STUDIES ON THE ISOLATION OF MESSENGER RNA

Thesis by

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ABSTRACT

The rapidly-labeled RNA fraction of E. coli has been purified approximately 15-fold on benzoylated DEAE cellulose columns (BC). It is metabolically unstable (as shown by a pulse/chase experiment) and is considered to represent mRNA. The yield of pulse-labeled RNA is about 70% and comprises 4-5% of the RNA of the cell.

The true size distribution of this RNA, determined by sedimentation in a denaturing solvent (99% DMSO), does not change during purification. This result indicates that neither degradation nor selection for molecules of a particular size has occurred. Upon sedimentation of the final preparation in DMSO, the distribution of pulse label is the same as that of RNA mass, indicating nearly complete separation from longer-lived RNA components.

The isolation of globin-specific mRNA from rabbit reticulocytes has been attempted, both by a modification of BC chromatography and by the previously published sucrose gradient method of Marbaix, Burny, and Chantrenne, but the results (in both cases) were inconclusive. The final preparation sedimented heterogeneously, with an estimated mean sedimentation coefficient ($s_{20,w}$) of 8.4 S. Tests of the possible identity of this material as mRNA by biological assay in a cell-free protein synthesizing system have not been attempted.

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GENERAL INTRODUCTION

The messenger RNA theory is important because it provides a biochemical rationale for the one-gene, one-enzyme hypothesis (1). In its original form, it was proposed by Jacob and Monod as a result of studies on the inducible enzyme systems of bacteria (2). These studies had revealed the existence of a transient state in the transmission of genetic information from the genotype, as encoded in the base sequence of DNA, to the phenotype, as expressed through the properties of enzymes and other proteins. Studies by Hershey (3) and others (4,5) on RNA metabolism in phage-infected bacteria suggested that the expression of genes was related to RNA turnover. Jacob and Monod hypothesized that a new class of RNA molecules, which they called messenger RNA (mRNA), acted as the transmitter of genetic information from DNA to protein. Their hypothesis applied to prokaryotic systems, but it has subsequently been generalized to eukaryotes as well (6). For a review of the early history of the hypothesis, see reference (7).

A good hypothesis should not only reduce to common terms as many diverse observations as possible, but should also make predictions which are testable by direct and rigorous experiments. The mRNA hypothesis was a good one because it led to predictions concerning the synthesis, structure, and utilization of the messenger molecules. These are listed below, with a brief summary of the experimental evidence bearing on each.

1) Messenger RNA is synthesized as a complementary copy of a strand of DNA.

Enzymes capable of catalyzing the synthesis of RNA from a DNA template have been sought and found in many types of cells (8). Those from E. coli and M. lysodeikticus are the best characterized (9). The idea of complementarity is derived from the DNA structure proposed by Watson and Crick (10). It provides a chemical mechanism assuring fidelity of transcription. Support for the idea of complementarity has been provided by the hybridization technique (11,12) and by experiments with RNA polymerase (13,14).

2) The genetic information is encoded in the base sequence of the messenger RNA.

That the information for the synthesis of specific polypeptides can in fact be encoded in the base sequence of polyribonucleotides has been shown by the coding experiments of Nirenberg et al. (15). They showed that synthetic polyribonucleotides can specifically direct polypeptide synthesis in a cell-free protein-synthesizing system from E. coli. The accomodation of genetic information in the structure of RNA is also shown by the occurrence of RNA as the genetic material of certain viruses (16). Experiments with chemical mutagens show that the base sequence is the determinant of protein specificity in the undisrupted cell as well (17,18).

3) Messenger RNA is synthesized and degraded more

rapidly than the bulk of cellular RNA.

The hypothesis obliges genetic information to pass through RNA in order to get from DNA to protein. It follows that mRNA synthesis must precede changes in the protein composition of a cell. Messenger RNA must also be degraded (or otherwise inactivated) rapidly in order to stop quickly the synthesis of proteins which are no longer needed. Therefore, mRNA is expected to "turn over" rapidly. Rapidly labeled RNA fractions are conveniently detected by exposing the cells to a brief "pulse" of radioactive precursor (5). The basic observation relating pulse-labeled RNA to the messenger function was that the base composition of the pulse-labeled RNA synthesized in phage-infected cells resembles that of phage, rather than host, DNA (19). Pulse-labeling has since become an operational definition of mRNA in prokaryotic systems (20).

In many of the tissues of higher organisms the cells do not divide rapidly and probably have stable protein compositions. It is not obvious why mRNA should turn over rapidly in such cells. Pulse-labeled RNA fractions have been found in them, but their identity as mRNA is not so straightforward as in the case of bacterial systems (21,22). Polyribosomal RNA fractions which almost certainly represent at least a portion of the total cellular mRNA have also been identified, but are metabolically rather stable. There are also some special examples of mRNA which have

metabolic stability approaching that of bulk RNA, such as globin mRNA in mammalian reticulocytes (23) and mRNA formed during sporulation in bacteria (24).

4) Messenger RNA preparations should be heterogeneous in molecular weight.

Since the genetic information is encoded in the linear sequence of bases along the mRNA chain, the length of the chain (and therefore its molecular weight) should be proportional to the length and molecular weight of the polypeptide chain for which it codes. Polypeptide molecular weights vary considerably (25); thus the prediction. The existence of polycistronic mRNAs further extends the molecular weight range expected for mRNA (26,27). Sucrose gradient velocity sedimentation has provided evidence for size heterogeneity of mRNA (28,29).

5) Messenger RNA should be found associated with ribosomes.

Pulse-labeled RNA has been shown to associate with ribosomes, both <u>in vivo</u> (30) and <u>in vitro</u> (31). The discovery of polyribosomes further substantiates this prediction (32).

The satisfaction of these predictions provided strong support for the mRNA hypothesis. However, these results do not constitute direct proof of the hypothesis because its central proposition is that the RNA molecules in question carry a message. Thus a rigorous proof of the hypothesis

requires that a unique molecular species of RNA, transcribed from DNA by RNA polymerase, be isolated and shown to contain encoded in its nucleotide sequence the requisite information for directing the synthesis of a specific natural polypeptide. Eventually this may become possible by direct chemical determination fo the nucleotide sequence of the suspected mRNA. This sequence would then be translated into an amino acid sequence by use of the amino acid code (15). This predicted sequence would be shown to correspond exactly with the amino acid sequence of a naturally occurring polypeptide. However, the longest polyribonucleotide for which the sequence has been determined to date is the 5 S rRNA of E. coli, which contains 120 nucleotides (33). This would correspond to a polypeptide containing only 40 amino acids, whereas most polypeptide chains are larger than this (25).

Another method for verifying messenger assignments is to determine the coding properties of the polyribonucleotide in question by a biological assay, such as the stimulation of an appropriate cell-free protein synthesizing system. A cell-free system must meet three criteria in order to be applicable to the testing of the mRNA hypothesis. The first is that the system be capable of responding specifically to the addition of natural RNA. This eliminates some eukaryotic systems, for example the reticulocyte system, which only respond aberrantly to natural RNA preparations (34). The second criterion is that the background rate of

incorporation (in the absence of added RNA) be low enough to permit accurate measurement of the stimulation. The third, and most important, is that the protein, the stimulation of which is under assay, be heterologous to the cells from which the system was derived. This is necessary in order to be able to conclude that the information for specific protein synthesis was introduced into the system by the added RNA. If the genes for the particular protein being stimulated occur in the cells from which the system was derived, it becomes difficult to exclude rigorously the possibility that the addition of RNA is merely stimulating the expression of genetic information previously resident within the system. Effects of this type have been noted. However, the mechanism by which they are produced is unknown (34,35). It is thus possible that their production depends on the molecular weight, secondary structure, or some other property of the added polyribonucleotide. Therefore, the addition of types of RNA other than mRNA (in particular rRNA) cannot be accepted as controls to show that spurious stimulation effects are not occurring. It appears that the only way to demonstrate mRNA activity using cell-free systems which is free from debilitating criticisms is to obtain the system from cells which are genetically unrelated to the source of the putative mRNA.

The E. coli system best satisfies these criteria.

Preincubating it results in the loss of endogenous mRNA activity without destroying the ability of the ribosomes to participate in protein synthesis (36). A system with a low background rate of incorporation and capable of responding to added polyribonucleotide is thereby obtained. It has been employed in several studies to demonstrate the presence of specific mRNA activity in RNA preparations from viruses and bacteria. A cell-free system derived from rat liver has been used to demonstrate messenger activity in RNA prepared from insect larvae. Two other notable reports concerning messenger activity in RNA preparations from higher organisms have appeared, although neither may be taken as proof of the hypothesis. Summaries of these several reports are given below.

1) The RNA isolated from the coliphage f2 has been shown to direct the de novo synthesis of phage coat protein in an E. coli cell-free system (37,38). The same has been shown for the closely related phages MS2 and R17 (39,40). These viral RNAs also direct the synthesis of at least one other protein which has not been characterized in detail (39,41). The coat protein product has been identified both by direct analysis ("fingerprinting") (38) and by its ability to combine with the phage RNA in vitro (40). No coat protein synthesis is observed if the RNA is obtained from phages carrying an amber mutation in the coat protein gene. However, the debilitating effect of the amber muta-

tion can be overcome by the addition of extracts from permissive host cells (40). That the complex process of genetic suppression can be duplicated *in vitro* shows that the phage RNA is acting *in vitro* as it does *in vivo*.

A valid though far-fetched objection to the designation of these experiments as proof of the mRNA hypothesis may be based upon the contention that uninfected *E. coli* contains the phage genes in a temperate state (42). This contention, while unprecedented in the case of an RNA phage, is nonetheless difficult to disprove rigorously. It has been vitiated by the demonstration that phage f2 RNA can also direct the synthesis of homologous coat protein in a cell-free system derived from chloroplasts of the protozoan *Euglena gracilis*, which is unlikely to be a host for a bacteriophage (43).

2) The RNA isolated from alfalfa mosaic virus has been shown to direct the *de novo* synthesis of homologous coat protein in an *E. coli* cell-free system (44).

3) The RNA isolated from satellite tobacco necrosis virus has also been shown to direct the *de novo* synthesis of homologous coat protein in an E. coli system (45).

4) RNA isolated from *E. coli* cells infected with phage T4 has been shown to direct the synthesis of phage-specific lysozyme in an *E. coli* cell-free system (46). The appearance of enzyme activity was the criterion by which specific protein synthesis was detected. *In vitro* suppression of

lysozyme amber mutations has been demonstrated in this work as in the case of phage f2 RNA (47). From an analysis of the *in vitro* suppression data it was indirectly concluded that the enzyme was synthesized *de novo*. The sensitivity of the stimulation to inhibitors of protein synthesis substantiates this conclusion. However, a direct demonstration of *de novo* synthesis has not yet been accomplished.

The same objection raised in discussing the f2 results may be raised here. In the absence of a clear understanding of the origin of viral parasites, it is difficult to eliminate the possibility that the host cell retains viral genetic information. Hybridization experiments make this unlikely (11); nevertheless, these results would be more satisfactory if they were confirmed using a completely unrelated cell-free system.

5) RNA prepared by a hot phenol extraction method from the nuclei of epidermal cells of larvae of the blowfly *Calliphora* has been shown to induce the synthesis of the enzyme DOPA-decarboxylase in a cell-free system derived from rat liver (48). *De novo* synthesis was inferred from the fact that a control RNA sample, prepared in the same way from younger larvae which has not yet reached the stage of development at which the enzyme appears *in vivo*, did not cause the appearance of as much enzyme activity. Also, the stimulation is sensitive to inhibitors of protein synthesis. Again, however, a direct demonstration of *de novo* synthesis

would be preferable.

6) RNA isolated from the large myosin-synthesizing polyribosomes of embryonic chick leg muscle has been shown to stimulate the appearance of a myosin-like product in a homologous cell-free system (49). Since the system and the RNA were both derived from the same organism, it is not possible to consider this work as a rigorous proof of the RNA hypothesis. The results are, however, consistent with the hypothesis and support the postulation of its applicability to vertebrate systems.

7) A ribonucleoprotein particle from mammalian reticulocytes has been shown to convey the information for specific hemoglobin synthesis to a heterologous reticulocyte cell-free system (50). The effect is sensitive both to ribonuclease and to deproteinization. It may therefore be taken as supporting or contradicting the hypothesis, depending on whether one considers the genetic information to reside in the RNA or the protein portion of the particle. However, in view of the rigorous demonstrations of the existence of mRNA in lower organisms, these results are best considered as further support for the general validity of the hypothesis.

These results establish the applicability of the mRNA hypothesis to both DNA and RNA viruses, as well as to at least one eukaryotic system. The extensive literature on the satisfaction of various predictions of the hypothesis

in many additional systems (see reviews (7,22) for references) validates its generalization to the status of a theory applicable (perhaps with minor modifications) to all organisms.

Part I

REPETITION OF THE EXPERIMENTS OF MARBAIX, BURNY, AND CHANTRENNE

INTRODUCTION

Reticulocytes are an excellent material from which to attempt the isolation of specific mRNA. Approximately 90% of the protein synthesis occurring in these cells is that of the single protein, hemoglobin (51), the structure of which is known (52). It may be inferred that the total mRNA present is largely globin-specific. It must, of course, be remembered that the globin molecule consists of two closely related, but non-identical polypeptide chains (52). "Globin mRNA" will therefore be a mixture of two similar polyribonucleotides,* one containing the information for directing the synthesis of the α-polypeptide chain, and the other specific for the β-chain. Since each chain contains about 145 amino acids (52), the mRNAs will each contain about 145 nucleotide triplets. The average molecular weight of a nucleotide triplet is about 1,000 daltons each. A polyribonucleotide of this molecular weight is expected to have a sedimentation coefficient of about 8 S in dilute neutral aqueous salt solutions (53). Because reticulocytes do synthesize some other proteins than hemoglobin (51), a preparation of total reticulocyte mRNA should contain a small amount of non-globin-specific mRNA. There is evidence for the existence in reticulocytes of polyribosomes much larger

* It is known that the genes for the α- and β-chains are unlinked (55). Therefore two mRNAs are expected.

than the predominant globin-synthesizing pentamers (54). Thus total reticulocyte mRNA may be expected to contain some molecules considerably larger than 8 S.

Reticulocytes also offer a number of technical advantages. The cells can be prepared in large quantities, and naturally occur in liquid suspension (viz., blood), obviating the need for grinding or mincing. They contain little ribonuclease (56,57,58). They can be lysed gently in hypotonic solutions (23), which is important because violent methods of cell disruption can degrade polyribosomes (59) and therefore cause strand breaks in mRNA. Furthermore, this method of hemolysis can be controlled so that only the erythroid cells are lysed, and not the white cells (60). White cells not only contain non-globin-specific mRNAs, but RNase (61).

Unfortunately, the RNA of reticulocytes cannot be satisfactorily pulse-labeled, because the cells contain no nucleus and synthesize no RNA.* Label can be incorporated if the radioactive material is injected into the anemic rabbit shortly before exsanguination in order to label the nucleated precursor cells in the bone marrow which subsequently become reticulocytes (64,65). However, this procedure results in heavy labeling of the rRNA in addition

* Except, of course, for the turnover of the -C-C-A end of tRNA (63).

to any labeling of mRNA. By this means, the specific activity of RNA sedimenting in the 8 S region of sucrose gradients can be made eight times higher than that of rRNA (64). The amount of mRNA expected to be found in the cells can be calculated from the expected molecular weight given above, the known molecular weight of rRNA (66), and the known content and size of polyribosomes (67). Such a calculation indicates that mRNA should comprise less than 1% of total cellular RNA. Even if the mRNA specific activity is eight times higher than that of rRNA, there will still be more than ten times as much label incorporated into rRNA as into mRNA. The label thus cannot be considered as marking mRNA for the purpose of characterizing fractionation procedures. One is left in the paradoxical situation of having a system which seems ideal for the isolation of specific mRNA, but in which there is no simple way to follow the course of its purification.

At the time that this work was begun, several unsuccessful attempts to isolate globin mRNA had been reported (68,69,70,71). A series of papers from the laboratory of H. Chantrenne reported the isolation of a 9 S minor RNA component from rabbit reticulocyte polyribosomes (54,72; also see 73). This material satisfied a number of properties predicted for the globin mRNA, but subsequent attempts to verify its identity as mRNA by cell-free tests met with failure (73).

The method of isolation used in these papers was based on the assumption that the globin mRNA has a sedimentation coefficient of approximately 9 S in dilute neutral aqueous buffers. The protein and RNA components of polyribosomes were dissociated by treatment with SDS.* The resulting mixture was fractionated by preparative velocity sedimentation in sucrose gradients, and the small amount of material sedimenting between the peak of sRNA (4 to 6 S) and the smaller rRNA component (18 S) was collected and isolated. This material was subjected to several more successive rounds of sucrose gradient centrifugation. A component sedimenting at about 9 S, representing 1 to 2% of the starting material, was detected and isolated in this way.

This method suffers from two drawbacks. First, it will select for any minor RNA component sedimenting around 9 S, be it mRNA, a degradation product of rRNA, or an undescribed RNA of unknown function. Second, the extensive handling and long exposure to sucrose, even the best grades of which may contain traces of RNase (74), raise the possibility that this RNA might be a degradation artifact.

At the time that the first reports of this work appeared, it was deemed sufficiently noteworthy to bear repetition. The results obtained are presented below.

* Abbreviation used: SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

See Part II for descriptions of sources and grades of reagents, synthesis of resin, purification of urea, phenol extraction, ethanol precipitation, analytical ultracentrifugation, and miscellaneous analytical methods.

Reticulocyte Preparation and Lysis

Rabbit reticulocytes were prepared, collected, and washed as described by Lingrel and Borsook (75), except that phenylhydrazine hydrochloride was dissolved in NKM, blood was not taken from the chest cavity, and cells were washed twice for ten minutes at 5,000 x g in cold NKM. Cells were lysed and stroma removed according to Marks et al. (23), except that 3 volumes of hypotonic solution were added to the washed, packed cells followed by 1/3 volume of hypertonic solution.

Lithium Chloride Extraction

Ribosomal and soluble RNAs were prepared by the method described by Barlow et al. (74), except that 10 M LiCl stock solutions were used which were always buffered with 0.01 M Tris-Cl, pH 7 to 8.

