NSG-479

SPECIFIC NUCLEOSIDE TRIPHOSPHATASES IN CRUDE EXTRACTS OF ESCHERICHIA COLI

GPO PRICE $ _____

CFSTI PRICE(S) $ _____

Hard copy (HC) 2.00

Microfiche (MF) .50

N65-29387 (ACCESSION NUMBER) 30 (PAGES) CR 59104 (NASA CR OR TMX OR AD NUMBER) 1 (THRU) 04 (CATEGORY)

FACILITY FORM 602

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SUMMARY

1. The distribution and general properties of not previously reported triphosphatases in crude extracts of Escherichia coli have been studied.

2. All four triphosphates are hydrolyzed by the extracts. The rate of release of inorganic phosphates is highest from ATP, followed by CTP, GTP and UTP, in that order.

3. Eighty to ninety percent of the total enzyme activities are sedimentable in a sucrose density gradient. The specific activity (per mg protein) is generally highest in the polysome fraction.

4. The enzymes can be released from the polysomes by treatment with ribonuclease in the cold.

5. The overall as well as the tube by tube distribution of each activity is affected in a different and characteristic way, independent of the others, by variations of pH and salt concentration in the extraction buffer or the gradient, showing that four triphosphatases are involved.

6. The enzymes have an alkaline pH optimum, require Mg^{++} for maximum activity, are not inhibited by fluoride and azide, and are not stimulated by dinitrophenol, Na^{+}, K^{+}, or amino acids.

7. Correlation of the peaks of enzyme activity for the four substrates throughout the gradient showed that they do not coincide with each other. The CTPase is definitely not bound to the same macromolecular component as the ATPase and GTPase activities.

INTRODUCTION

In the preceding paper (1) it was shown that in crude extracts of E. coli, as well as in isolated ribosomes, natural polysomes had a high protein content compared to single ribosomes, and that this excess protein was released to the supernatant when polysomes were destroyed. It was further shown that the RNA to protein ratio profile showed well-defined and periodically-spaced peaks, indicating the presence of a series of partially overlapping components differing in their relative RNA and protein content. It seemed of interest to investigate further the nature of this "extra" protein by determining whether or not it had enzymatic activity and, more specifically, to see whether it represented the adsorption of some specific enzymes, rather than of a large spectrum of enzymes, from the supernatant.

The choice of the kind of enzymatic activities to analyze first was guided largely by considering which enzymes would be likely to be pertinent to the function of the structures richest in this "extra" protein, namely the polyribosomes. The first to come to mind were the amino acid activating and "transfer" enzymes, but because of the difficulty experienced by early workers in the field of amino acid incorporation in washing the ribosomes completely free of these activities (2), it was assumed that these enzymes were probably present. Others were enzymes which might be indirectly concerned with protein synthesis, by controlling the supply of energy,

for example, or which might be downright detrimental, such as the phosphodiesterase credited with destroying mRNA (3). A preliminary investigation showed that the latter activity was present overwhelmingly in the supernatant, but that the ribosomes were rich in nucleoside triphosphatase activity.

It is well-established that ribosomal amino acid incorporating systems in general, and that from E. coli in particular, require a triphosphate-regenerating system for good activity. Although various phosphate donors and kinases have been used, they all function by addition of phosphate to the nucleoside diphosphate. The amino acid activating reaction, however, presumably the greatest user of ATP, does not yield ADP, but AMP, which cannot be used by the regenerating system (4). The implication is that in all ribosomal systems there takes place a reaction which rapidly destroys ATP to give not AMP, as the activation reaction, but ADP, which can be regenerated into ATP.

Despite this implication, however, there has been very little explicit mention of the presence of such triphosphatase activity in ribosomal systems. Webster reported the presence of ATPase and GTPase activities in pea seedling ribosomes (5), but did not study either the distribution or detailed properties of these enzyme(s). Furthermore there is GTPase activity associated with the so-called transfer enzyme from various sources (2, 5-7), which is known to be associated with crude ribosomes, as well as being found in the soluble fraction (2).

There are numerous reports on di- and triphosphatase activities in microsomes of animal origin (8-12), but most of these activities seem to be localized in the membranes of the endoplasmic reticulum, rather than

on the ribosomes. Moreover, as will be shown in other publications, the properties of these enzymes are very different from the ribosomal triphosphatases studied in this laboratory, the most general difference being that the former seem to be inhibited by KF and/or azide, whereas the latter are not affected by either agent.

The only bacterial triphosphatase studied in any detail seems to be an ATPase in the membranes of Streptococcus faecalis (13).

In Escherichia coli, apart from the above mentioned GTPase activity (2) there seems to be no information on nucleoside triphosphatases. The purpose of the present investigation was to study in a detailed fashion the distribution of these enzymes in soluble and particulate fractions of crude extracts separated by means of sucrose density gradient centrifugation.

Partial reports on the distribution and behavior of these enzymes in crude extracts of E. coli have appeared (14, 15).

METHODS AND MATERIALS

The methods and buffers used in the preparation of crude extracts and ribosomes of E. coli B, sucrose density gradient (SDG) centrifugations, and the analyses for RNA and protein were as described in the preceding paper (1).

Phosphate Analyses:

Inorganic phosphate was determined by the following modification of the method of Delsal and Manhouri (16).

Reagents:

Molybdate-acetate: 43 parts of acetate buffer (45 g sodium acetate .3 H_2O in 1 liter 2 N acetic acid, Baker and Adamson, A.R.); 1 part 37 %

formaldehyde (Mallinckrodt, A.R.) and 5 parts of 5 % ammonium molybdate .4 H_2O (Mallinckrodt, A.R.). A fresh mixture is prepared daily from stock solutions stored in the cold.

Stannous chloride: 5.935 g $SnCl_2$.2 H_2O (Matheson, Coleman and Bell, A.R.) dissolved in 25 ml of concentrated HCl (Baker and Adamson, A.R.). This stock solution can be kept in the refrigerator for one month. It is diluted 1:100 with distilled water just before use.

