturation are more drastic than others, but reveal the same proportions of 16 and 22S material, we feel that the broken bonds were present in the original preparation, and not introduced during the treatments. This may also explain why the theoretical ratio of 28S to 16S is not achieved. However, since 28S material persists in all of these experiments, we feel that it is a single polyribonucleotide chain, and not an aggregate of 16S subunits. Moreover, the 22S RNA is probably a degradation product and not a fundamental unit of reticulocyte ribosomal RNA.

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