Sucrose Gradients

Gradient solutions were prepared from Mallinkrodt AR grade sucrose. Purified bentonite (76) was added to a concentration of about 1 mg./ml. and the solution stirred

until all lumps had become dispersed. Bentonite was removed by centrifugation for 60 minutes at 10,000 x g. The treated solutions were stored frozen at -20° C.

Most sucrose gradients were prepared using the standard two-chambered mixing device (77). For large-scale fractionations, gradients were centrifuged in the fixed angle Spinco No. 30 rotor, rather than in a swinging bucket rotor. Twelve gradients were formed simultaneously in the rotor tubes by dividing the output from a large two-chambered gradient forming device among twelve matched pieces of Technicon pump tubing using a Technicon Proportioning Pump. The sample was layered onto the gradients using the same equipment. After centrifugation, the contents of the tubes were combined by inserting a glass capillary attached to the end of one of the pieces of pump tubing to the bottom of each of the twelve centrifuge tubes and reversing the direction of pumping. Fractions were collected by a fraction collector from the combined flow from the tubes. Although resolution is poorer in the fixed angle rotor than in the swinging bucket rotor, Figure 1 shows that this method does separate RNA species according to sedimentation rate.

Dialysis

Dialysis tubing was prepared according to Muench and Berg (78).

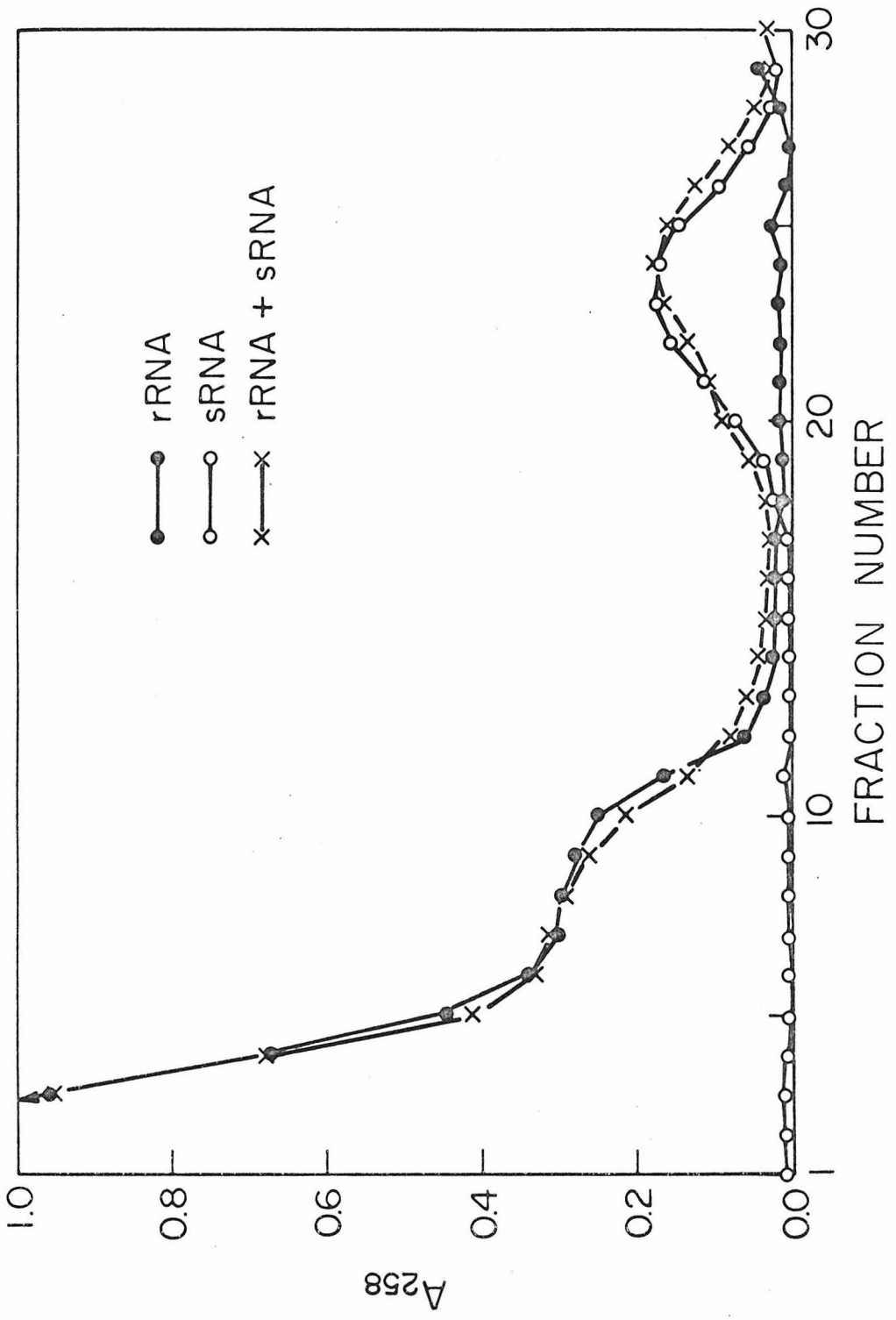

rRNA
sRNA
rRNA + sRNA
A258
1.0
0.8
0.6
0.4
0.2
0.0
1
10
20
30
FRACTION NUMBER

Figure 1

Figure 1. Sucrose gradient velocity sedimentation in a fixed angle rotor.

5 to 20% linear sucrose gradients in 0.02 M K_3PO_4, pH 6.8, 30 ml. total volume per tube, were formed in Spinco No. 30 rotor tubes as described in Materials and Methods. 5 ml. samples containing (1) 20 mg. rRNA; (2) 2.5 mg. sRNA; (3) 20 mg. rRNA + 2.5 mg. sRNA, dissolved in 0.08 M KCl, 0.0015 M $MgCl_2$, 0.01 M Tris-Cl, pH 7.5, were layered onto the gradients. They were centrifuged for 10 hours at 30,000 rpm (78,000 x g) at 5° C. Reticulocyte rRNA and sRNA were prepared by the LiCl method. The concentration ratio used approximates that found in lysates. Fractions were diluted with water before determination of optical absorbance.

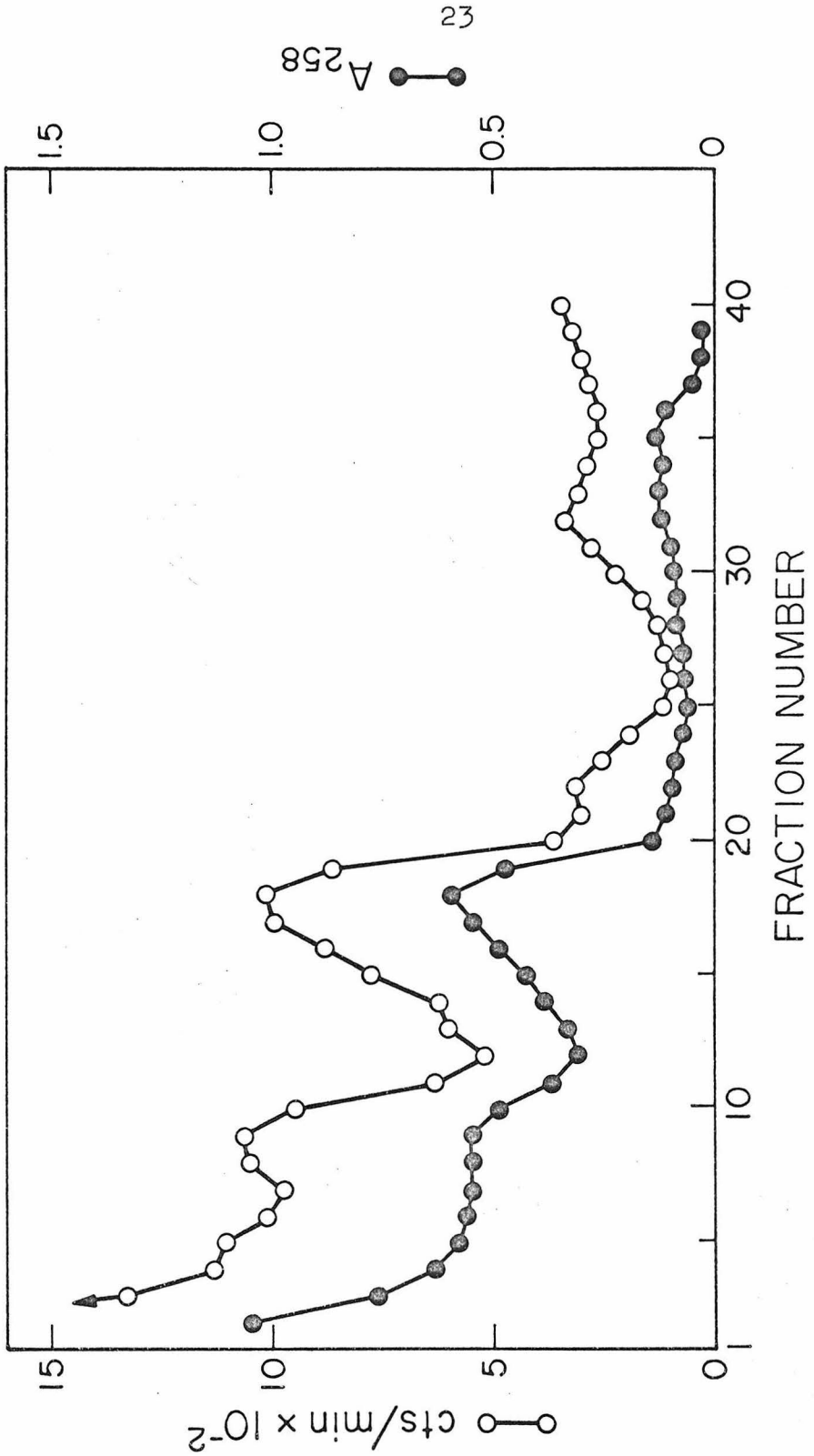
RESULTS

^{32}P-labeled RNA Preparation

Three rabbits were made anemic, and each was injected with 5 mC. neutralized, carrier-free ^{32}P-orthophosphate in the marginal ear vein 16 hours before exsanguination. Reticulocytes were collected and stroma-free lysates prepared. A "polysome-enriched pellet" (64) was obtained by centrifugation of the lysate at 30,000 rpm for 1.5 hours at 5° C. in the Spinco No. 30 rotor. The pellets were rinsed with a few ml. cold 0.08 M KCl, 0.0015 M $MgCl_2$, 0.01 M Tris-Cl, pH 7.5, and resuspended in this buffer with the aid of a loose-fitting homogenizer. (This procedure had been found to cause no degradation of polyribosomes [data not shown].) Polyribosomal RNA was dissociated from protein by treatment with SDS, 0.5% (w/v) final concentration, for 5 minutes at 37° C. and then fractionated by sucrose gradient velocity sedimentation as shown in Figure 2. Note the slight shoulder on the radioactivity profile between fractions 20 and 25, in agreement with the results of Marbaix and Burny (64).

Large-Scale Preparation of 8 S RNA

A "polysome-enriched pellet" was prepared from the reticulocytes of six anemic rabbits by centrifuging stroma-free lysate at 30,000 rpm for 2 hours at 5° C. in the Spinco


A258
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1.0
0.5
0
40
30
20
10
1
FRACTION NUMBER
15
10
5
0
cts/min x 10-2

Figure 2

Figure 2. Fractionation of reticulocyte ^{32}P-labeled RNA.

4.1 ml. SDS-treated ribosome suspension containing 23.4 mg. RNA was layered onto each of three 8 to 20% linear gradients of sucrose in 0.005 M Tris-Cl, pH 7.4, formed in Spinco No. SW25.2 rotor tubes (50 ml. per tube). The tubes were centrifuged at 24,000 rpm for 30 1/4 hours at 4° C. Fractions were collected by dripping from the tube bottoms; drops were removed at regular intervals during the fractionation of one tube for determination of radioactivity and optical absorbance. The direction of sedimentation is from right to left.

No. 30 rotor. Pellets were rinsed, resuspended, and treated with SDS as before, except that the incubation time was 10 minutes. This extract was fractionated by sucrose gradient velocity sedimentation in the Spinco No. 30 rotor as described in Materials and Methods. The results are shown in Figure 3a. Fractions were combined as indicated and dialyzed against several changes of 0.001 M Na_4EDTA, pH 7. In order to avoid any losses during lyophilization, the material in the dialysis sacs was concentrated by placing them in 50% (w/v) polyvinylpyrrolidone (PVP) in water until collapsed. The sacs were emptied, rinsed out with a small volume of 0.0001 M Na_4EDTA, pH 7, and nucleic acids precipitated with ethanol followed by redissolution and reprecipitation. This precipitate was dissolved in 1 ml. distilled water and again fractionated on a sucrose gradient. The results are shown in Figure 3b. Fractions were collected and combined to give three samples. Each was dialyzed against several changes of 0.001 M Na_4EDTA, pH 7, and precipitated with ethanol. These precipitates were each dissolved in 2 ml. 0.01 M Na_3PO_4, 0.0001 M Na_4EDTA, pH 7, and analyzed by band velocity sedimentation in the analytical ultracentrifuge. The most rapidly and least rapidly sedimenting peaks from the sucrose gradient gave sedimentation coefficients ($S_{20,w}$) of 15 and 4.4, respectively (data not shown). Material from the middle sucrose gradient peak gave a very broad, heterogeneous peak, as shown in Figure 4.

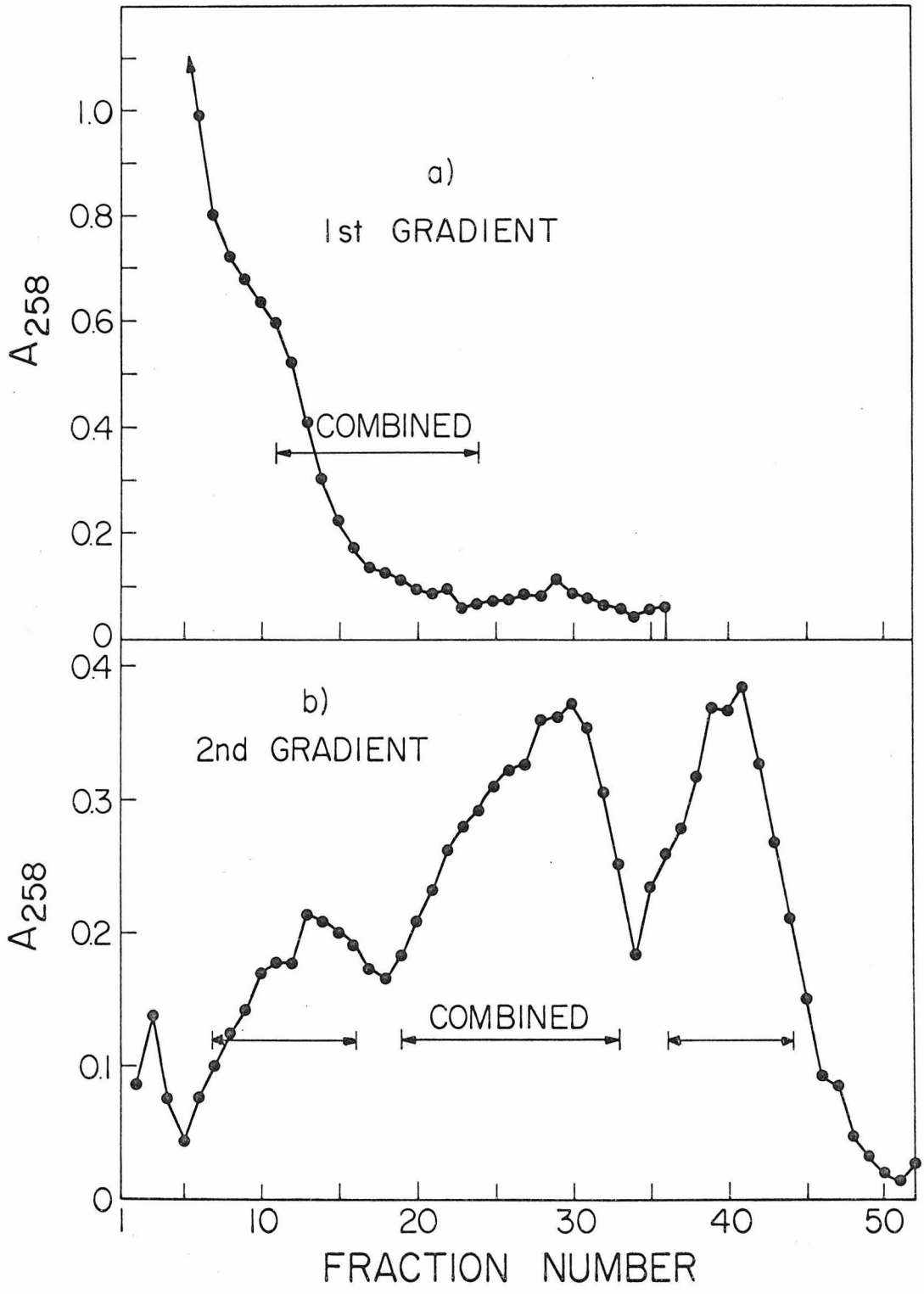
a)
1st GRADIENT
COMBINED
A258
1.0
0.8
0.6
0.4
0.2
0
b)
2nd GRADIENT
COMBINED
A258
0.4
0.3
0.2
0.1
0
1
10
20
30
40
50
FRACTION NUMBER

Figure 3

Figure 3. Large-scale fractionation of reticulocyte RNA.

Both fractionations were made in 5 to 20% sucrose gradients in 0.01 M K_3PO_4, 0.001 M Na_4EDTA, pH 6.8. The direction of sedimentation is from right to left.

(a) 5 ml. samples of SDS-treated ribosome suspension containing 10 mg. RNA each were layered using a Technicon Proportioning Pump onto each of twelve 30 ml. gradients formed simultaneously in Spinco No. 30 rotor tubes using the pump. The tubes were centrifuged at 30,000 rpm for 10 hours at 3° to 5° C. Fractions were collected simultaneously from the twelve tubes by pumping; a small aliquot was removed from each and diluted with water for determination of optical absorbance.

(b) 6 mg. RNA in 1 ml. water was layered onto a 25 ml. gradient formed in a Spinco No. SW25.1 rotor tube. This was centrifuged at 25,000 rpm for 30.5 hours at 4° C. Fractions were collected by dripping from the tube bottom; drops were removed at regular intervals and diluted with water for determination of optical absorbance.