Procedure:

1. Add 4.0 ml of ice-cold molybdate-acetate reagent to the phosphate-containing sample (0.2 ml) and mix rapidly in a Vortex mixer.

2. Add 0.2 ml of ice-cold stannous chloride exactly 90 seconds later, and again mix in the Vortex mixer.

3. Determine the optical density at 735 mμ in a Zeiss PMQ II spectrophotometer exactly 3 minutes after the addition of the stannous chloride. Readings are taken against distilled water.

4. Blanks of distilled water, buffer, or other appropriate solutions are taken through the same procedure and subtracted from the O.D. reading of the sample.

Under these conditions the standard curve is linear throughout, with a slope of 0.256 μmoles of P_i per O.D. unit. The standard curve can be hit with an accuracy of not less than 3 % with a single determination, and within 1 % with duplicates, provided certain precautions, described below, are taken.

Because of the sensitivity of the method, the phosphate content of the reagents has to be rigorously controlled. It was also found that ordinary distilled or de-ionized water has a variable phosphate content,

leading to spurious results. Glass-bi-distilled water is therefore used for the preparation of all reagents, sucrose and buffers for the gradient, as well as the incubation mixtures. It is also essential that glassware be scrupulously clean. Tubes scrubbed with Haemosol, thoroughly rinsed with ordinary distilled water, then once with bi-distilled water, drained and dried upside down in an oven, were satisfactory; acid-washed tubes gave erratic results. Under these conditions, a distilled water blank should read less than 0.1 O.D. units, and be reproducible to within ± 0.005 O.D. units.

For reliable results, it is essential that all operations be rigorously timed, especially between addition of the stannous chloride and the optical density readings. The optical density changes continuously, but under our conditions the changes were found to be minimal between 2.5 and 3.5 minutes after initiation of color development.

While most of the standard methods for phosphate determination were tried, the present method was found to be the most satisfactory for meeting the requirements for the present investigation, for the following reasons: The use of a molybdate-acetate reagent instead of the usual molybdate-HCl, prevents the precipitation of protein, eliminating time-consuming and error-introducing deproteinization with TCA or perchloric acid prior to phosphate determination. (A slight turbidity is sometimes observed when the sample contains more than 100 μg of protein, but this can be adequately corrected by taking the enzyme alone through the same procedure.) The use of the higher pH, as well as ice-cold reagents and short contact times prevents non-specific hydrolysis of labile phosphates and eliminates the necessity for extractions with organic

solvents. With manipulations thus reduced to a minimum, the method is not only extremely fast, but also highly reproducible. It covers the whole optical density range of the spectrophotometer, eliminating repeated dilutions of unknown samples. It is very sensitive, since as little as 10 mμmoles of inorganic phosphate (0.3 μg P) can be determined with an accuracy of 10 %, with the above-mentioned accuracy of 1-3 % prevailing for samples containing 1 μg P or more. Furthermore, with some sacrifice in speed and convenience, but little in accuracy, the sensitivity can be increased five or ten-fold, by cutting all volumes by a corresponding factor and reading the optical density in micro-cuvettes.

Enzyme Assays:

Nucleoside phosphatase activities were determined by measuring the release of inorganic phosphate from the appropriate substrates.

The incubation mixture contained 0.05 M Tris - HCl buffer, pH 8.2, 5 mM $MgCl_2$, 5 mM KF, and one of the four ribonucleoside triphosphates at the following concentrations: ATP, 2 mM, and GTP, UTP, and CTP, 1 mM.

ATP was purchased from the California Biochemical Corporation, while GTP, UTP and CTP were from Pabst. The substrates were dissolved and neutralized with NaOH to pH 7.5 in large batches, and stored in small aliquots in the freezer. Under these conditions, there was no change in inorganic phosphate content during several months; there was, however, a substantial increase in P_i if the substrates were stored in the unfrozen state, or if the incubation mixtures were prepared on the previous day and stored in the cold. Substrates were discarded if the P_i blank approached 10 % of the nucleotide concentration.

For the assay, 0.1 ml of double-strength incubation mixture was pipetted into chilled tubes. At zero time 0.1 ml of the ice-cold SDG fractions was added, and the tubes incubated at 28 - 29° C for 10 or 20 minutes, as indicated. (The Mg concentration of the incubation mixtures was adjusted to take into account the Mg content of the SDG, but the 5 mM Tris buffer contained in the gradient was ignored.) The final pH of the incubation mixture, after addition of enzyme, was usually 8.1.

At the end of the incubation, the reaction was stopped by adding the ice-cold molybdate-acetate reagent, and proceeding with the phosphate determination as described above. Appropriate blanks were taken through the same procedure.

When a large number of assays has to be performed, it is convenient to set up the operations on a production line basis, with three operators. Operator 1 mixes the enzymes with the substrate in the cold, and starts the incubations on the half-minute, at the rate of one tube per minute. Operator 2 removes the tubes from the water bath, adds the molybdate-acetate reagent, and 90 seconds later, the stannous chloride; the first operation is performed every minute on the half-minute, and the second, every minute on the minute. Operator 3 determines the optical density, again reading one tube every minute, exactly on the minute. In this way, 200 assays can be run comfortably in one day, but only half that many by two people.

Because of the very large number of assays involved in the present investigation, it became impractical to run assays in duplicate as a matter of routine. Elaborate control experiments were therefore performed

to determine the reliability of a single assay under our conditions. Since an assay is susceptible to more errors than a simple analytical determination, the controls were designed to test the following sources of errors: variability of enzyme action and contribution to the blanks of the sucrose gradient and of the material (ribosomes or crude extract).

In the first test, duplicate assays were run all along a gradient (40 tubes). The average deviation among duplicates was 0.005 O.D. units and the standard error ± 0.003 O.D. units.

In the second test a blank SDG was prepared and centrifuged as usual. Single phosphate determinations were then made on every tube, and the deviations of these single determinations from the average were determined. The average deviation was ± 0.005 O.D. units, and the maximum deviation was 0.015. Hence, there is no contribution to the O.D. by the sucrose in the gradient.

In the third test, single tube by tube determinations were made on SDG-separated, purified ribosomes and crude extracts, respectively, without the addition of substrate. In the case of ribosomes, the standard deviation from the mean was only ± 0.005 O.D. units, which is very little higher than that of the gradient alone. In the analyses of ribosomes, no enzyme blanks were therefore run, and the reliability of the assay is ± 0.015 O.D. units, corresponding to ± 4 mμmoles per sample, or ± 0.04 μmoles per ml of gradient fraction.

In the case of crude extracts, the contribution by the material was very appreciable, so that tube by tube enzyme blanks were determined routinely. Since the reagents contribute equally to the enzyme and substrate blanks, the total blank was calculated by adding the difference

between the substrate and reagent blanks (measuring the contribution of the substrate alone) to the enzyme blank. In the absence of enzyme activity, the total blank thus calculated agreed within the limits of error of the method with the incubated gradient fractions.

RESULTS

Assays for triphosphatase activity on a number of extracts separated by SDG centrifugation showed that a considerable amount of activity is present in all fractions of the gradient. The hydrolysis of each of the four ribonucleoside triphosphates (XTPs) follows a distinct and characteristic pattern, independent of the distribution of activities towards the other XTPs and each activity sediments in a series of well-defined peaks (see also (15)).

As is the case for the distribution of RNA and protein, described in the preceding paper (1), there is a certain amount of variability in the distribution of the enzymes from extract to extract. There is also, however, a large amount of consistency in the patterns of distribution, which vary in a characteristic fashion with such factors as the pH of the extracts, and the presence or absence of KCl.

Influence of pH:

The distributions of triphosphatase activities in two different crude extracts, prepared and centrifuged in 0.01 M Mg acetate and 0.005 M Tris buffer at pH's of 8.1 and 7.4, as well as the corresponding patterns for the specific activities (μmoles P_i liberated per mg protein), are shown in Figs. 1 and 2, respectively. Despite minor variations, the patterns are rather similar, both in total as well as in specific activities.

In the preceding paper (1) it was shown that at a pH of 7.1, the amount of protein in the heavy region of the gradient is larger than at 8.1. Nevertheless, there is relatively more enzyme at the more alkaline pH, as can be seen from Fig. 3, which shows a comparison of total and specific activity patterns for the same extract at the two pH's. While the total activities are practically identical, the specific activities are higher at pH 8.1. This is also seen in Table I. At pH 7.4, on the other hand, the specific activities of enzymes in the polysome fractions are the same as those at pH 7.1, even though there is more protein at the lower pH. This means that at pH 7.4, triphosphatases are displaced along with other proteins. These data show that the "extra" protein is not homogeneous, and that differential displacement of certain proteins can occur with relatively minor changes in conditions, such as an increase in pH from 7.4 to 8.1.

Influence of KCl:

In the previous paper (1) it was shown that the addition of KCl to the extracts had a much more pronounced effect on the relative distribution of RNA and protein than had a change in pH. The patterns of triphosphatase distribution in the extracts were, similarly, markedly influenced by the addition of salt.

In order to visualize the overall changes in distribution of the enzymes, the gradient was divided into four fractions, and activities were averaged over each fraction. (Alternatively, the tubes in each fraction were pooled prior to assays and analytical determinations; the results were the same.) Fraction I (roughly the first 17 tubes of the gradient) comprised polysomes and part of the 100s peak; Fraction II (the next 4-5 ml)

comprised the main peak, i.e., 70-100s particles, fraction III (again 4-5 ml) is the light particle fraction, corresponding to 20-50s; and, finally, fraction IV, the last 2-3 ml on top of the gradient, is the supernatant.

Fig. 4 shows summaries of the effect of KCl on the distribution of the four enzymes. Each curve represents the average of 2-4 experiments. The distribution of RNA and protein are included for reference purposes. It is seen that the distribution of the enzymes does not follow that of total protein; with one exception, 80-90 % of the total activity being sedimentable. Furthermore, the distribution of each enzyme activity follows its own characteristic pattern, and is affected by KCl in its own way. In general, except for ATPase, the presence of KCl lowers the percentage of enzyme bound to the polysome fraction, while at the same time (with the exception of UTPase) increasing the proportion of enzyme bound to sedimentable particles.

A summary of specific activities (per mg protein) for the four enzymes in the presence and absence of KCl is given in Fig. 5. It is seen that, with only one exception (CTPase in Mg-Tris buffer), the specific activities are highest in the polysome fraction, and always very low in the supernatant. In addition to causing shifts in the relative activities of the different fractions, however, there was some inhibition of total activity in the presence of KCl; thus, the average specific activities (in mμmoles P_i per mg protein per min) of crude extracts in the absence of KCl were 32.5 for ATP, 28 for GTP, 27 for CTP, and 17 for UTP, while in the presence of KCl the corresponding values were 31, 12, 21, and 8.

In addition to changes in the average distribution and specific activities of the enzymes among the four fractions, the inclusion of salt

also causes characteristic changes in the detailed patterns of enzyme activity along the gradient. Typical patterns for the distribution of total and specific activities for the four enzymes in Tris-Mg-KCl buffer are given in Fig. 6. These should be compared with Figs. 1 and 2. Particularly noteworthy is the peak sharpening effect of KCl. This is well illustrated in Fig. 7 which shows an ATPase assay of the same extract centrifuged in the presence and the absence of KCl. The same figure also illustrates the inhibiting effect of KCl on the UTPase activity (see above), particularly that in the polysome region.

From these data it is evident that the addition of KCl to crude extracts of E. coli produces very complex effects, of which the redistribution of the four triphosphatase activities may be only a secondary manifestation.