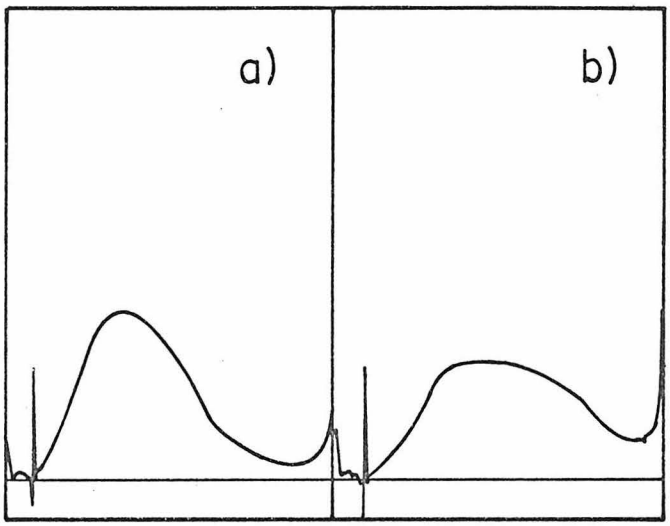

Figure 4

Figure 4. Analytical band velocity sedimentation of 8 S RNA fraction.

Output traces from the Photoelectric Scanner of the Spinco model E analytical ultracentrifuge, operating at 2650 Å, were made (a) 64 minutes and (b) 92 minutes after reaching the operating speed of 56,100 rpm. 5 μg. RNA was centrifuged through 0.01 M Na_3PO_4, 0.0001 M Na_4EDTA, 90% (v/v) D_2O, pH 7 (nominal) at 20° C. The direction of sedimentation is from left to right.

The sedimentation coefficient ($S_{20,w}$) calculated for the peak position was 8.4 ± 0.1 and that calculated for the inflection point (as estimated by eye) on the leading edge of the peak was 11.3 ± 0.4. The final yield of the heterogeneously-sedimenting 8.4 S material was approximately 0.7%, based on the total amount of RNA in the "polysome-enriched pellet."

DISCUSSION

The observation of a slight increase in the specific activity of RNA in the 8 S region of sucrose gradients (first experiment) confirms the results of Marbaix and Burny (64). However, the broad distribution of sedimentation rates found by analytical band velocity sedimentation for the material isolated in the second experiment is at variance with their results. Their preparations were analyzed by boundary velocity sedimentation and by sucrose gradient velocity sedimentation (79), not by band velocity sedimentation. But even though direct comparisons cannot be made, it is felt that the material isolated in the second experiment here is so heterogeneously sedimenting as to permit the conclusion that a real difference exists.

The most likely explanation for the broad sedimentation pattern observed is that degradation of rRNA has occurred. rRNA is the suspected source of the 8 S material

because many of the molecules in the preparation sediment more rapidly than 8 S, and the only major RNA components of the cells larger than this size are rRNA. Of course, it is possible that the preparation does contain the component found by Marbaix and Burny, but that it is too contaminated with rRNA degradation products for a discrete peak in the 8 to 9 S region to be observable.

The indications of degradation, lack of any direct test of identity as mRNA, and selective nature of the isolation procedures prompted the abandonment of centrifugal methods in favor of column chromatography. For the purposes of characterizing new mRNA isolation procedures, E. coli, the mRNA of which can be conveniently identified by pulse labeling techniques, was chosen as a model system.

Part II

PURIFICATION OF E. COLI PULSE LABELED RNA ON BENZOYLATED DEAE CELLULOSE COLUMNS

(Manuscript to be submitted for publication)

The studies reported in this section were carried out in collaboration with Mr. John Sedat, largely in the laboratories of Professor Robert L. Sinsheimer.

THE PURIFICATION OF *E. COLI* PULSE LABELED RNA BY BENZOYLATED DEAE-CELLULOSE CHROMATOGRAPHY

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Running title: Purification of Pulse Labeled RNA

Summary

The rapidly-labeled RNA fraction of E. coli has been purified approximately 15-fold on benzoylated DEAE cellulose columns (BC). It is metabolically unstable (as shown by a pulse/chase experiment) and is considered to represent mRNA. The yield of pulse labeled RNA is about 70% and comprises 4 to 5% of the RNA of the cell.

The true size distribution of this RNA, determined by sedimentation in a denaturing solvent (99% DMSO), does not change during purification. This result indicates that neither degradation nor selection for molecules of a particular size has occurred. Upon sedimentation of the final preparation in DMSO, the distribution of pulse label is the same as that of RNA mass, indicating nearly complete separation from longer-lived RNA components.

1. Introduction

Since the existence of messenger RNA has been established, there have been numerous attempts to purify this RNA (for example, by sucrose gradient centrifugation [Attardi, 1963], MAK column chromatography [Asano, 1965], preparative hybridization [Bautz & Reilly, 1966], and counter current distribution [Kidson & Kirby, 1964]). However, numerous difficulties have been encountered in attempts to devise a purification scheme that will not be selective in view of the expected heterogeneity of size distribution, base composition and base sequence, and the small fraction of total RNA (≈4%) concerned. Other difficulties, such as the lack of any simple relation between sedimentation constant and molecular weight of RNA (Boedtker 1968) and the aggregation of mRNA with rRNA (Asano, 1965; Hayes, Hayes & Guerin, 1966), have complicated the use of sucrose gradients; the problems of hybridization kinetics (Britten & Kohne, 1968) and the difficulty of specific DNA preparation (so that tRNA and rRNA genes are absent) have limited the scope of DNA-RNA hybridization. None of these procedures for the purification of mRNA seems to be generally applicable.

We have approached the problem of mRNA purification by the use of benzoylated (naphthoylated) DEAE cellulose (BNC) chromatography* (Gillam, Millward, Blew, von Tigerstrom,

Wimmer & Tener, 1967; Sedat, Kelly & Sinsheimer, 1967). This resin is characterized by high capacity, high recovery, and yields sharp, well-resolved peaks. Resolution on this resin seems to depend upon (at least at pH 7.5) preferential interaction of the single-stranded regions of the nucleic acids with the aromatic rings bound to the cellulose. Chromatography on BNC has proved useful in the study of replicating MS2 RNA intermediates (Kelly & Sinsheimer, 1967), the purification and study of intracellular λ DNA (Kiger & Sinsheimer, 1969), and of ØX intracellular replicating DNA (both single- and double-stranded) (Sinsheimer, Knippers & Komano, 1969; Knippers, Razin, Davis & Sinsheimer, in preparation). In principle, a structural difference in mRNA might permit it to be separated from the other RNAs of the cell.

We have made use of the pulse label technique to label selectively the rapidly synthesized mRNA (Gros, Gilbert, Hiatt, Attardi, Spahr & Watson, 1961), with the understanding that this fraction could include some intermediates in rRNA synthesis (Mangiarotti, Apirion, Schlessinger & Silengo, 1968) and will bias the results against any messengers which are not synthesized so rapidly. In most experiments we have made use of a double label technique: $^{32}PO_4^{=}$ is used as a short pulse label, and ^{3}H-5-uracil is used to label the culture for a few generations prior to the pulse. The ^{3}H counts are thus a direct measure of the

RNA mass (as is also A_{260}), weighted of course by any difference in pyrimidine content per molecule.

We have made use of sedimentation in 99% DMSO (in which RNA is completely denatured at 25°C. [Strauss, Kelly, & Sinsheimer, 1968]) to attempt to ascertain at each step of the purification whether any degradation or selection has occurred. This procedure is also useful to determine the size distribution of the pulse and mass labels. The distribution of the pulse label after a chase was studied to determine the metabolic fate of the RNA fraction.

Purified mRNA should be very useful in the study of gene expression, metabolic control, and protein synthesis. There is as yet little information concerning the structure of mRNA or detailed study of its function in in vitro systems.

2. Materials and Methods

(a) Materials

(i) Benzoylated (naphthoylated) DEAE cellulose (BNC) and benzoylated DEAE cellulose (BC) were prepared essentially according to Gillam et al. (1967). However, 5 lb. of anhydrous ether/50 g resin was used to wash the resin further after the benzoylation procedure. The resin was sized through a U.S. sieve No. 80 and then the fines were decanted. Since slight variations in the elution patterns appeared

when using various early batches of resin, later batches were stored, moist, at -20°C. or suspended in water at 4°C.

(ii) Reagent grade urea (Baker & Adamson or Mallinckrodt) was found to degrade high molecular weight RNA in solution. Figure 1a shows an analytical ultracentrifuge pattern of a band sedimentation analysis in 90% D_2O (vide infra) of E. coli rRNA (previously shown to consist only of 23 S and 16 S components in an estimated mass ratio of 2:1) which had been treated for 10 min. at 20° C. with 8 M urea in 0.1 M CH_3COOH, pH 3.5. Several degradative hits/molecule (1 x 10^6 avograms) occurred in 10 min. Attempts to purify the urea by passage through an IRC-50, Chelex 100 column at pH 5.0 (to remove RNase I and heavy metal ions) were unsuccessful as judged by the above assay method. It was found, however, that Mann high purity urea (Mann Research Laboratories, 136 Liberty St., New York) does not degrade rRNA, as is shown in Figure 1b. Ribosomal RNA, treated for 10 hr. at 20° C. with an 8 M solution of Mann urea (passed through a 50 mμ Millipore filter to remove particulate impurities) in 0.1 M CH_3COOH, pH 3.5 (pH adjusted with HCl), and then subjected to analytical band velocity sedimentation through 99% DMSO (vide infra), showed, it was estimated, less degradation than 0.1 hits/molecule. Each lot of urea was subjected to the above test before use.

(iii) Earlier lots of DMSO were obtained from J. T. Baker Chemical Co.; later lots were Spectroquality Reagent

from Matheson Coleman & Bell, Cincinnati, Ohio. d_6-DMSO (containing 38,000 ^3H cts/min/ml.) was obtained from BioRad, Richmond, California.

(iv) RNase-free sucrose was obtained from Schwarz BioResearch, Orangeburg, N.Y.

(v) E. coli MRE 601 (RNase I$^-$) was obtained from Dr. M. Heisenberg. E. coli THU, HF4704, and TPA and TPG media are described in Lindqvist & Sinsheimer (1967). ØXam3 is described in Hutchison & Sinsheimer (1966).

(vi) $^{32}PO_4^{\equiv}$, carrier-free in H_2O, was obtained from Nuclear Consultants, Glendale, California. ^3H-5-uracil (20 to 27 C/mM) or ^3H-6-uracil (15 to 27 C/mM) and uracil-2-^{14}C (56 mC/mM) were obtained from Schwarz BioResearch, Orangeburg, N.Y.

(b) Methods

(i) Preparation of $^{32}PO_4$ pulse-^3H uniformly labeled RNA extracts.

1 liter of TPA medium was inoculated with an overnight culture of E. coli MRE 601 and grown to 1 x 10^8 cells/ml., at which time 2 mC of ^3H-5-uracil (2γ/ml. uracil) were added. After the culture had reached 4 to 6 x 10^8 cells/ml., 10 mC of neutralized $^{32}PO_4$ were added; 45 sec. later (0.035 generation), NaN_3 was added to a concentration of 0.01 M. The cells were then centrifuged, and quickly resuspended in 10 ml. of 0.02 M Tris-Cl, pH 7.5. One ml.

of 1 mg/ml. lysozyme in 0.25 M Tris-Cl, pH 8.1, plus 1 ml. of 7.5 μg/ml. EDTA were rapidly added and the mixture warmed to room temperature for 3 min., after which SDS was added to 0.5% (final concentration) to lyse the cells. One volume of redistilled phenol, saturated with 0.02 M Tris-Cl, pH 7.5, was added and the mixture placed on a Vortex mixer for a total mixing time of 3 min. (1 min. mixing, then 2 to 3 min in an ice bath). After centrifugation, the aqueous layer was removed and re-extracted with 1/2 volume of 0.01 M Tris-Cl, pH 7.5; the combined aqueous layers were extracted with cold anhydrous ether 3 times; 1/10 volume of 3 M Na acetate, pH 5.0, plus two volumes of 95% ethanol were added to precipitate the nucleic acids. After approximately 10 hr. at -20° C., the precipitate was centrifuged, dissolved in 5 ml. of 0.02 M Tris-Cl, pH 7.5, and stored at -20° C.

(ii) Preparation of ^{3}H-uracil pulse-^{14}C-uracil uniformly-labeled, chased RNA extracts.

One liter of TPG + 4 μg/ml. uracil + 5 μg/ml. thymidine + 20 μg/ml. ℓ-histidine was inoculated with E. coli THU, and the cells were grown at 37° C. to 1 x 10^{8} cells/ml., after which 200 μC of uracil-2-^{14}C were added. After the culture had reached 3 to 4 x 10^{8} cells/ml., 2 mC of ^{3}H-5-uracil were added for 45 sec.; 1/2 of the culture was immediately treated with NaN_3 (0.01 M) and the nucleic acids extracted as in (i). To the other half of the culture was

added sterile, unlabeled cytidine plus uridine to 10 mg/ml. each. The cells were then filtered on a large, cold, 142 mm, 0.45 HA Millipore filter, and washed with 0.5 l. of ice-cold TPG + 10 mg/ml. each cytidine and uridine. Finally the filtered, washed cells were resuspended in 500 ml. of warm TPG + 10 mg/ml. each cytidine and uridine. The culture was grown for one generation (60 min. at 37° C.) and then the nucleic acids extracted as in (i).

(iii) Preparation of RNA extracts from ØXam3-infected cells, pulse labeled with ^{3}H-uracil, and from uninfected cells, pulse labeled with $^{32}PO_4$.

This culture of E. coli HF4704 was prepared essentially as in (i). One-half of the culture was infected at 5 x 10^8 cells/ml. with ØXam3 (m = 5). Twelve to 15 min. after infection, ^{3}H-6-uracil (2 /ml. uracil) was added for 45 sec., after which the cells were treated with NaN_3. The centrifuged cells from the two halves of the culture were combined and the nucleic acids extracted as in (i).

(iv) Fractionation on BC column at pH 7.5.

Approximately 1/2 of the nucleic acid from (i) was diluted with 10 volumes of 0.3 M NaCl, 0.02 M Tris-Cl, pH 7.5, 0.001 M EDTA and applied by gravity to a 2.5 cm x 5 cm column of BC, previously washed with 0.002 M Tris-Cl plus 0.3 M NaCl buffer. In some cases the extreme viscosity of the sample required stirring up the resin in order to increase the flow rate. The column was then washed with 0.3 M NaCl buffer until the unabsorbed ^{32}P counts (see Results)

were washed out. 200 ml. of a linear gradient of NaCl from 0.3 M to 0.7 M and of DMSO from 0 to 30% (v/v) (all solutions contained 0.02 M Tris-Cl, pH 7.5, plus 0.001 M EDTA) was applied at a flow rate of approximately 1 ml./min. Absorbance was monitored through a Gilford Absorbance Monitor, and 3 ml. fractions were collected. At the end of the gradient, the column was washed with 8 M urea (see Materials [ii]) in 0.1 M CH_3COOH, pH 3.5, until the pH reached 3.5 and the ^{32}P counts thus eluted were washed out (see Results). 100 ml. of a subsequent linear gradient from 0 to 1.0 M NH_4Cl in 8 M urea plus 0.1 M CH_3COOH, pH 3.5, was applied at a 1 ml./min. flow rate; at the termination of this gradient, a solution containing 1 M NH_4Cl plus 2.5% SDS plus 8 M urea, pH 3.5, was passed through the column to remove additional ^{32}P pulse counts. The individual RNA peaks from both gradients were precipitated by adding 1/10 volume of 3 M Na acetate, pH 5.0, and 2 volumes of 95% ethanol, and stored at least 10 hr. at -20° C. After centrifugation, the pellets were dissolved in 0.02 M Tris-Cl, pH 7.5.

(v) Fractionation on BC columns at pH 3.5.

In order to remove rRNA from the RNA peaks eluted in the prior fractionation at pH 7.5, an additional fractionation was performed entirely at pH 3.5. The RNA from the pH 7.5 fractionation was diluted 10 times with 8 M urea in 0.1 M CH_3COOH, pH 3.5, and applied by gravity onto a 1.5 x 4 cm BC column, previously washed with the 8 M urea

buffer until A_{260} was low and constant. Additional 8 M urea buffer was used to wash in the sample; then 100 ml. of a linear gradient of NH_4Cl from 0 to 1 M in 8 M urea buffer was applied at a flow rate of 1 ml./min. The eluted RNA was precipitated with ethanol as in (iv).

(vi) Sedimentation.

Analytical sedimentation in aqueous solvents was performed as described by Strauss & Sinsheimer (1968). Analytical sedimentation through 99% DMSO was also described by Strauss et al. (1968), except that when the solution layered onto DMSO had an ionic strength greater than 0.1, a lower speed was employed during the establishment of the diffusion gradient. Preparative sedimentation in 99% DMSO was performed as described by Strauss et al. (1968), with slight modifications. 5 ml. linear gradients were established between 99% d_6-DMSO (containing 10% sucrose and 0.001 M EDTA, pH 7.0), and a mixture of 0.1 volume fraction 99% d_6-DMSO with 0.90 volume fraction 99% DMSO (containing 0.001 M EDTA, pH 7.0). Onto this was layered 5 μl RNA + 90 μl 99% DMSO + 5 μl dimethylformamide. Centrifugation was performed at 64,000 rev./min. in a Spinco L2-65B ultracentrifuge for 11 hr. at 27° C. Fractions were collected and precipitated with TCA for counting.

(vii) Miscellaneous.

Hybridization of RNA (from ØX-infected cells) with ØX RF DNA was done essentially by the procedure of Gillespie

& Spiegelman (1965) in a volume of 0.3 ml. with a 13 mm filter containing 2 to 3 μg of ØX RF (prepared by the procedure of Komano & Sinsheimer, 1968) at 66° C. for 24 hrs.

For radioactivity counting, samples were usually dried onto glass filters and counted in a toluene-based scintillation fluid (Liquifluor, Nuclear Chicago, Inc.) in a Beckman LS-200 scintillation counter. Readings were subsequently corrected for background and overlap. Some samples, especially those from the sucrose gradients, were counted directly in Bray's scintillation fluid (Bray, 1960).