Effects of Ribo- and Deoxyribonuclease:

As shown in a previous publication (1), treatment of crude extracts with very small amounts of ribonuclease in the cold caused a displacement of ATPase from the heavy components to the lighter ones, indicating that the enzymes were bound to the heavy structures by a labile RNA. Fig. 8 shows a summary of the effect of RNAase on the distribution of RNA, protein, ATPase and CTPase of one extract. Treatment with the enzyme caused a reduction of the specific activity (mμmoles P_i/mg protein/min) of the polysome fraction of ATPase from 126 to 76, and of CTPase from 45 to 3.

Treatment of extracts with DNAase has no obvious effect on the distribution of enzymes. The point was not investigated in detail, however, and extracts were routinely treated with this enzyme, since it has been shown that oligodeoxynucleotides can bind to ribosomes (17) and that DNA can form polysome-like structures (18, 19).

General Properties of the Triphosphatases:

Although a detailed account of the properties of the triphosphatases is not in order here and will be given in a separate publication, a few remarks are necessary to substantiate the claims made in this paper.

The discussion on the assay procedure showed that the inorganic phosphate liberated from the substrates can be determined with a high degree of accuracy and that the amount of P_i liberated under a given set of conditions is reproducible. For the assay to be quantitatively valid, however, it is also necessary that the amount of product formed be proportional to the rate of the enzyme reaction; i.e., for a given amount of enzyme the reaction should be linear with time, and for the chosen time it should be proportional to enzyme concentration. Since in the present case, the amount of protein per assay tube varies over a range of a hundredfold along the gradient, it could conceivably be difficult to satisfy these criteria by using one set of standard conditions throughout. It could further be possible that the requirements for maximum activity of the particulate and soluble enzymes might be different. These points were investigated by using pooled enzyme fractions I-IV (see above).

It was found that all fractions had the same requirements. Unlike most XTPases described in the literature, they were unaffected by fluoride, azide, and dinitrophenol. They were completely inactive in the presence of EDTA, and required a divalent metal for activity; while Mg^{++} was most effective, Mn^{++} and Ca^{++} could also serve as activators. Within a five-fold range (2-10 mM) the concentration of Mg^{++} was not critical. Na^{+} and K^{+} have generally little effect, although the UTPase is markedly inhibited by K^{+}. The enzymes showed a broad pH optimum between 7.5 and 8.5.

All enzymes showed complex kinetics with a marked dependence on substrate concentration. Lineweaver-Burk plots show minima corresponding to optimum substrate concentrations; but although these are a function of enzyme concentration, for the relative activities encountered in the gradient, they are close to the substrate concentrations used in the assay, i.e., 2 mM for ATP, and 1 mM for GTP, UTP and CTP.

The enzymes are quite stable when stored in the frozen state, the most labile one being the ATPase on the polysomes.

The activity of the enzymes is somewhat enhanced by dialysis, the enhancement being more pronounced in the supernatant than in the heavier fractions. From kinetic studies it appears that some inhibitor(s) is present in crude extract, which means that there is an apparent decline in activity with increasing concentration of enzyme. Again, this is more pronounced in the top part of the gradient. In the range of activities encountered, the reaction rate was practically linear up to 10 minutes but was 10-20 % lower at 20 minutes.

The effect of these various factors on the distribution of enzyme, under unfavorable circumstances (poor resolution of the gradient, shallow peaks) can be seen in Fig. 9. This shows an assay for ATPase with 0.1 ml fraction, compared with an assay with only 0.05 ml, run three weeks later. While there is a slight inactivation of the enzyme in the heavy fractions and some apparent increase in activity in the lighter fractions assayed at the lower concentration, the errors are relatively minor and do not alter in any way the picture of enzyme distribution.

Correlation of Different Enzyme Peaks:

Since in most cases the four enzyme activities appear in well-defined peaks, one might ask whether or not the location of these peaks coincides for all activities. Furthermore, since the RNA to protein ratio profile

(see preceding paper (1)) shows peaks and valleys corresponding to two kinds of qualitatively different components, one might inquire about the relationship of these with the peaks of enzyme activities.

Because of the complexity of the patterns and the close spacings of the peaks, it is not possible to obtain an unequivocal answer to these questions through simple inspection of a few diagrams. The crudeness of the fractionation affects the apparent resolution not only by pooling two or more enzyme peaks which are less than 1 ml apart, but also by pooling parts of peaks and valleys, thereby shifting the apparent maxima and minima, and flattening or even obliterating peaks. This in turn means small absolute differences between peaks and valleys, so that a relatively small experimental error could conceivably shift the apparent position of the maximum.

In order to eliminate these uncertainties as much as possible, the enzyme peaks from all gradients, regardless of conditions and resolution, were correlated with each other and with the peaks of RNA and protein. The results, given in Table II, show that all enzymes are definitely not carried on the same macromolecular components. While the peaks of ATPase and GTPase activities coincide in most cases, and are also in the position of the protein-rich components (valleys in the RNA to protein ratio profiles), the peaks of CTPase activity always correspond to the RNA peaks and are almost never to be found in the same tubes as the ATPase and GTPase activities. UTPase seems to be carried on both types of components.

DISCUSSION

The highly individualistic distribution patterns of the four XTPase activities, demonstrated both in the tube by tube assays (Figs. 1-3, 6, 7), as well as in assays of pooled fractions (Figs. 4, 5, 8), show that we are dealing with four individual enzymes rather than with one non-specific

triphosphatase. This fact is brought out even more strongly in the following paper on the fate of the different XTPases during purification and reconstitution of the ribosomal system (20). It constitutes the first clear demonstration in the literature for the existence of specific phosphatases for all four ribonucleoside triphosphates.

The enzymes are referred to as triphosphatases, because so far it has not been possible to demonstrate any cofactor requirement. Specifically, repeated efforts to demonstrate any stimulation of triphosphatase activity by amino acids have been entirely unsuccessful. Despite some resemblances, the main portion, at least, of our enzymatic activities is therefore not due to the nucleoside triphosphate-dependent peptide synthetases or "amino acid incorporating enzymes" which liberate inorganic phosphate from all four triphosphates in the presence of amino acids and RNA, purified from Alcaligenes faecalis by Beljanski (21), and whose presence in E. coli have been reported by Nisman (22). The insensitivity of our enzymes to fluoride and azide, as well as their lack of stimulation by either Na^+ or K^+, further distinguishes them from the membrane-bound ATPases associated with cation transport, which also show hydrolytic activity towards all four triphosphates (8, 9, 11, 12, 23).

The lack of any demonstrable cofactor requirement so far does by no means rule out the possibility that such does, in fact, exist, and that hydrolysis of triphosphates represents a degenerate activity of these enzymes. Many phosphoryl transferases exhibit such degenerate hydrolytic activity, which is generally increased by denaturation or "aging" phenomena, such as the oxidation of SH groups. Examples of this type are the latent ATPase of mitochondria, which is "unmasked" by aging (24, 25), and glyceraldehyde-3-P dehydrogenase (26), as well as the triphosphatases of the ion transport

systems (27). In the latter cases there is a great preponderance of hydrolysis over transport, thought to be due to the "uncoupling" during isolation of multi-enzyme systems which are tightly integrated in vivo. It is probable that part, at least, of the GTPase activity in our extracts is identical to that associated with the transfer of amino acids from sRNA to ribosomes (2), which recently has been reported to be more specifically concerned with the binding of sRNA to ribosomes (28, 29), and in which case also hydrolysis is at least 50 times greater than the binding (29).

Since the triphosphatase activities are assayed in crude extracts, it is quite likely that many enzymes may contribute to the observed activity, since any enzyme using a triphosphate as a cofactor in such a way as to lead to the liberation of inorganic phosphate is, operationally speaking, a triphosphatase. However, the clean separation of the various activities during purification of the ribosomes, as reported in the following paper (20) suggests a relatively great homogeneity of each activity. This illusion of homogeneity is undoubtedly due to the very high specific activity of these enzymes. The most active polysome fractions, for example, hydrolyze as much as 1 μmole of ATP per minute per mg total protein, or at least 2 μmoles per mg "extra" protein. The activity of crystalline alkaline phosphatase of E. coli, towards ATP is about 20 μmoles per min per mg protein (30), but the amount of this enzyme associated with ribosomes of fully derepressed cultures was reported to be a mere 0.02 μmole per min per mg RNA (31). (Our cultures were grown in the presence of phosphate, and no alkaline phosphatase activity could be demonstrated by the method of Garen and Levinthal (32) with ten times the protein concentration used in the triphosphatase assays.) The activity of other enzymes known to be present in ribosomes, and which could conceivably contribute to the amount of inorganic phosphate liberated, is similarly negligible. The activity of

polynucleotide phosphorylase, for example, which could liberate phosphate from the diphosphates produced by true triphosphatases seems to be of the order of 0.1 mμmole per min (33). Furthermore, pyrophosphate was not hydrolyzed, and no C^{14}-ATP was incorporated into any acid-insoluble product under our conditions. This effectively rules out the possible indirect contribution to our assays of the enzymes of RNA metabolism and amino acid activation, which cause a pyrophosporolytic split of ATP.

The overall distribution of these enzymes in the crude extract is worthy of note, since 80 % or more of the total activity is sedimentable. In some animal cells a similarly high percentage of the total ATPase activity is sedimentable (11), and is located almost entirely in the microsomal membranes. This case differs from ours, however, in that the ATPase could not be solubilized by RNAase (11). The lability of the enzyme-ribosome bond to pancreatic ribonuclease as well as nucleases in the crude E. coli extract (15) is a unique feature of the E. coli triphosphatases, and makes their case different from that of E. coli ribonuclease, which is also found entirely in the ribosomal fractions (34), but which remains tightly bound to the 30s ribosomes throughout the purification of these particles (34, 35).

Since the proportion of triphosphatases which are sedimentable seems to be directly proportional to the care with which a given extract has been prepared, it seems likely that in vivo these enzymes are entirely particle-bound. In that sense, they can be termed ribosomal enzymes. They are not ribosomal enzymes, however, in the usual sense which requires that they be part of the structural proteins of the ribosomes. However, with the recent casting of doubt by Neu and Heppel (36) on the ribosomal localization of the E. coli ribonuclease, which up to now had been considered the only truly ribosomal enzyme, the whole concept of enzymes as

part of the basic ribonucleoprotein matrix of ribosomes appears suspect. A better criterion for defining a ribosomal enzyme might be on the basis of ribosomal function, regardless of the strength of the enzyme-ribosome bond. The idea that such functional associations may occur through specific RNA "adaptors" is an attractive one, but whether or not our triphosphatases are ribosomal enzymes by the functional test, remains to be seen.

The data presented in this paper not only reflect on the separateness of the four triphosphatases, but also strongly corroborate the conclusions reached in the preceding paper (1) on the heterogeneity of ribosomes. The very pronounced peaks in the distribution of specific enzyme activity along the gradient (Figs. 1 and 3) show that not all particles bind a given enzyme, and the correlation data presented in Table II show that there are at least two qualitatively different components, only one of which binds CTPase. One might say that one has a kind of chromatography in reverse, in which the ability for specific binding of certain enzymes is used to discern a heterogeneity in the adsorbant.

This clear demonstration of the qualitative heterogeneity of sedimentable ribosomal species, however, is not necessarily a demonstration of the intrinsic heterogeneity of ribosomes; i.e., of the so-called ribosomal RNAs and structural proteins. Adsorption is a surface phenomenon, and hence alteration of the ribosomal surface could conceivably alter its specificity. Whatever the cause, however, the recognition that qualitative differences can and do exist between ribosomal species which are very similar by gross chemical and physicochemical criteria, is of great importance.

ACKNOWLEDGEMENTS

This investigation was supported by Grant No. G 19532 from the National Science Foundation and Grant No. GM-07924-02 from the National Institutes of Health. It was also aided by Grant No. NSG 479 from the National Aeronautics and Space Agency, through the Space Sciences Laboratory, University of California, Berkeley, and the courtesy of Drs. Hardin B. Jones and Thomas H. Jukes, whose interest and help I gratefully acknowledge.