Several precautions were taken to minimize contamination with RNase. All glassware was heat-sterilized and plastic centrifuge bottles were only used once. Pipettes were disposable (Falcon Plastics) as were the fraction collector tubes. Concentrated buffers were brought to a specified pH with an RNase-free combination electrode, stored cold, and diluted just before use. Great care to use plastic disposable gloves whenever near the RNA or during the procedures was essential.

3. Results

(a) The chromatography of ^{32}P pulse-^{3}H uniformly-labeled nucleic acid extract on BC

The chromatography of ^{32}P pulse-^{3}H-uracil uniformly-labeled nucleic acid extract from RNase I⁻ *E. coli* 601 on

BC at pH 7.5 is shown in Figure 2. A large fraction of the ^{32}P counts applied did not absorb on the BC column; these have been shown to be soluble in TCA and ethanol. The linear gradient (0.3 to 0.7 M NaCl, 0 to 30% DMSO in 0.02 M Tris-Cl, pH 7.5) eluted two major ^{3}H-labeled RNA peaks. The first, small one consisted of tRNA (Gillam et al., 1967) and DNA (Sedat et al., 1967). The second ^{3}H peak was largely undegraded rRNA, as determined by analytical band velocity sedimentation. The ^{32}P counts eluted in this gradient were found in the trailing edge of the large rRNA peak.

The additional gradient of 0 to 1 M NH_4Cl in 8 M urea plus 0.1 M CH_3COOH, pH 3.5 (see Materials [ii]) eluted a small amount of ^{3}H (mass) label but a larger amount of the ^{32}P label, equaling in some cases the amount of ^{32}P eluted in the first gradient. Analytical band velocity sedimentation in D_2O and DMSO showed that the large rRNA peak from the first gradient and the small peak eluted in the second gradient were undegraded rRNA with the 23 S and 16 S components in a mass ratio of 2:1. We have labeled these fractions $rRNA_I$ and $rRNA_{II}$, respectively.

The additional ^{32}P radioactivity that remained on the column could be removed by the use of 2% SDS in 1 M NH_4Cl plus 8 M urea, pH 3.5. This material did not appear to be RNA, although it was precipitated by TCA. It resisted degradation by RNase, DNase, pronase, or alkali (0.3 N). There

was no label in this material when the cell culture had been pulse-labeled with ^{3}H-uracil. The nature of this fraction is further complicated by the finding that the ^{32}P counts banded in a CsCl equilibrium gradient at a density of 1.52. These ^{32}P counts may represent an intermediate of lipid or carbohydrate metabolism.

In another experiment in which a pulse of ^{3}H-thymidine replaced the usual $^{32}PO_4$ and ^{3}H-uracil labels, a large peak of ^{3}H counts appeared under the first small peak in Figure 2 in the first gradient; no other ^{3}H counts were eluted until after the peak of $rRNA_{II}$ in the second gradient. ^{3}H label was also eluted when the SDS plus 1 M NH_4Cl plus 8 M urea was applied (in this case the ^{3}H counts and A_{260} banded at a density of 1.70). These results are consistent with those obtained by Kiger & Sinsheimer (1969) for λ replicating DNA; Knippers, Whalley & Sinsheimer (in preparation) for ØX RF replicating DNA; and Rohwer (personal communication) for E. coli replicating DNA.

Slight variations were occasionally seen in the patterns of Figure 2. Also, early batches of BNC and especially BC showed considerable subfractionation of the tRNA-DNA peak, and the large $rRNA_I$ peak was split into the 16 S and 23 S components (see Fig. 1, Sedat et al., 1967). In addition, a large amount of ^{32}P pulse label (equal in quantity to that eluted with $rRNA_{II}$ in the second gradient), but no ^{3}H (mass) label was eluted from the column when the

8 M urea, 0.1 M CH_3COOH, pH 3.5, was used to wash the column and lower the pH. These counts did not precipitate with TCA or ethanol, as in the case of the counts eluted during sample application.

The yield of RNA fractionated on BC at pH 7.5 is summarized in Table 1. A large fraction of the rRNA (83% of the mass) was eluted in the first gradient ($rRNA_I$), together with 60% of the pulse (ignoring the unbound ^{32}P counts of the sample eluate). Only 3% of the RNA mass was eluted as $rRNA_{II}$ in the second gradient, together with 16% of the pulse. The fraction of ^{32}P pulse counts eluted with $rRNA_I$ or $rRNA_{II}$ varied; at times the ratio was 1:1. A surprising amount of the $^{32}PO_4$ pulse label (23%) was eluted with the SDS plus 8 M urea, although this does not represent nucleic acid. The recovery of the ^{3}H counts (based on the amount adhering to the column) was approximately 99%, while that of the ^{32}P pulse averaged 90 to 95%.

There are significant ionic effects on the first column fractionation. Substitution of 0.02 M phosphate, pH 9.6, buffer (see Hayes et al., 1966) for 0.02 M Tris-Cl, pH 9.6, did not change the elution pattern of the ^{3}H label but shifted almost all the RNA ^{32}P pulse counts to the $rRNA_I$ peak, leaving little if any ^{32}P with $rRNA_{II}$; also, the ^{32}P counts were found coincidentally distributed with the ^{3}H mass of $rRNA_I$ instead of on the trailing edge.

(b) Is there any difference between the pulse RNA in $rRNA_I$ and $rRNA_{II}$?

As is shown in Figure 2, the ^{32}P pulse label distributed into two fractions both of which contained rRNA. To determine whether this fractionation of the mRNA was meaningful, cells were infected with ØX174 bacteriophage and the distribution of ØX mRNA studied by means of hybridization to ØX RF. A mixed mucleic acid extract from infected cells pulse labeled with ^{3}H-uracil and uninfected cells pulse labeled with $^{32}PO_4$ was chromatographed on BC as in Figure 2, and RNA samples from the $rRNA_I$ and $rRNA_{II}$ components were separately sedimented on sucrose gradients. Each fraction from each gradient was assayed for the ability to hybridize to ØX RF filters and to ØX DNA filters. Figure 3 shows the result of this experiment.

The sedimentation distribution of the total pulse label is the same in $rRNA_I$ and in $rRNA_{II}$ and is the same in infected and uninfected cells. Also, the proportion of the total pulse label from infected cells which hybridized with ØX RF and the sedimentation distribution of the hybridizable label were the same (within experimental error) in both $rRNA_I$ and $rRNA_{II}$. This experiment, together with the DMSO sedimentation results presented in (d), indicated that little, if any, selective fractionation of mRNA had taken place on the BC column.

Furthermore, when $rRNA_I$ and $rRNA_{II}$ were separately re-chromatographed on a BC column at pH 7.5, each was eluted in two peaks similar to those of Figure 2. The fact that in Figure 3 (also see Fig. 15, Sinsheimer, 1968) hybridizable pulse label appears in the fractions sedimenting faster than does ØX DNA (21 S) could be a consequence of the aggregation of the mRNA to rRNA as reported by Asano (1965), Hayes et al. (1966), and Sedat & Sinsheimer (in preparation).

(c) Fractionation of pulse labeled RNA at pH 3.5 on BC

The results of the ØX hybridization experiment indicated that there was no apparent selection of mRNA in $rRNA_I$ and $rRNA_{II}$, and analytical and preparative velocity sedimentation of these fractions demonstrated that the major mass components were undegraded 16 S and 23 S rRNA. In order to separate the pulse label from the rRNA an additional fractionation step on BC entirely at pH 3.5 in 8 M urea was employed. Figure 4 shows the result of chromatography of either $rRNA_I$ or $rRNA_{II}$ in 8 M urea at pH 3.5. No radioactivity flowed through the column during sample application and a single peak, greatly enriched for the pulse label, was eluted during the 0 to 1 M NH_4Cl gradient. Table 2 provides the proportions of the total mass and pulse labels eluted when $rRNA_I$ and $rRNA_{II}$ were chromatographed at pH 3.5 on BC. Only a small fraction of the applied mass

was eluted with approximately 60% of the pulse label (this has been as high as 75% in some pulse experiments). $rRNA_{II}$ was initially enriched for pulse label, and chromatography of this fraction resulted in an eluate with an increased proportion of the applied mass.

The radioactivity remaining on the column could not be eluted with any of the gradients employed with the first column at pH 7.5. The only means yet discovered to dislodge the residual RNA has been partially to degrade it by passage of 8 M urea (Baker & Adamson only) in 0.1 M CH_3COOH, pH 3.5, plus 2% SDS, followed by distilled water, which brings off the rRNA largely depleted of ^{32}P pulse label. As usual with the use of unpurified urea, an estimated 5 to 10 hits/molecule were found in the rRNA. For this chromatographic procedure the pH must be lower than 4.5, NaCl will not substitute for the NH_4Cl, and dimethylurea will not substitute for urea.

The BC resin appears to age on standing for $\geq$ 6 months and older batches allowed increasing amounts of rRNA (especially 16 S) to elute with the pulse counts.

The peak eluted at pH 3.5 on BC was RNA as judged by its complete hydrolysis (to TCA-solubility) by RNase. No unusual spots of radioactivity were found after electrophoresis of the nucleotides from a 0.3 N KOH hydrolysate.

Rechromatography of the RNA eluted at pH 3.5 resulted

in a pattern identical to that of Figure 4. Greater than 85% of both pulse and mass labels applied was eluted (except for the above-mentioned case when the use of aged resin resulted in elution of some rRNA).

(c) Velocity sedimentation analysis in sucrose gradients

The results of preparative velocity sedimentations, in neutral sucrose gradients, of ^{32}P pulse-^{3}H-uracil uniformly-labeled, fractionated RNA from the BC columns are shown in Figure 5. Part (a) of this figure is the velocity pattern of $rRNA_I$; especially noticeable is the large fraction of ^{32}P pulse label sedimenting faster than the 23 S rRNA. From the pulse experiment in ØX-infected cells (see Fig. 3) and the analytical sedimentation patterns presented (also see Results, part [d]), both $rRNA_I$ and $rRNA_{II}$ are very similar with respect to distribution of mass and pulse label. The sedimentation patterns of the RNA eluted from the BC column in the pH 3.5 fractionation of both $rRNA_I$ and $rRNA_{II}$ appear in (b) and (c) respectively. The 16 S and 23 S rRNA components are largely gone, except for the very small shoulders in the regions where these rRNA components sediment; there is still a small amount of tRNA at 4 S (Gillam et al., 1967). (The presence of tRNA in $rRNA_I$ and $rRNA_{II}$ is a consequence in part of trailing from the tRNA peak and in part of retarded elution of acylated tRNA

bearing aromatic amino acids.)

The ^{3}H (mass) label now approximately parallels the pulse label in both gradients. When the tRNA present in the RNA analyzed in (b) and (c) was removed by rechromatography at pH 7.5 as described in Materials and Methods (b [iv]), and the product sedimented again, the distribution pattern was as shown in (d). There is a general correlation of the pulse and mass labels; when the sedimentation profile of the original $rRNA_I$ (Fig. 5a) is compared to that of the final preparation (Fig. 5d), it is seen to be essentially similar except for the disappearance of the pulse counts which initially sedimented ahead of the 23 S rRNA. This result, in addition to the ØX result, again suggests that the fast pulse label could be due to mRNA-rRNA aggregation.

(d) Velocity sedimentation in 99% DMSO

Such aggregation effects, in addition to the well-established observations that the measured S values are not simply related to RNA molecular weight, decrease the utility of sucrose gradient analyses of RNA preparations. Sedimentation of RNA through 99% DMSO has been shown to be independent of prior secondary structure and the dependency of S on molecular weight is regular and known (Strauss et al., 1968). Figure 6 illustrates the result of sedimentation through 99% DMSO of RNA from different stages of the

fractionations described here. In Fig. 6a, $rRNA_I$ was used; in Fig. 6b, $rRNA_{II}$; and in Fig. 6c, the RNA eluted from two successive fractionations at pH 3.5 of an $rRNA_I$ fraction. The results of these 99% DMSO gradients indicated that there was no apparent degradation as judged by the mass ratio (2:1) for the 23 S and 16 S rRNA in both $rRNA_I$ and $rRNA_{II}$. The size distribution of the pulse label was the same in the initial (a) as in the final (c) preparation, making it unlikely that selection or degradation had occurred. In (c) there is a good correlation of the pulse and mass labels, disregarding the slower-sedimenting tRNA component. Comparison of the distribution of pulse label on the sucrose gradients and in the DMSO gradients indicates a good agreement except for the pulse label sedimenting ahead of the 23 S marker; since this component is absent in DMSO, it probably represents aggregation as suggested above.

(e) Fate of the ^{3}H-uracil pulse- and ^{14}C-uracil uniformly-labeled RNA during a "chase"

The experiments described above were performed with a ^{32}P pulse- and ^{3}H uniform-label and the good correlation of the pulse and mass counts in the final preparation suggested that the mRNA had been purified to its actual mass (~3 to 5% of the total RNA mass of the cell). As a further test of this conclusion, a culture of *E. coli* was labeled with ^{14}C-uracil for one generation and then with a 45 sec. pulse

of ^{3}H-5-uracil. The culture was then split; one-half was killed with NaN_3, while the label in the other half was chased by adding a large excess of unlabeled uridine and cytidine, filtering off the cells, and resuspending them for one generation of growth in excess unlabeled uridine and cytidine. The nucleic acids were then extracted, and fractionated on columns of BC at pH 7.5 and pH 3.5. Successful change of medium was indicated by the lack of further incorporation of the ^{14}C or ^{3}H labels; also, the specific activity of the ^{14}C decreased by one-half during the chase, indicating uninterrupted cell growth. Equivalent amounts of RNA (A_{260}) from the two half cultures were sedimented on sucrose gradients (Fig. 7). In Figure 7a, the RNA from the ^{3}H pulse-^{14}C uniformly-labeled control half of the culture shows the usual heterogeneous sedimentation distribution of ^{3}H pulse label with corresponding A_{260} and ^{14}C label. The sedimentation distribution of the RNA from the chased half of the culture is seen in Figure 7b. The ^{3}H label has largely disappeared, indicating success of the chase. The ^{14}C counts have also decreased; however the ratio of ^{14}C/^{3}H as compared to that of the control indicates there is substantial ^{14}C label that does not chase or does so very slowly. Much of this is probably tRNA, but it is surprising that the distribution of persistent ^{14}C label sedimenting ahead of tRNA is very similar to that of the control.

4. Discussion

(a) The basis for fractionation on BC

At pH 7.5 the BC and BNC columns appear to separate nucleic acids on the basis of their secondary structure (see Sedat *et al.*, 1967). It is, however, unexpected that such chromatography fractionates *E. coli* pulse labeled RNA into two peaks (in 0.3 M NaCl, 0.02 M Tris-Cl, pH 7.5). It was thought that this result might be explained by the aggregation of mRNA to rRNA, but even after two successive fractionations at pH 3.5 to remove rRNA, rechromatography at pH 7.5 distributes the pulse label (both in position and amount) identically as in Figure 2. Whatever the basis of this fractionation of the pulse label, it is very sensitive to a change in buffer, as to 0.02 M PO_4, pH 9.6, in 0.3 M NaCl.**

The fractionation of mRNA from rRNA at pH 3.5 is not well understood. The requirements for urea (and not dimethylurea), for NH_4Cl (and not NaCl), and that the pH $\leq$ 4.5 seem to have no obvious basis; equally bizarre is the ability to bring off the rRNA with H_2O (after the SDS wash in the presence of a degrading 8 M urea).

There are several observations to suggest that the interaction of the column with nucleic acid is not an equilibrium reaction as in most ion exchange chromatographic procedures. First, the separations and resolution are not

significantly improved by an increase in height of the column (even by a factor of 20), and no change is noted by mixing up the column bed during the loading process (the capacity is $\geq$ 100 A_{260}/ml. bed volume at pH 7.5). Second, we find that the fractionation with respect to position and peak appearance is very sensitive to the conditions of application, suggesting that the RNA molecules are fixed on the BC column (decreasing their degrees of freedom) in a state dependent on the solvent in which they were applied and that this state determines the necessary ionic conditions for elution.

We find no evidence that there is any selectivity for different messengers across peaks eluted from BC, at any pH, as evidenced by the similar sedimentation patterns of different fractions and the constant ratio of ØX ^{3}H-pulse label to ^{32}P-uninfected pulse label.

(b) Proof that the pulse label represents mRNA

The pulse label (in $rRNA_I$ and $rRNA_{II}$) from ØX-infected cells (and not from uninfected cells) contains messenger RNA that hybridizes specifically to ØX RF. (That it does not hybridize to ØX DNA indicates that the complementary strand of the RF is the site for transcription [with a preference better than 10^6:1] in agreement with previous work on ØX mRNA [Hayashi, Hayashi & Spiegelman, 1963]). The sedimentation distributions of the purified pulse labeled

RNA are in good agreement with many previous studies of the sedimentation distribution for the heterogeneous E. coli mRNA (cf. Bremer & Yuan, 1968). The total mass of the final pulse labeled RNA preparation (mass label now coincident with pulse label distribution) is in general agreement with recent estimates for the mass of E. coli mRNA: 3% (Salser, Janin & Levinthal, 1968); 1.5 to 3% (Leive, 1965); 3% (Mangiarotti & Schlessinger, 1967); 2.2% (Mueller & Bremer, 1968). The chase experiment indicates that the pulse label is chased out, as expected, in accord with other observations (see the discussion by Salser et al., 1968). It is also of interest that the pulse labeled RNA isolated on BNC in this way from phage-infected cells contains messenger RNA fully active in the stimulation, in cell-free systems, of the synthesis of specific, biologically active proteins of the phage T4 (T. Young, personal communication).