I would like to thank Dr. J. Fiala for preparing the extracts and Dr. H. L. Young and the Misses Sara P. Goolsby and Estelle Cook for help with the assays.

Table I

EFFECTS OF pH ON THE DISTRIBUTION OF TRIPHOSPHATASE ACTIVITIES

		Percent of total Activity in fraction				Specific activity (mμmoles/mg/min)				
Substrate	pH	I	II	III	IV	I	II	III	IV	Total
ATP	7.1	48	22	23	7	127	43	28	10	42.5
	7.4	42	17.5	19.5	21	125	63	27	12	41
	8.1	43	23	23	12	153	44.5	25	16	41.5
GTP	7.1	-*	-	-	-	-	-	-	-	-
	7.4	38.1	15.9	22.3	23.2	64	44	25	14	28
	8.1	27.6	18	18.7	35.5	76	26	16	38	31
CTP	7.1	-	-	-	-	-	-	-	-	-
	7.4	30.2	28.1	20.3	21.6	47	73	21	12	27
	8.1	17.4	45.5	22.3	14.7	45	63	18	15	30
UTP	7.1	26	21	35	19	17	9.5	11.5	7.5	10.5
	7.4	27.5	22	34	16.5	29	22.5	18.5	6.5	15.5
	8.1	35	15	29	21	42.5	10	11.5	10	14

* Dashes mean not determined.

All extracts and gradients were 0.01 M in Mg^{++} and 0.005 M is Tris-HCl.

Table II

PERCENT POSITIVE CORRELATION OF TRIPHOSPHATASE PEAKS OVER THE WHOLE GRADIENT

Peaks:	ATPase	GTPase	UTPase	CTPase
ATPase		79 ± 17	44 ± 17	5 ± 5
GTPase			65 ± 15	0 ± 0
UTPase				22 ± 10
RNA	18 ± 9	11 ± 13	68 ± 18	100 ± 0
Protein	80 ± 6	91 ± 9	52 ± 14	10 ± 10

The percent correlation was calculated by dividing the number of coinciding peaks x 100 by the number of total peaks. It is given ± the average percent deviation.

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LEGENDS FOR FIGURES

Fig. 1. Distribution of triphosphatase activities of an E. coli extract analyzed on a SDG gradient containing 0.01 M Mg acetate and 0.005 M Tris, pH 7.4.

Total activities are plotted on the left (10 min assay) and specific activities on the right. X – X, ATPase; o...o, GTPase; ● – ●, CTPase; (UTPase values were too low in this experiment).

Fig. 2. Distribution of triphosphatase activities of an E. coli extract analyzed by SDG centrifugation in 0.01 M Mg acetate and 0.005 M Tris, pH 8.1.

Total activities are shown in the lower part (20 min assay), specific activities in the upper part of the graph. X – X, ATPase; o...o, GTPase; ● – ●, CTPase; ▲ – ▲, UTPase. The broken line shows distribution of RNA.

Fig. 3. Comparison of total (upper) and specific activities (lower) of ATPase (crosses) and UTPase (triangles) of the same extract at two different pH's.

Solid lines, pH 8.1; broken lines, pH 7.1.

Fig. 4. Overall distribution of RNA, protein, and enzymes in the presence and absence of KCl.

o – o, 0.01 M Mg acetate, 0.005 M Tris, pH 7.4; ● – ●, 0.0175 M $MgCl_2$, 0.086 M KCl, 0.005 M Tris, pH 7.4.

Fig. 5. Effect of KCl on the specific activities of four triphosphatases. Legend as in Fig. 4.

Fig. 6. Distribution patterns of total (upper) and specific (lower) activities of XTPase in Tris-KCl buffer.
Legends as in Fig. 2.

Fig. 7. Effect of KCl on the specific activity patterns of ATPase (X) and UTPase (▲) in the same extract.

Solid lines, Tris-Mg buffer; broken lines, Tris-Mg-KCl buffer.

Fig. 8. Effect of ribonuclease on the distribution of RNA, protein, ATPase and CTPase in crude extract of E. coli.

Solid lines, control; broken lines, RNAase-treated (5 μg/ml at 0°).

Fig. 9. Effect of storage and enzyme concentration on the ATPase assay.

Circles represent results obtained with 0.1 ml of gradient fractions, crosses, with 0.05 ml assayed three weeks later. The broken line in the upper diagram represents distribution of protein in the extract; on the lower, the RNA to protein ratio profile.

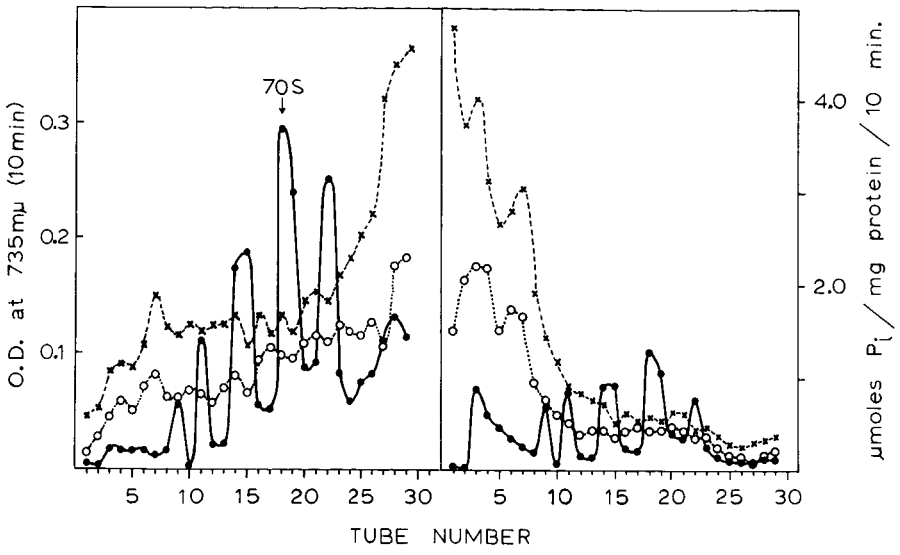


Figure 1

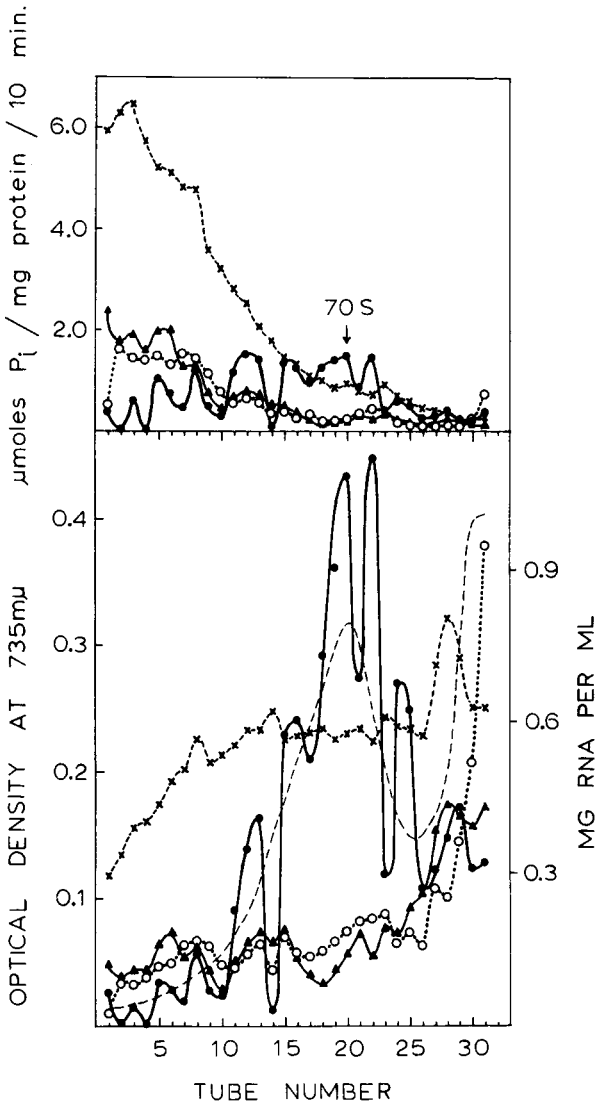
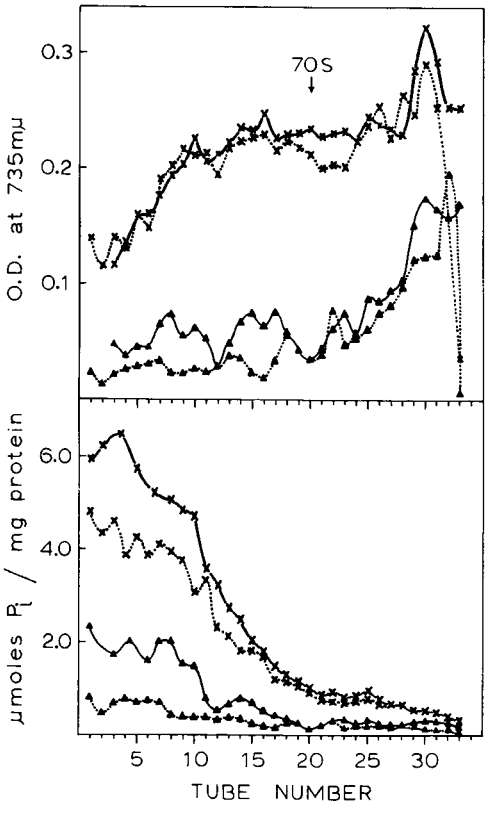


Figure 2



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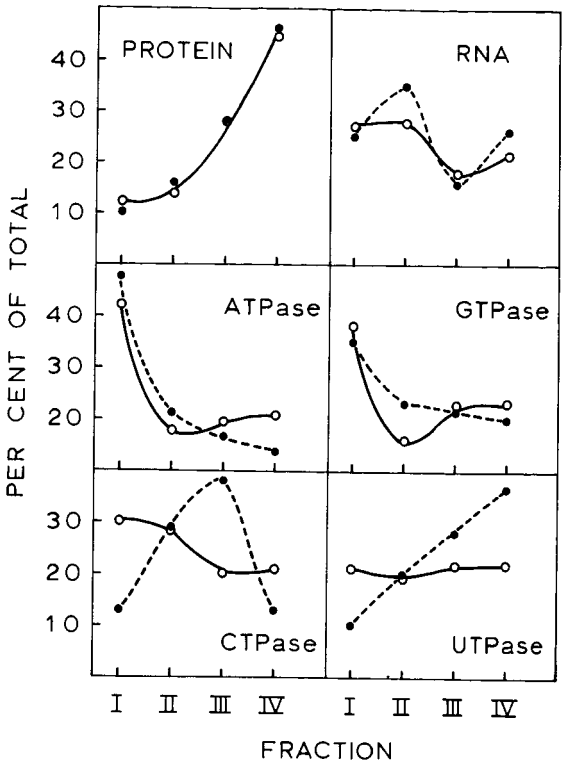