(c) Possible selection of mRNA fractions

It is very difficult to ascertain if there has been selective enhancement of any mRNA fraction during the preparation of the total heterogeneous mRNA. One major uncontrolled process wherein selection could arise is in the lysis and extraction with phenol. It is known that more of the single-stranded replicating DNA is retained in the phenol layer (Ganesan & Lederberg, 1965; Hanawalt & Ray, 1964), and pulsed RNA or DNA is found bound to the inter-

face of phenol layers in extracts of animal cells (Kidson, Kirby & Ralph, 1963). However, the high yields of ØX mRNA, tryptophan operon mRNA, lac operon mRNA and lambda phage mRNA by the phenol extraction procedure indicate that this is probably not a significant objection. Another step in which unwanted selection could arise is during the BNC fractionation. Since the recovery of ^{3}H-mass counts is > 95% (Fig. 2), it is unlikely that selection of this RNA is significant; the recovery of the ^{32}P pulse label is over 90%. Much of the firmly bound ^{32}P pulse label is in a non-nucleic acid component, which can be eluted with SDS. No selection of pulse label into $rRNA_I$ or $rRNA_{II}$ is evident in the profiles of ØX RF-hybridizable RNA. Rechromatography of previously fractionated (see Fig. 5a) pulse label, at pH 3.5 or pH 7.5 on BNC, results in a firm binding of approximately 10% of the ^{32}P pulse; however, from DMSO velocity gradients there appears to be no selection of a particular component. All the fractionation steps seem to have very similar or identical leading edges in DMSO gradients which may be the best evidence that the BNC chromatography is not selective.

(d) Problems of RNA degradation

If it is accepted that a mass ratio of rRNA components < 2:1 indicates degradation during the preparation, then from the DMSO sedimentation (where masked hits are unfolded)

little degradation is evident. Of course, even with the use of RNase I^- mutants it is still possible that there might be polysome degradation (but not ribosome) during the lysozyme-EDTA lysis before addition of SDS and phenol.

The degradation of RNA by 8 M urea was unexpected, requiring the use of a highly purified reagent.

(e) Purity of mRNA

One of the best indications of purity of the mRNA preparation is the observation in DMSO (or sucrose) gradients that the mass counts closely parallel the pulse counts as a function of the mRNA size (in DMSO). It is extremely unlikely that this is a coincidence but more probably reflects the purification to the actual mass of the mRNA. This mass, allowing for losses on the columns and correction for the tRNA contaminant, agrees with the reported value of 3%. Another argument for purity of this fraction comes from the observation that the ratio of pulse to mass label in the sedimentation region between rRNA and tRNA (near the mean molecular weight of mRNA) in the $rRNA_I$ sedimentation in DMSO (Fig. 6a, tube 30) is the same as in the final purification (Fig. 6c, tube 30). This observation is also true for $rRNA_{II}$.

There are, however, some indications that some impurities may exist in the final preparation. First, the ratio of pulse to mass in $rRNA_I$ is approximately one-half of that

in $rRNA_{II}$, and this is still true for the RNA eluted at pH 3.5 (after removal of ribosomal RNA) from both of these fractions (see tube 30 in Figures 6a, 6b, and 6c; tube 35 in Figures 5b and 5c). This is not understood but it might represent an impurity. Second, it is possible to see small shoulders in the mass counts in Figures 5b, c, d in the region where one would expect rRNA; these are reduced on rechromatography at pH 3.5 or pH 7.5, but not eliminated.

The most important indication of impurity comes, however, from the pulse/chase experiment. The pulse/chase labeling sequence results in the loss of approximately 97% of the pulse label from the final mRNA preparation, but only about 68% of the mass label. (The contribution of tRNA contaminating these fractions was eliminated by comparing the values for individual points in the DMSO gradients of the final preparations. These points were taken from the peak of the pulse label distribution, well ahead of the tRNA peak). An excess of mass label implies that the molecules containing it were synthesized before addition of the pulse label. Three ways in which such molecules might appear in the final mRNA preparation have been discerned. First they might be some minor RNA component, perhaps a degradation product of rRNA, which happens to co-elute with mRNA. The second possibility is that some fraction of the total mRNA synthesized by the cell is metabolically stable. Long-lived mRNA synthesized before addition of pulse label

would contribute to the mass label, but not to the pulse label, and would not chase. However, the fraction of total mRNA which would have to be postulated to be stable ($\geq$ 0.3) in order to account for the observed effect seems too large. A third possibility is that the unchasable mass label comes from cells which have died during the initial period of labeling with mass label. (It is, of course, then necessary to assume that all mRNA in dead cells remains undegraded.) It should be noted that the cultures were filtered, which may have caused some cell death. However, general experience with the strains used indicates that the procedures employed cause very little loss of viability. It is unlikely that the observed effect stems solely from any one of these hypothetical mechanisms; it is perhaps attributable to a combination of all three operating simultaneously.

It appears therefore that it is in general difficult to decide the purity of the mRNA. Though it has been purified 15-fold, more work will have to be done to decide this point.

(f) Implications of this research

The finding that the mRNA pulse label elutes as a single peak at the final pH 3.5, resolved from rRNA, but with a size distribution identical to that present in the starting RNA, implies that there is some common feature,

structural or chemical, in mRNA molecules permitting them to act as a group during BNC fractionation.

Our sedimentation velocity patterns in DMSO imply that very little (if any) mRNA is larger than 1 x 10^6 avograms, enough RNA for at most 10 cistrons. The peak of the mRNA distribution is at an S corresponding to M $\simeq$ 72,000. (The corresponding M for ØX messenger RNA is 116,000.)

These low average values of messenger size raise the possibility of a selective degradation of messenger RNA (and not of ribosomal RNA) during isolation. However, the isolation, as previously mentioned, of functional messenger RNA from infected cells by these methods argues qualitatively against the likelihood that extensive degradation has occurred.

Our results on the mechanisms of fractionation on BNC or BC suggest that a new chromatographic effect is operative such that the elution (recovery and position) of the nucleic acid is strongly influenced by the conditions that prevailed when it was applied on the column; it is as if the degrees of freedom are fixed once the nucleic acid is bound to the column and can only be changed by elution when the ionic conditions are right.

This research was supported in part by a grant GM13554 from the U.S. Public Health Service.

Footnotes

*Abbreviations used: BNC, benzoylated (naphthoylated) DEAE cellulose; BC, benzoylated DEAE cellulose; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.

**It should be mentioned that reticulocyte rRNA is not completely eluted during BC chromatography unless a 0 to 4 M urea gradient is substituted for the 0 to 30% DMSO gradient (Lyon, unpublished observations).

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Figure Legends

Fig. 1. Analytical velocity sedimentation of rRNA, treated either (a) with 8 M urea (Baker & Adamson) or (b) 8 M urea (Mann, passed through V/M Millipore filter). Aliquots of the rRNA peak eluted from a BNC column at pH 7.5, and previously shown to be undegraded by velocity sedimentation in DMSO, were treated (a) with 8 M urea (Baker & Adamson) in 0.1 M Tris-Cl, pH 8, for 10 min. at 25° C. and then sedimented into D_2O, or (b) were treated with 8 M urea (Mann, passed through V/M Millipore filter) in 0.1 M acetate, pH 3.5, for 10 hr. at 25° C. and then sedimented in 99% DMSO. Sedimentation is from left to right.

Fig. 2. Fractionation of ^{32}P pulse labeled, ^{3}H-uracil uniformly labeled nucleic acid extract on BNC column at pH 7.5.

O——O ^{32}P (pulse) counts/min.

●——● ^{3}H (mass)

Fig. 3. Sedimentation in a sucrose gradient of (a) $rRNA_I$ or (b) $rRNA_{II}$ from both ØXam3-infected cells, pulse labeled with ^{3}H-uracil for 45 sec. at 15 min. after infection, and from uninfected cells, pulse labeled with ^{32}P (phosphate) for 45 sec.

●——● Total ^{3}H cts/min. in each fraction

o——o Total ^{32}P cts/min. in each fraction

Δ——Δ ^{3}H cts/min. hybridized to ØX RF in each fraction

▲——▲ ^{32}P cts/min. hybridized to ØX RF in each fraction

□——□ ^{3}H cts/min. hybridized to ØX viral DNA in each fraction

■——■ Position of label in viral ØX DNA in another sedimentation tube

Fig. 4. Chromatography of $rRNA_I$ (isolated from ^{32}P pulse-, ^{3}H mass-labeled nucleic acid extract on BNC at pH 7.5) on BNC column at pH 3.5 in the presence of 8 M urea. $rRNA_{II}$ behaves similarly.

o——o ^{32}P (pulse) cts/min.

●——● ^{3}H (mass) cts/min.

Fig. 5. Sedimentation through a sucrose gradient of different RNA fractions eluted from BNC columns. Sedimentation from right to left at 5° C. at 25,000 rev./min. for 12 hr., through a 5 to 20% sucrose gradient in 0.02 M Tris-Cl buffer, pH 7.5.

o——o ^{32}P (pulse label) cts/min.

●——● ^{3}H (mass label) cts/min.

(a) $rRNA_I$ from BNC column at pH 7.5

(b) The RNA eluted from a BNC column at pH 3.5, after application of $rRNA_I$

(c) The RNA eluted from a BNC column at pH 3.5, after ap-

plication of $rRNA_{II}$

(d) An aliquot of RNA similar to that used in (b) was re-chromatographed on BNC at pH 7.5 and the peak eluted from this column in the second gradient sedimented as above.

Fig. 6. Sedimentation through 99% DMSO gradients of ^{32}P pulse-, ^{3}H-uracil uniformly-labeled RNA fractions from BNC column.

O——O ^{32}P (pulse) cts/min.

●——● ^{3}H (mass) cts/min.

(a) $rRNA_I$

(b) $rRNA_{II}$

(c) $rRNA_I$ after repeated (2x) chromatography on BNC column at pH 3.5

Fig. 7. Sedimentation of RNA eluted from BNC column at pH 3.5 ($rRNA_I$ applied). The RNA of the cells was uniformly labeled with ^{14}C-uracil and pulse labeled (45 sec.) with ^{3}H-uracil. At the end of the pulse, one-half of the culture was chilled on ice and the other half incubated in the absence of label for one generation of growth (chase).

Equal amounts (A_{260}) of the RNA, extracted from the two halves of the culture and chromatographed, were sedimented at 10° C. for 8 hr. at 40,000 rev./min. through a 5 to 20% sucrose gradient in 0.02 M Tris-Cl buffer.

A_{260}

^{3}H (pulse) cts/min.

^{14}C (mass) cts/min.

Δ Ratio $^{14}C/^{3}H$ in each fraction

(a) Immediately at end of 45 sec. pulse

(b) After chase for one generation of growth

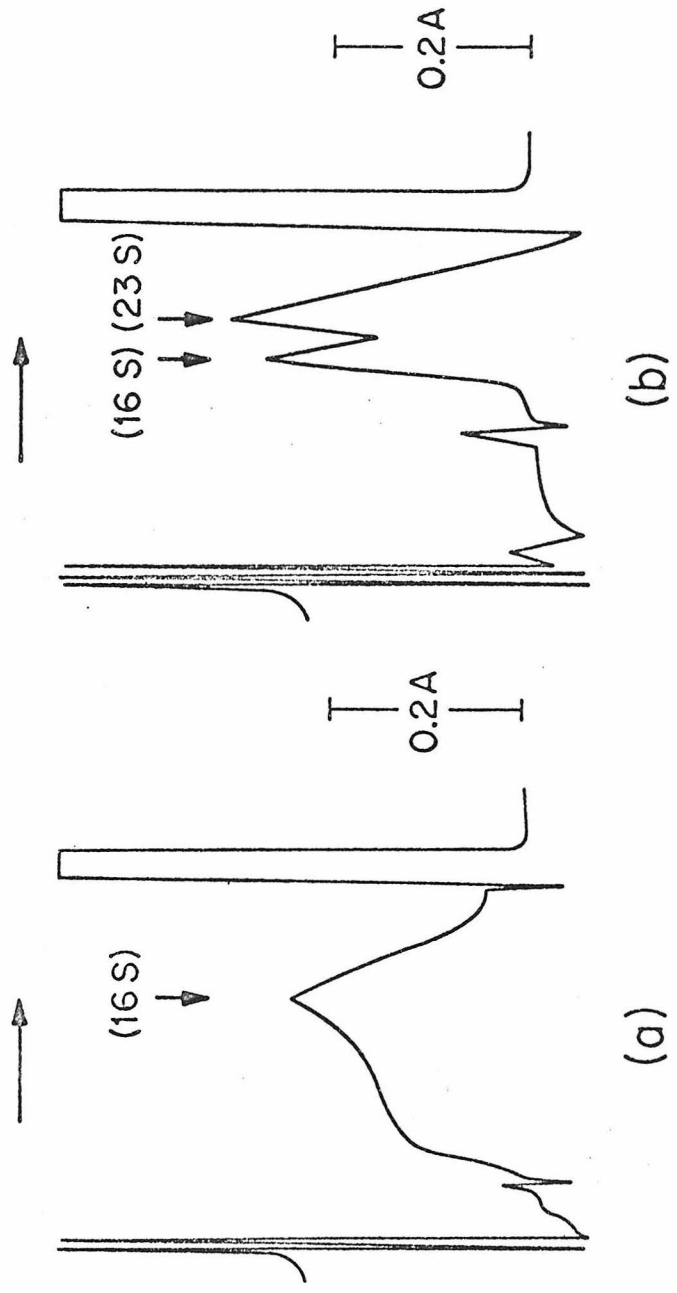

Figure 1

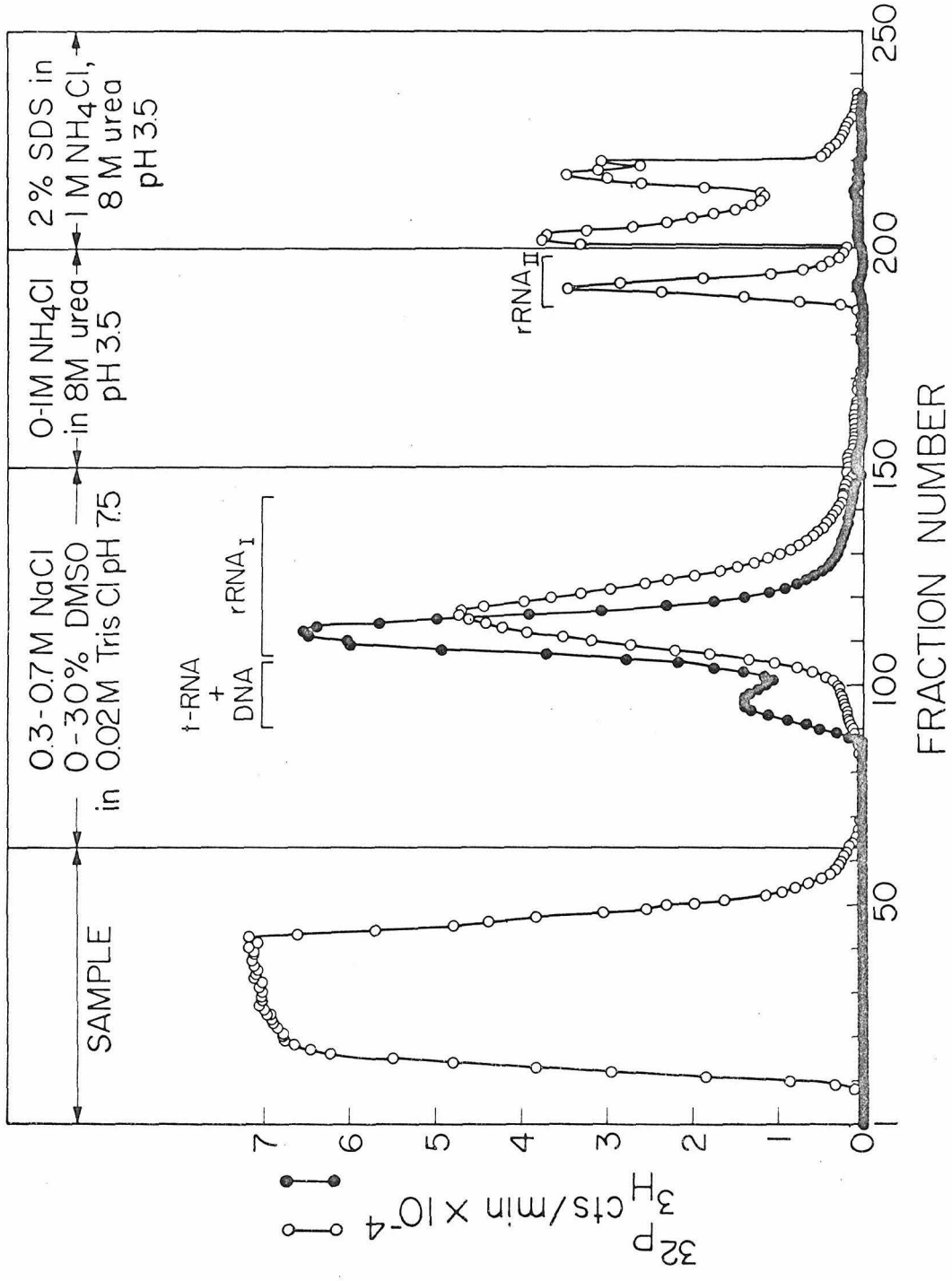
SAMPLE
0.3-0.7 M NaCl
0-30% DMSO
in 0.02M Tris Cl pH 7.5
0-1M NH4Cl
in 8M urea
pH 3.5
2% SDS in
1 M NH4Cl,
8 M urea
pH 3.5
t-RNA
+
DNA
rRNAI
rRNAII
32P
3H cts/min x 10-4
FRACTION NUMBER
0
1
2
3
4
5
6
7
50
100
150
200
250

Figure 2

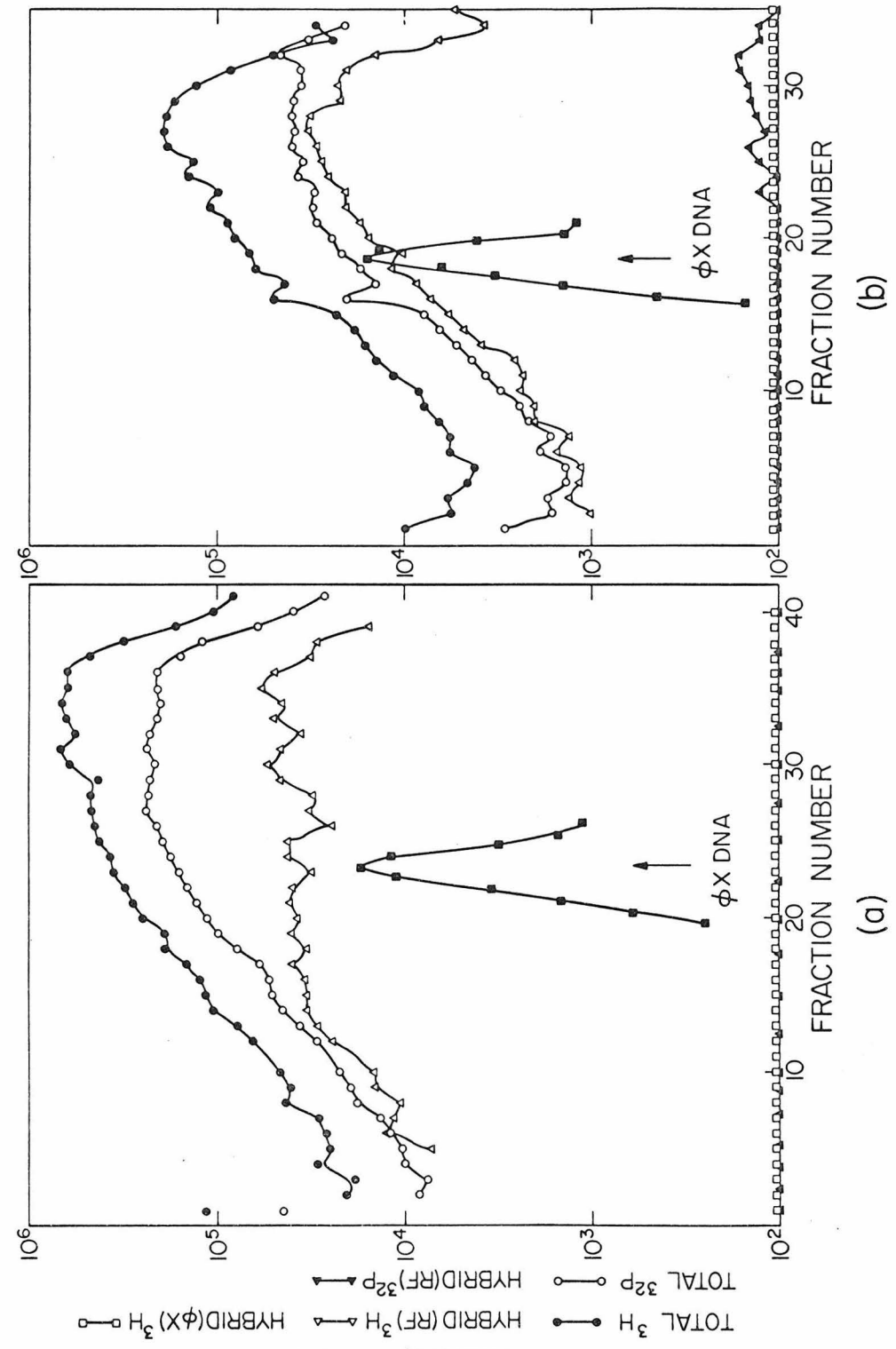
COUNTS/MINUTE
TOTAL 3H
TOTAL 32P
HYBRID (RF) 3H
HYBRID (RF) 32P
HYBRID (ΦX) 3H
ΦX DNA
FRACTION NUMBER
(a)
(b)

Figure 3

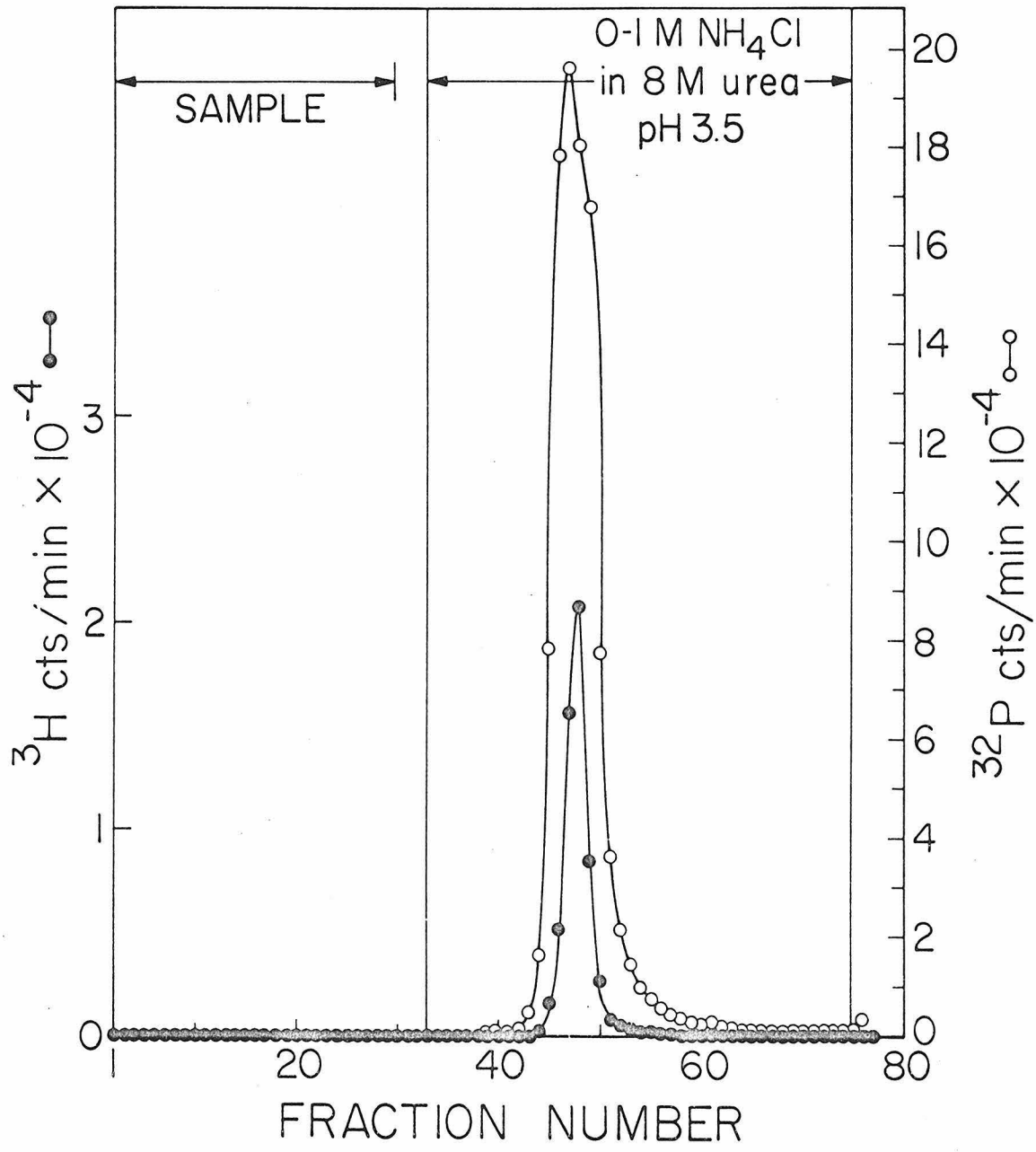
SAMPLE
0-1 M NH_4Cl
in 8 M urea
pH 3.5
^3H cts/min $\times 10^{-4}$
^{32}P cts/min $\times 10^{-4}$
FRACTION NUMBER
0
1
2
3
0
2
4
6
8
10
12
14
16
18
20
20
40
60
80

Figure 4

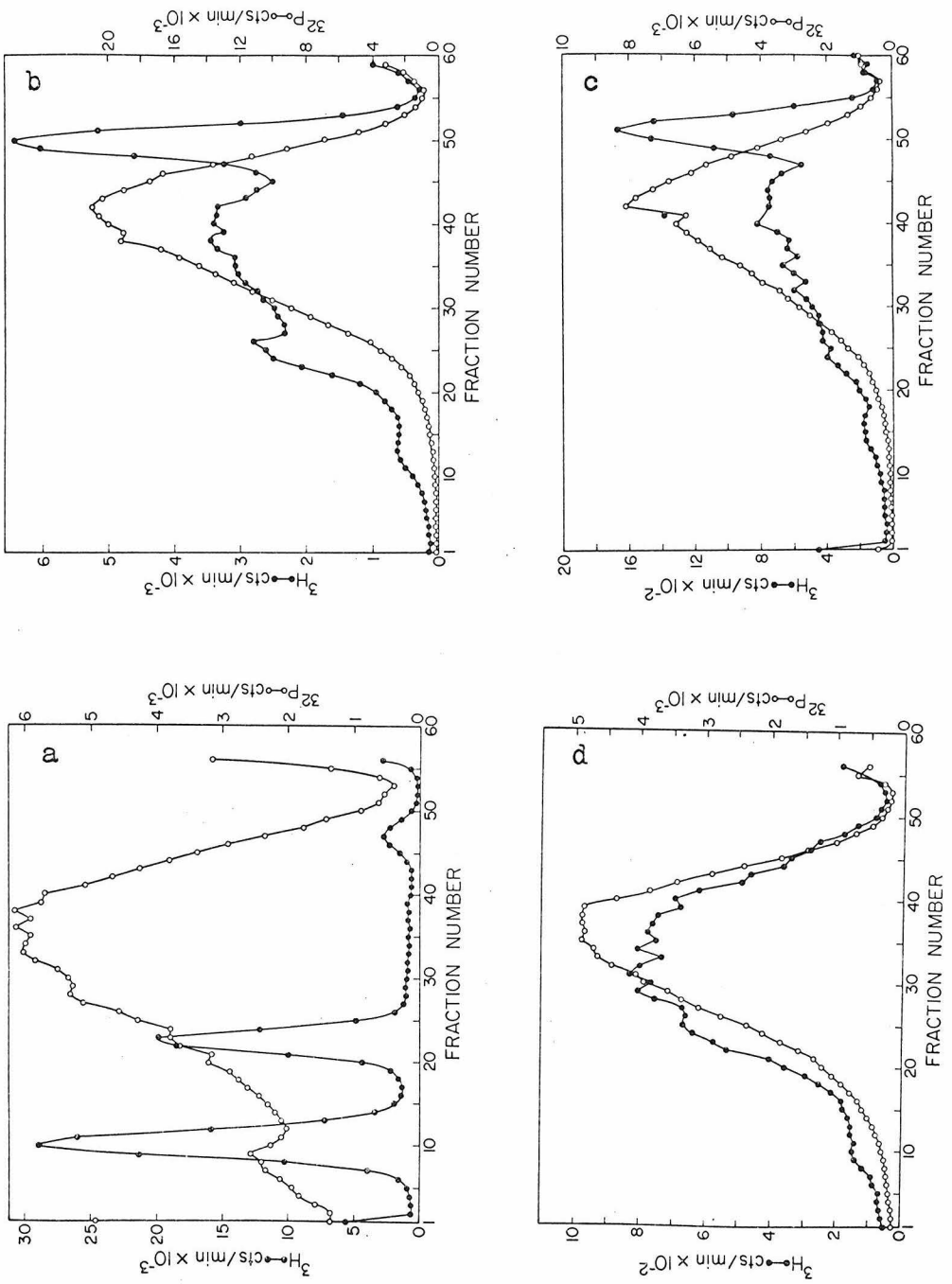
a
b
c
d
FRACTION NUMBER
FRACTION NUMBER
FRACTION NUMBER
FRACTION NUMBER
^{3}H—•cts/min x 10^{-3}
^{32}P—○cts/min x 10^{-3}
^{3}H—•cts/min x 10^{-3}
^{32}P—○cts/min x 10^{-3}
^{3}H—•cts/min x 10^{-2}
^{32}P—○cts/min x 10^{-3}
^{3}H—•cts/min x 10^{-2}
^{32}P—○cts/min x 10^{-3}

Figure 5

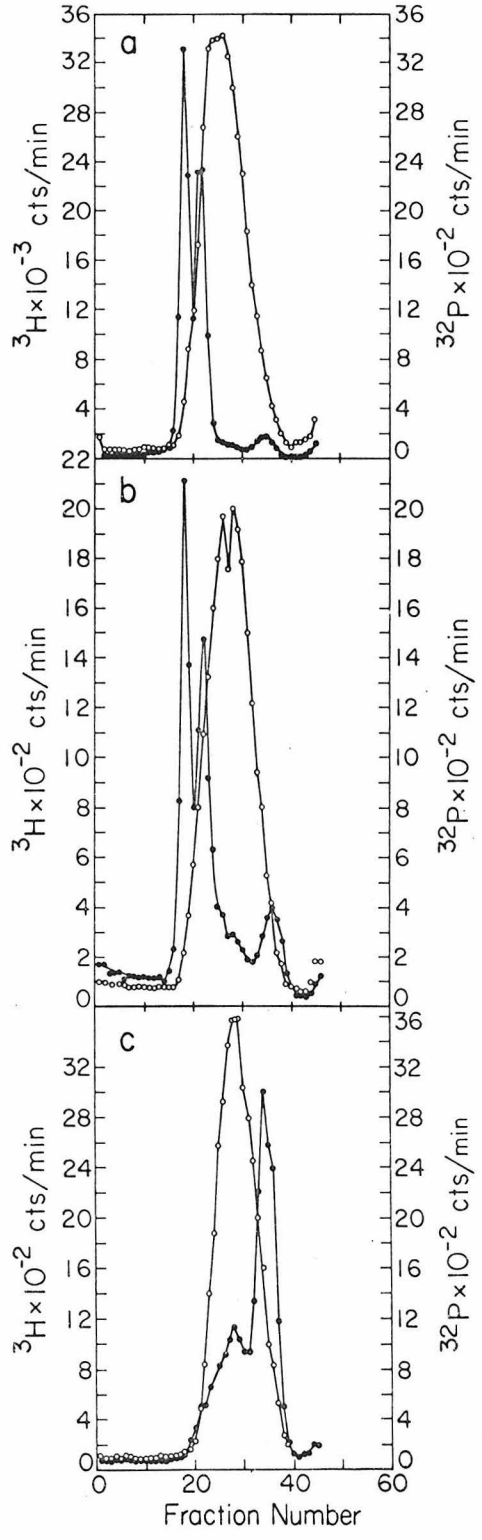
a
b
c
$^{3}H \times 10^{-3}$ cts/min
$^{32}P \times 10^{-2}$ cts/min
$^{3}H \times 10^{-2}$ cts/min
$^{32}P \times 10^{-2}$ cts/min
Fraction Number
0
20
40
60

Figure 6

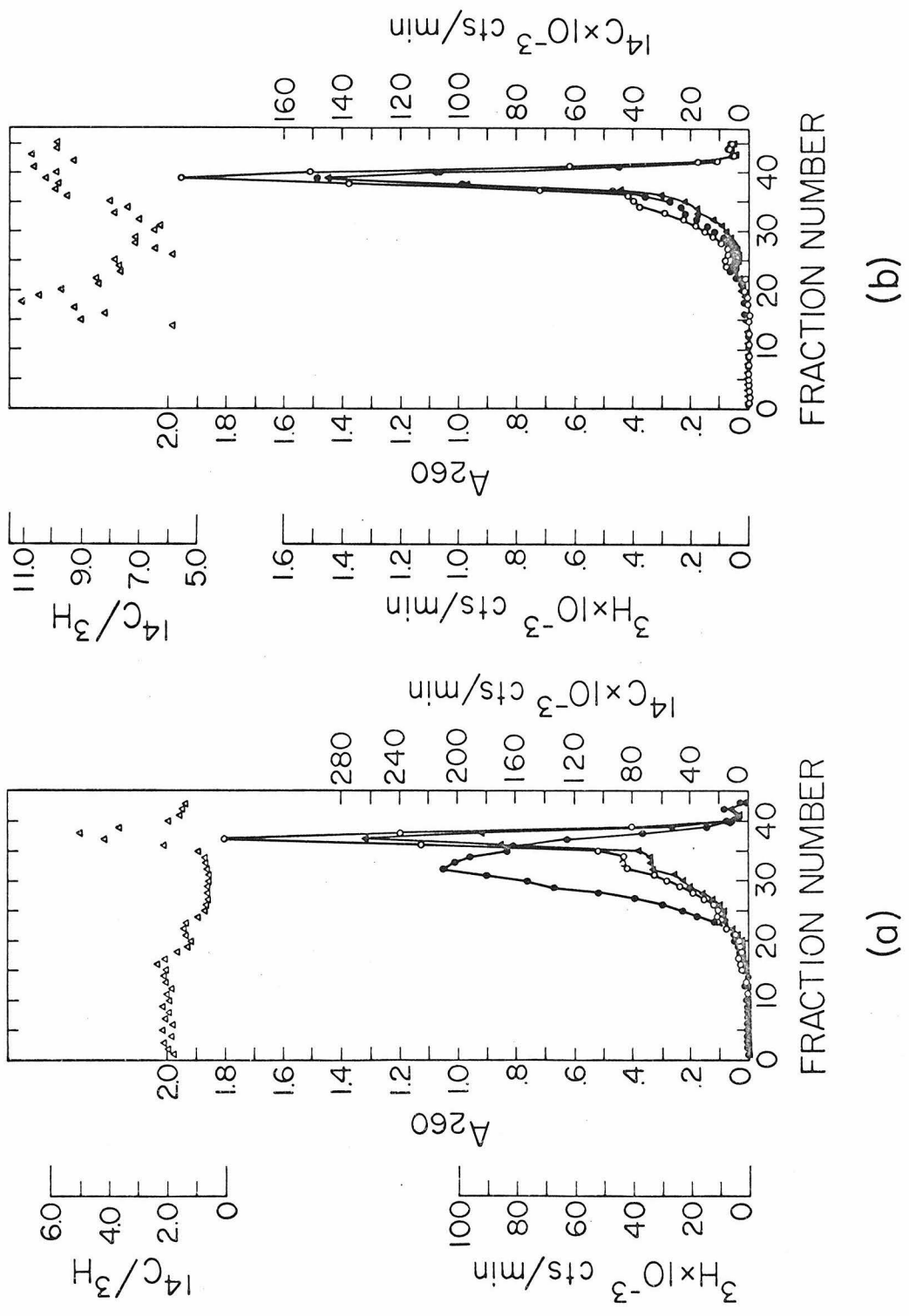
(a)
FRACTION NUMBER
A260
$^{14}C \times 10^{-3}$ cts/min
$^{3}H \times 10^{-3}$ cts/min
$^{14}C/^{3}H$
(b)
FRACTION NUMBER
A260
$^{14}C \times 10^{-3}$ cts/min
$^{3}H \times 10^{-3}$ cts/min
$^{14}C/^{3}H$

Figure 7

TABLE 1

$_{p}$H 7.5 BNC FRACTIONATION

PEAK	FRACTION OF THE MASS	FRACTION OF THE PULSE	PULSE/MASS
t-RNA + DNA	0.11	0.02	0.20
r-RNA$_{I}$	0.83	0.60	0.85
r-RNA$_{II}$	0.03	0.16	4.00
SDS	0.02	0.23	≥12.50

TABLE 2

$_{p}$H 3.5 BNC FRACTIONATION

RNA APPLIED	FRACTION OF THE MASS ELUTED	FRACTION OF THE PULSE ELUTED	PULSE/MASS
r-RNA$_{I}$	0.10	0.60	5.9
r-RNA$_{II}$	0.23	0.80	10.9

Part III

BENZOYLATED DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF RETICULOCYTE RNA

INTRODUCTION

The methods developed in the previous section were next applied to RNA preparations from rabbit reticulocytes. The *E. coli* results show that the BC column distinguishes mRNA on the basis of some class-specific property, presumably some feature of the secondary structure of mRNA in solution. This structural feature might result from some aspect of base sequence common to all mRNAs, and related to their function. On the other hand, it could stem from some special functional requirement found only in *E. coli* mRNA (or, more likely, all prokaryotic mRNA). It is thus of interest to see whether eukaryotic RNA, such as reticulocyte RNA, behaves similarly to *E. coli* RNA on BC columns.