Figure 4

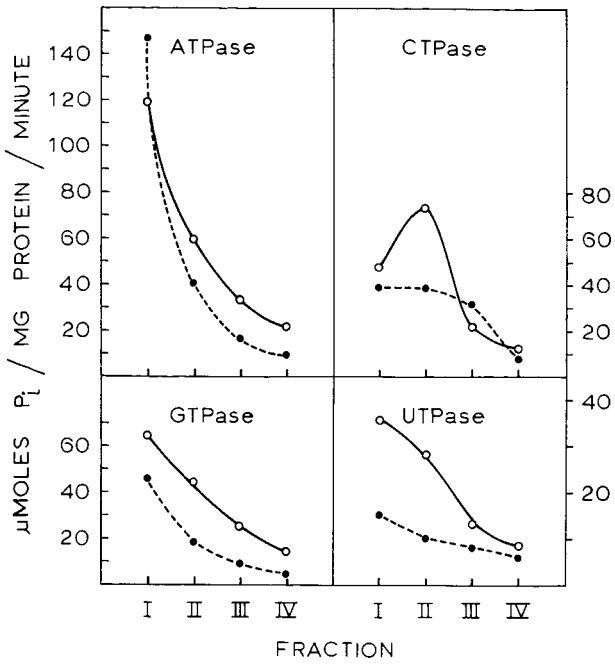

Figure 5

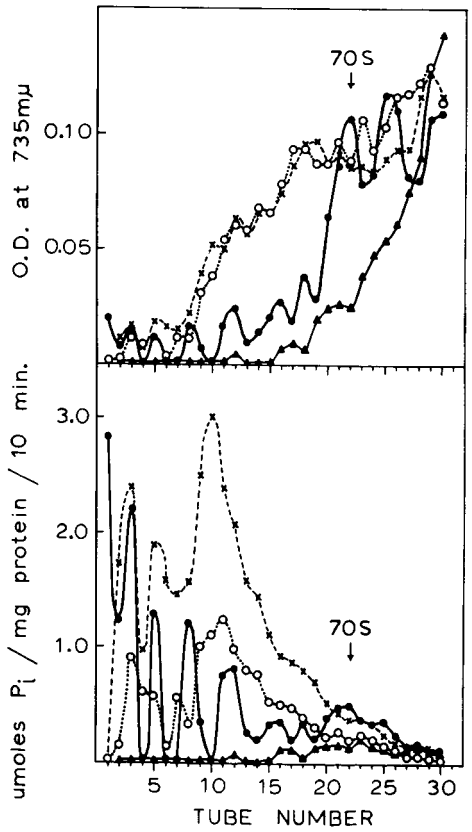

Figure 6

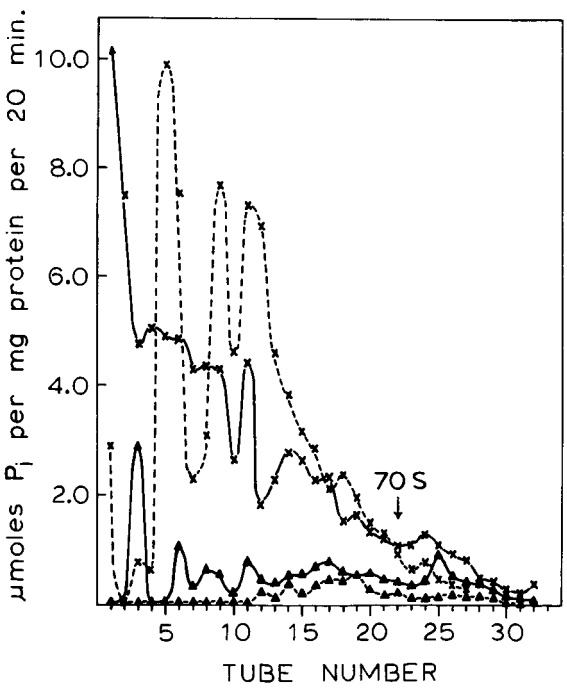

Figure 7

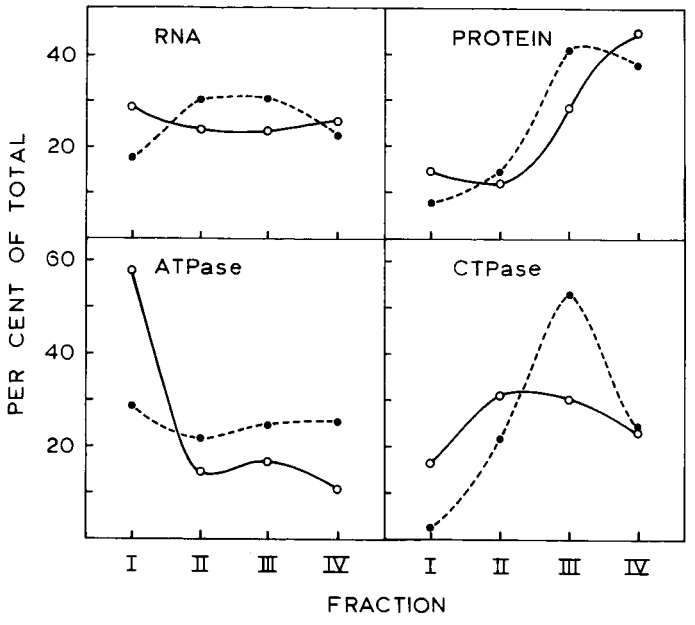

Figure 8

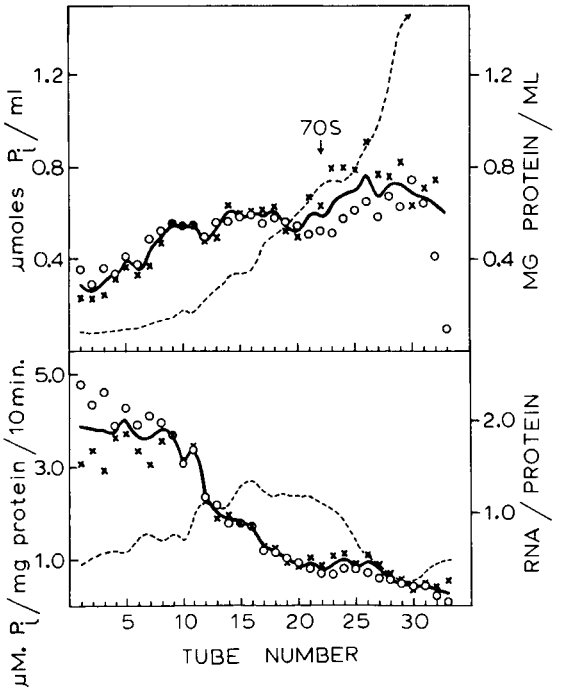

Figure 9