MATERIALS AND METHODS

All materials and methods used in this section have already been described in the Materials and Methods section of either Part I or Part II.

RESULTS

Modification of pH 7.5 BC Chromatography

It was found that reticulocyte rRNA, unlike *E. coli* rRNA, could not be eluted from BNC or BC by neutral aqueous salt gradients. The inclusion of urea in the elution buff-

ers resulted in its elution, however, as is shown in Fig. 5. The apparently low recoveries of rRNA (as judged by the relative areas under the peaks of sRNA and rRNA) are probably anomalous. In another experiment, 1.79×10^4 cpm (760 μg) ^{32}P-reticulocyte rRNA, prepared by the LiCl method, was applied to a 0.5 cm^2 x 12 cm BNC column which had been equilibrated with 0.3 M NaCl, 0.001 M Na_4EDTA, 0.02 M Tris-Cl, pH 7.5. It was eluted at a flow rate of 0.3 ml./min. by applying a linear gradient (30 ml. in each chamber of a two-chambered gradient-forming device) between this buffer and 0.7 M NaCl, 4 M urea, 0.001 M Na_4EDTA, 0.02 M Tris-Cl, pH 7.5. 1.39×10^4 cpm (78% of the applied radioactivity) was recovered in the eluted material. The resin was removed from the column, spread on planchets, and the radioactivity was determined in a Nuclear Chicago gas-flow geiger counter. 1.14×10^3 cpm remained bound to the resin, corresponding to 8% of the starting material. Thus recovery of rRNA (under these elution conditions, at least) is satisfactorily high.

The inclusion of other denaturing agents, such as DMSO, in place of urea, gave low and variable yields. Soluble RNA was eluted by neutral aqueous salt gradients alone, as in the case of E. coli sRNA. Its position of elution was shifted to slightly lower salt concentrations when urea was included in the elution buffers. The arrow on Fig. 5b shows the position at which the reticulocyte sRNA peak would have been expected to elute, had urea been omitted

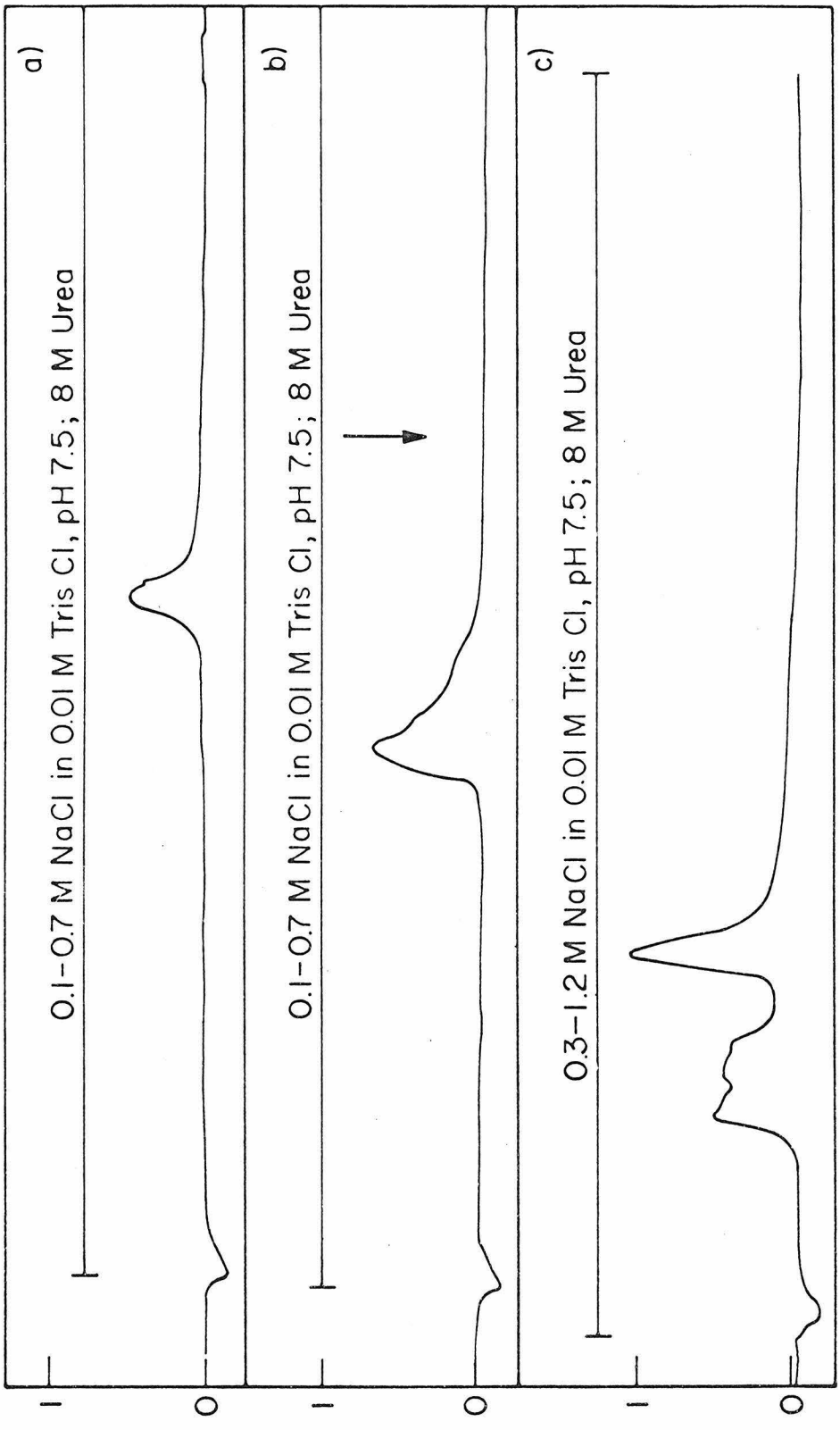
a)
0.1–0.7 M NaCl in 0.01 M Tris Cl, pH 7.5; 8 M Urea
b)
0.1–0.7 M NaCl in 0.01 M Tris Cl, pH 7.5; 8 M Urea
c)
0.3–1.2 M NaCl in 0.01 M Tris Cl, pH 7.5; 8 M Urea
1
0
1
0
1
0
A254

Figure 5

Figure 5. Modified pH 7.5 BC chromatography.

Elution gradients were formed between the high and low concentration buffers indicated on the figure by a two-chambered linear gradient forming device, 50 ml. per chamber. Samples were diluted 1/10 in the low concentration buffer, applied to a 0.5 $cm.^2$ x 12 cm. BNC column which had been equilibrated with low concentration buffer, and eluted at a flow rate of approximately 0.5 ml./min. Optical absorbance at 2540 Å was continuously monitored with an Instrument Specialties Company UV flow monitor.

(a) Chromatography of 450 μg. reticulocyte rRNA, prepared by the LiCl method. Diluted sample volume: 1 ml.

(b) Chromatography of 260 μg. reticulocyte sRNA, prepared by the LiCl method. Diluted sample volume: 1 ml. The arrow marks the expected elution position of the sRNA, had urea been omitted from the eluants.

(c) Chromatography of 450 μg. rRNA + 260 μg. sRNA. Diluted sample volume: 2 ml.

from the eluants. This position was calculated from a series of earlier experiments (data not shown) in which the salt concentration (in the absence of denaturant) required to elute the sRNA peak was measured.

A simultaneous elution gradient in NaCl and urea concentration, viz., 0.3 to 0.8 M NaCl, 0 to 4 M urea, 0.02 M Tris-Cl, 0.001 M Na_4EDTA, pH 7.5, was eventually found to give optimum separation of the sRNA and rRNA peaks. In earlier experiments, including those shown in Fig. 5, unpurified urea was used. After the recognition of the degradative activity of such urea (see Part II), purified urea was used. Changing the urea did not alter the characteristics of the modified pH 7.5 BC chromatography.

Large-scale RNA Preparation and Column Chromatography

The precautions against RNase contamination observed in Part II were observed here. Ten rabbits were made anemic, blood was collected, the reticulocytes were washed and lysed, and the stroma was removed. The lysate was extracted with an equal volume of phenol saturated with 0.1 M NaCl, 0.01 M NaAc, 0.001 M Na_4EDTA, 0.1% (w/v) SDS, pH 5. The phases were separated and the phenolic phase re-extracted with an equal volume of buffer. The aqueous phases were combined, extracted with ether, and the nucleic acids precipitated with ethanol. The precipitate was collected by centrifugation, suspended in 100 ml. of the extraction

buffer, and again extracted with an equal volume of buffer-saturated phenol to eliminate residual contamination with denatured protein. The aqueous phase was collected, extracted with ether, and nucleic acids precipitated with ethanol. The precipitate was dissolved in 0.01 M NaAc, 0.001 M Na_4EDTA, pH 5. The yield was approximately 470 mg. RNA.

In order to determine if any degradation had occurred during isolation, the RNA preparation was analyzed by band velocity sedimentation in DMSO and in 90% D_2O. A representative trace from the latter analysis is shown in Fig. 6a; it can be seen that the mass ratio of 28 S rRNA to 18 S rRNA was approximately 2 to 1, indicating extensive degradation had not occurred (see Part II). The DMSO analysis gave the same result (data not shown).

Figure 7 shows the result of chromatography of this RNA preparation on BC at pH 7.5, modified as described above. The material eluting during the first half of sample application was not precipitable by ethanol; it may correspond to the orcinol-positive, low molecular weight material previously noted in reticulocyte lysates (80). The material eluting during the second half of sample application contained rRNA (as determined by analytical band velocity sedimentation) in addition to low molecular weight material. The chromatogram shown is the only time this phenomenon was observed. It is presumably due to over-

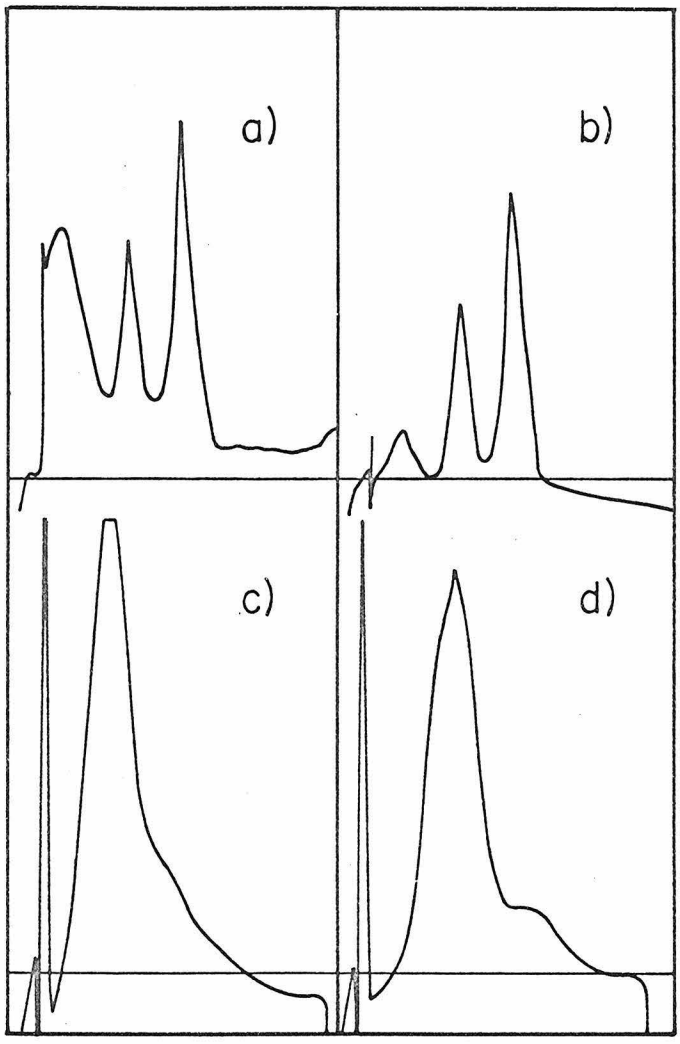

Figure 6

Figure 6. Analytical band velocity sedimentation of reticulocyte RNA.

(a) Unfractionated RNA preparation: 1.5 μg. RNA was centrifuged through 0.1 M NaCl, 0.001 M Na_4EDTA, 90% (v/v) D_2O, pH 7.2 (nominal) at 20° C. The output trace from the Photoelectric Scanning System of the Spinco model E analytical ultracentrifuge, operating at 2650 Å, was made 32 minutes after reaching the operating speed of 42,040 rpm.

(b) Combined $rRNA_I$ and $rRNA_{II}$ fractions from modified pH 7.5 system BC chromatography: 1.0 μg. RNA was centrifuged; the solvent, rotor speed, time, etc. were the same as in (a). Since the $rRNA_I$ fraction contained more than 50 times as much material as $rRNA_{II}$, the data reflect the composition of $rRNA_I$.

(c) and (d) Final "m"RNA fraction from pH 3.5 system BC chromatography: 0.84 μg. RNA was centrifuged through 99% (v/v) DMSO, 0.001 M Na_4EDTA at 20° C. The output traces from the Photoelectric Scanning System of the Spinco model E analytical ultracentrifuge, operating at 2750 Å, were made (c) 188 minutes and (d) 260 minutes after reaching the operating speed of 56,100 rpm.

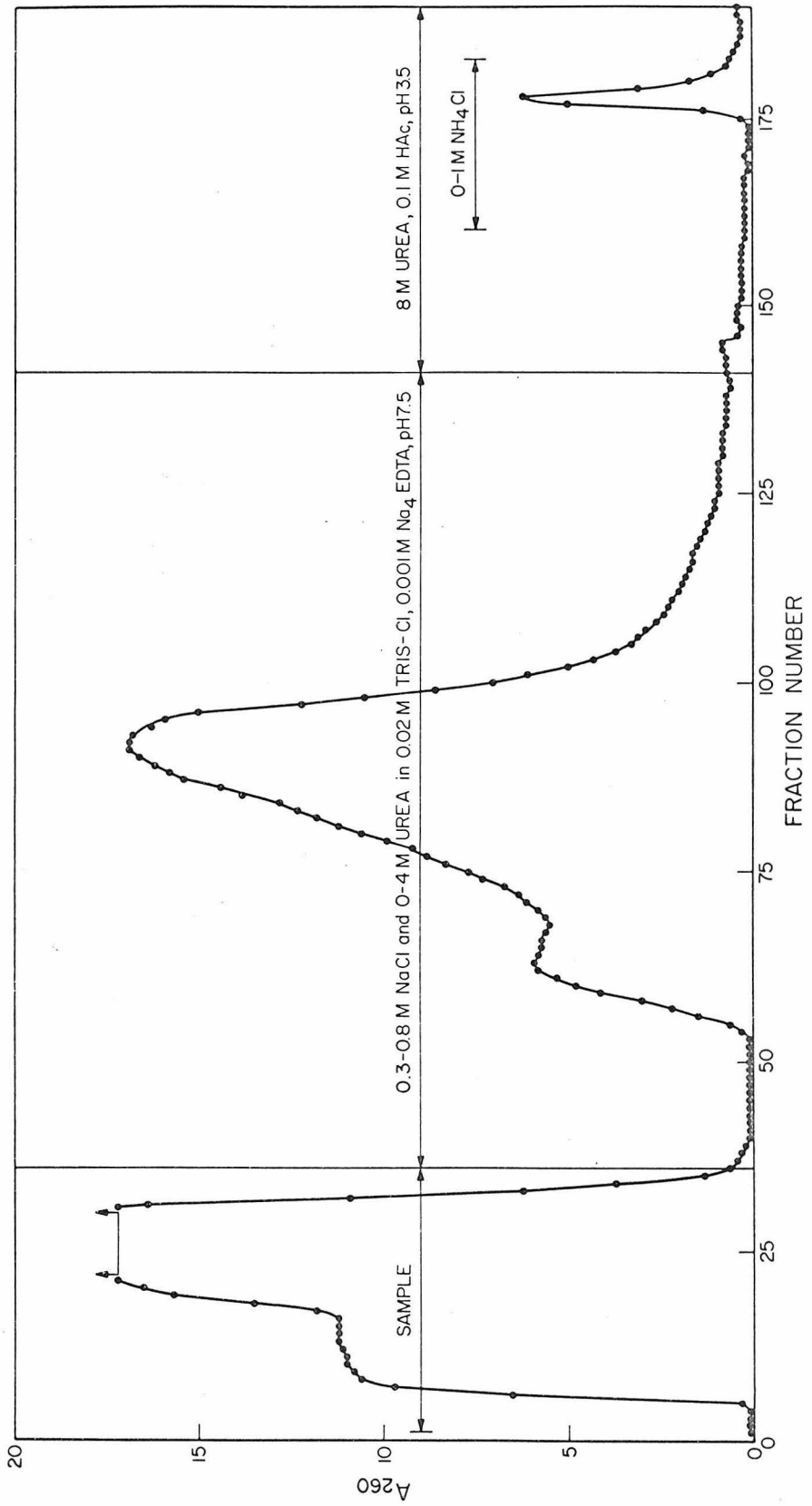
A260
FRACTION NUMBER
SAMPLE
0.3-0.8 M NaCl and 0-4 M UREA in 0.02 M TRIS-Cl, 0.001 M Na4 EDTA, pH7.5
8 M UREA, 0.1 M HAc, pH3.5
0-1 M NH4Cl

Figure 7

Figure 7. Modified pH 7.5 chromatography of reticulocyte RNA.

The sample, 470 mg. phenol-extracted reticulocyte RNA dissolved in 27.1 ml. 0.01 M NaAc, 0.001 M Na_4EDTA, pH 5, was diluted 1/10 with the low concentration pH 7.5 buffer (see figure) and applied to a 12.5 $cm.^2$ x 4 cm. BC column which had been equilibrated with low concentration pH 7.5 buffer. It was eluted as shown on the figure by two sequential linear concentration gradients formed by a two-chambered gradient forming device, 500 ml. per chamber in the first (pH 7.5) gradient, and 100 ml. per chamber in the second (pH 3.5) gradient, at a flow rate of 3 to 4 ml./min. Optical absorbance at 2600 Å was continuously monitored with a Gilford Absorbance Monitor.

loading of the column. The major peak eluted in the first gradient was shown by analytical band velocity sedimentation to be rRNA (see Fig. 6b), and the shoulder preceding it, sRNA. Absence of degradation during chromatography was indicated by the mass ratio of 23 S rRNA to 18 S rRNA, which had not changed (compare Fig. 6a and Fig. 6b). The small peak eluted by the second gradient (pH 3.5) also contained rRNA, as in the case of E. coli. Band velocity sedimentation analysis of this peak gave results comparable to Fig. 6b (data not shown).

Since the E. coli results show that both the $rRNA_I$ and $rRNA_{II}$ peaks contain mRNA, the major pH 7.5 gradient peak and the pH 3.5 gradient peak were combined. This sample was fractionated by BC chromatography entirely at pH 3.5. The results are shown in Fig. 8. Note that the single sharp peak is eluted at a NH_4Cl concentration of approximately 0.8 M, rather than approximately 0.5 M as in the case of E. coli. The sensitivity of the optical absorbance recorder was such that less than 0.1% of the applied sample could have eluted elsewhere than in the major peak and escaped detection.

The RNA in the peak obtained from pH 3.5 BC chromatography was concentrated by ethanol precipitation and dissolved in a small volume of 0.01 M NaCl, 0.001 M Na_4EDTA, pH 5. Figures 9a, b, and c show the result of band velocity sedimentation analysis in 90% D_2O of this material.

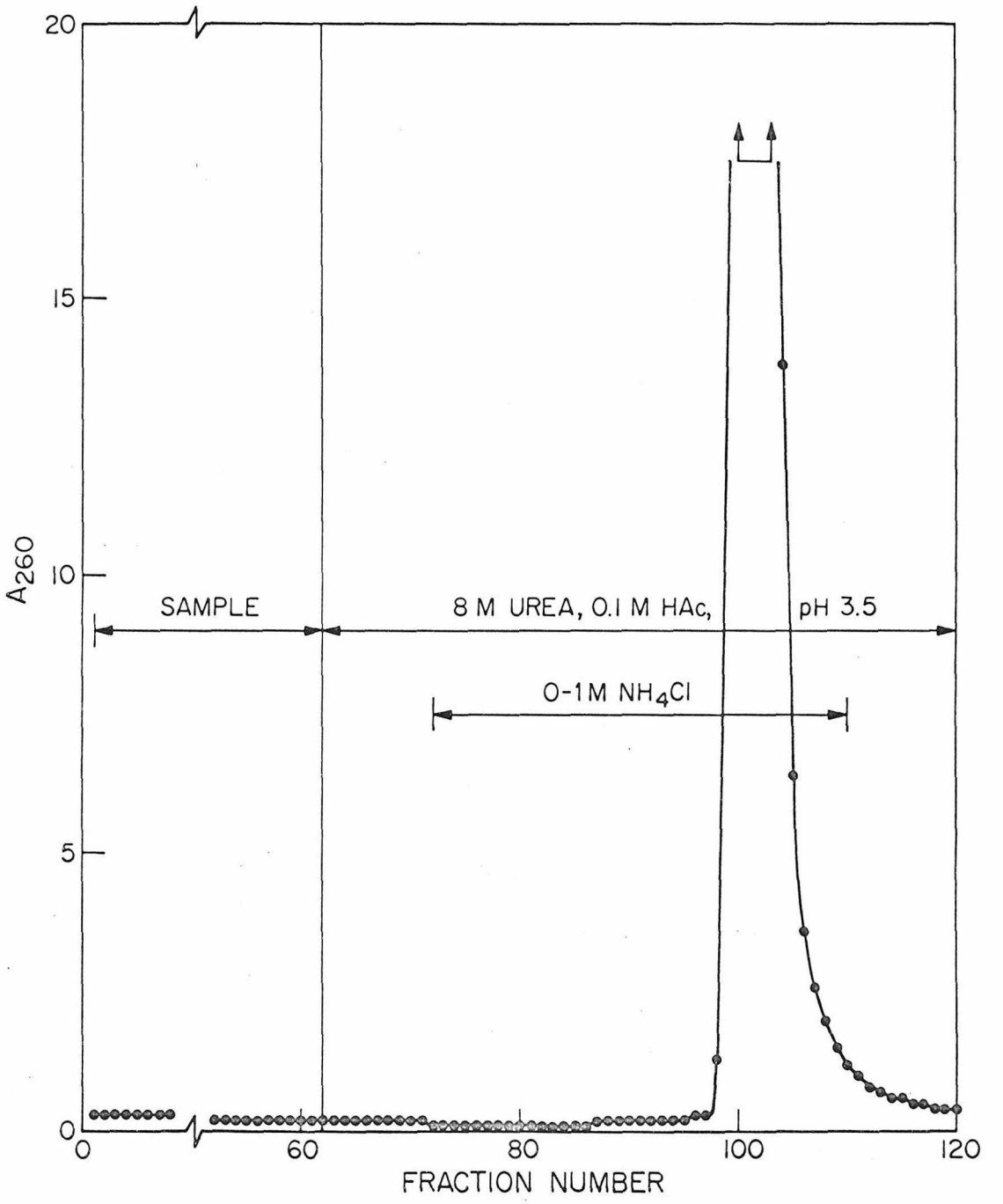
20
15
10
5
0
A_{260}
SAMPLE
8 M UREA, 0.1 M HAc,
pH 3.5
0-1M NH_4Cl
0
60
80
100
120
FRACTION NUMBER

Figure 8

Figure 8. BC chromatography, entirely at pH 3.5, of reticulocyte RNA.

The sample, 195 mg. reticulocyte RNA from a previous BC column (see text and Fig. 6) dissolved in 48 ml. 0.01 M NaAc, 0.001 M Na_4EDTA, pH 5, was diluted 1/10 with the low concentration pH 3.5 buffer (see figure) and applied to a 12.5 $cm.^2$ x 4.5 cm. BC column which had been equilibrated with low concentration pH 3.5 buffer. It was eluted by a linear concentration gradient formed by a two-chambered gradient forming device, 100 ml. per chamber, at a flow rate of 1 to 2 ml./min. Optical absorbance at 2600 Å was continuously monitored by a Gilford Absorbance Monitor.

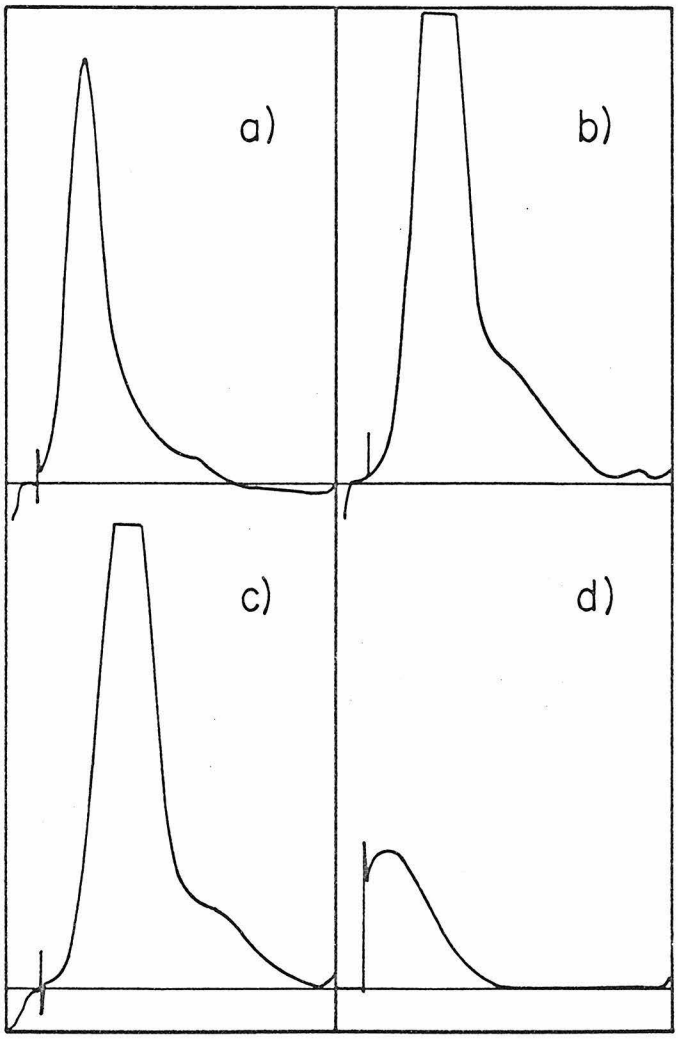

Figure 9

Figure 9. Analytical band velocity sedimentation of reticulocyte RNA.

(a), (b), and (c) Final "m"RNA fraction from pH 3.5 system BC chromatography: 2.9 μg. RNA was centrifuged through 0.1 M NaCl, 0.001 M Na_4EDTA, 90% (v/v) D_2O, pH 7.2 (nominal) at 20° C. The output traces from the Photoelectric Scanning System of the Spinco model E analytical ultracentrifuge, operating at 2650 Å, were made (a) 36 minutes, (b) 68 minutes, and (c) 88 minutes after reaching the operating speed of 50,740 rpm.

(d) Final "m"RNA fraction treated with RNase: 2.6 μg. RNA which had been treated for 20 minutes at 37° C. with 1 μg./ml. bovine pancreatic RNase (RNA concentration 185 μg./ml.) was centrifuged through 0.1 M NaCl, 0.001 M Na_4EDTA, 90% (v/v) D_2O, pH 7.2 (nominal) at 20° C. The output trace from the Photoelectric Scanning System of the Spinco model E analytical ultracentrifuge, operating at 2650 Å, was made 108 minutes after reaching the operating speed of 52,640 rpm.

The same results were obtained by band velocity sedimentation in 99% DMSO (Fig. 6c and d). Treatment with RNase degraded all components (see Fig. 9d). About 90% of the RNA was sRNA, sedimenting (in 90% D_2O) as a single peak with a sedimentation coefficient ($S_{20,w}$) of 4.8 ± 0.5S. A tiny fraction of the RNA sedimented as a discrete component at 18.6 ± 0.3S. The remainder, about 10% of the total, sedimented broadly between these two components. The midpoint of this shoulder, as estimated by eye, moved with a sedimentation coefficient ($S_{20,w}$) of 8.4 ± 0.4S.

The distribution of components in this RNA preparation was as follows. (The figures are derived from some small-scale chromatograms which were performed before the large-scale fractionation.) Of the initial preparation, 26% was low molecular weight material which eluted before application of the first (pH 7.5) gradient of the pH 7.5 column. The sRNA and $rRNA_I$ peaks contained 6.2% and 51% of the applied sample, respectively. The $rRNA_{II}$ peak contained 0.74%. Of the material applied to the pH 7.5 column, 84% was recovered; most of the 16% loss probably remained bound to the resin, although some was undoubtedly lost during precipitation and handling steps. When the combined $rRNA_I$ and $rRNA_{II}$ peaks, representing 52% of the starting material, were fractionated by BC chromatography entirely at pH 3.5, 1.7% of the RNA (calculated on the basis of the sample applied to the first, pH 7.5 column) was eluted. Since it

was estimated that only about 10% of this final preparation was contained in the broadly-sedimenting 8 S material, the overall yield of this component, based on the amount of RNA applied to the pH 7.5 column, was about 0.2%.

DISCUSSION

The requirement for urea in order to elute reticulocyte rRNA during BC chromatography at pH 7.5 indicates that there is a structural difference between this rRNA and that from E. coli. It is not clear what this structural difference might be since the effect of urea in BC chromatography is not understood. Presumably the urea is acting to destabilize the bonds formed between the rRNA molecules and the resin. This is consistent with the observation that sRNA is eluted at slightly lower salt concentrations in the presence of urea. The higher NH_4Cl concentration required for elution of $rRNA_{II}$ also suggests that the structure of reticulocyte rRNA results in more tenacious binding to BC than in the case of E. coli. However, the failure to elute both reticulocyte and E. coli rRNA when chromatographed entirely at pH 3.5 in urea remains unexplained.

As can be seen from the sedimentation patterns, the 8 S material in the final RNA fraction is very heterogeneous, as was the case in the experiments described in Part I. It again probably represents a degradation product of rRNA

rather than mRNA. While it is felt that the experimental procedures, including chromatography in 8 M urea solutions, do not cause degradation (the absence of any change in mass ratio of rRNA components supports this view), degradation may occur intracellularly. The reticulocyte is, after all, a moribund cell, as far as nucleic acid metabolism is concerned. Eventually it loses all RNA and becomes a megalocytic erythrocyte (65). It is reasonable to suppose that nucleases are involved in this process (81). There are known to be nucleolytic "hot spots" along the length of reticulocyte rRNA molecules (82). Fragments of discrete size might be produced intracellularly during the catabolism of ribosomes. A certain fraction of such material, which might happen to be around 8 S in size, might have properties such that it would be strongly bound to BC.

If this be the case, then where is the globin mRNA? The conditions of phenol extraction may have been such that it was not recovered in the aqueous phase in the first place. While in the case of E. coli there do not appear to be any gross changes in the distribution of individual mRNA species during chromatography, the failure of a particular mRNA to elute from the column (or to bind to it at all) would not have been detected. The globin mRNAs might be such molecules. Then again, the assumption that eukaryotic and prokaryotic mRNAs have similar properties may be incorrect, although the universality of mRNA function (83,84)

makes this unlikely.

ADDENDUM

Two reports have appeared since the writing of this thesis which bear upon it. They are "Synthesis of Rabbit Globin by a Bacterial Cell Free System," by D. G. Laycock and J. A. Hunt, Nature, 221, 1118 (1969), and "Isolation of an RNA with the Properties of Haemoglobin Messenger," by F. Labrie, Nature, 221, 1217 (1969).

In the report by Laycock and Hunt, a minor RNA fraction (1% of total RNA) was isolated by means of a novel salt fractionation procedure. This fraction contained roughly equal amounts of two components sedimenting in sucrose gradients at approximately 8 S and 14 S. The former was found to be located mainly in polyribosomes, the latter in monosomes. Both components had base compositions more similar to that of rabbit DNA than to those of rabbit tRNA or rRNA.

Both fractions were tested in a modified E. coli cell-free system for their ability to stimulate amino acid incorporation. In the standard system, neither component produced significant stimulation. However, if all twenty N-acetyl-aminoacyl-tRNAs were added to the cell-free system, significant stimulation was observed. It had earlier been observed by Lucas-Lenard and Lipmann (Lucas-Lenard, J., and Lipmann, F., Proc. Nat. Acad. Sci. Wash., 57, 1056 1967) that N-acetylphenylalanyl-tRNA acted as an initiator for poly-U-directed polyphenylalanine synthesis in the E. coli cell-free system. Laycock and Hunt considered the failure of the E. coli system to respond to added reticulocyte RNA

to be due to a failure of initiation, which could be overcome, as in the poly-U-directed case, by addition of N-acetyl-aminoacyl-tRNA. They further found that N-acetyl-valyl-tRNA alone was fully effective with the 8 S RNA component. Valine is the N-terminal amino acid of both rabbit globin chains. N-acetyl-phenylalanyl-tRNA was completely ineffective with 8 S RNA, and both the valyl and phenylalanyl derivatives were ineffective with the 14 S RNA component.

The radioactive product of stimulation by 8 S RNA was characterized by chromatography on DEAE cellulose and Sephadex columns after adding either rabbit globin or reticulocyte lysate as carrier. The radioactivity co-chromatographed with the authentic globin in the former case, and largely with hemoglobin in the latter. Furthermore, if, in the former case, hemin was also added, not only did a large percentage of the globin elute at a new position characteristic of hemoglobin, but approximately the same percentage of the radioactive cell-free product did so as well. Control RNA preparations from liver produced completely different patterns of stimulation.

The radioactive cell-free product was also characterized by chromatographic separation of tryptic peptides (after admixture with carrier rabbit globin). The radioactivity and ninhydrin-positive profiles were largely coincident when 8 S RNA had been used to program the system, but did not match when the RNA came from liver.

The second paper, by Labrie, reports the isolation of a minor reticulocyte RNA component which satisfied some of the properties predicted for globin mRNA. It was isolated by collecting the RNA sedimenting in the 4 to 18 S region of sucrose gradients (method of Chantrenne and coworkers) and effecting further fractionation by acrylamide gel electrophoresis. Seven minor components, with estimated sedimentation coefficients ranging from 7 S to 15 S, were detected in this way. The rate of labeling of the original sucrose gradient fraction was somewhat greater than the rate of labeling of rRNA (confirming the results reported by Chantrenne's laboratory and in this thesis). A particular minor component, designated 10 S, with an estimated molecular weight of 1.9×10^5 daltons, became labeled more rapidly than the others. This 10 S RNA was also more sensitive to degradation than the others. Nucleotide fingerprints indicated that the 10 S RNA was not a degradation product of rRNA.

The work reported by Laycock and Hunt establishes, for the first time, a reliable assay for globin mRNA function, thus permitting further research in this area to proceed with confidence. The demonstration by Labrie that the 4 S to 18 S region of sucrose gradients of reticulocyte RNA contains numerous minor components with various S values probably explains the broad band velocity sedimentation patterns observed in Part I of this thesis. If such minor

RNA components bound strongly to benzoylated DEAE-cellulose, the similar results in Part III might be explained.

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