GUANOSINE TRIPHOSPHATE METABOLISM IN RABBIT RETICULOCYTES

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ABSTRACT

GUANOSINE TRIPHOSPHATE METABOLISM IN RABBIT RETICULOCYTES

by Alex Bruce MacDonald

The role of guanosine triphosphate in protein biosynthesis is not as yet understood. A novel approach to the problem has been attempted by studying GTP metabolism in reticulocytes as reflected by hydrolysis, intermediates present in the reaction and binding of the nucleoside triphosphate to factors associated with ribosomes. Reticulocytes are the precursors of red blood cells in which protein synthesis takes place in the absence of RNA synthesis. The separation of the release reaction from the incorporation reactions in a cell-free system from reticulocytes, gives rise to a GTP dependent release reaction suitable for this type of study.

The immediate products of GTP hydrolysis under releasing conditions are GDP and inorganic phosphate. The hydrolysis is not dependent upon ribosomal integrity and can be "uncoupled" by pretreating ribosomes with RNase.

GTP binds to ribosomes in a reaction that appears protein dependent since RNase pretreatment of the ribosomes

decreases the ribosomal binding slightly while pronase pretreatment totally inhibits the ribosomal binding. GDP will bind to ribosomes to the same extent as GTP. The reaction will take place at 4^{o} and is partially inhibited by ATP. GMP does not bind. No increase in binding was demonstrable in polysomes as opposed to monosomes or 60S subunits as opposed to 40S subunits.

An intermediate arising from incubation of (^{32}P) GTP with ribosomes has been shown to be phosphoprotein in nature but has not been characterized.

The isolation of a specific GTPase from the supernatant fraction from which the ribosomes were sedimented was attempted with some success. The GTPase had no detectable ATPase or nucleoside diphosphokinase activity. The enzyme required magnesium, sulfhydryl reagents and catalyzed the hydrolysis of GTP at an optimium pH of 9.0. No transfer of radioactivity was detectable when the GTPase was incubated with (^{14}C) GTP or (^{32}P) GTP.

GUANOSINE TRIPHOSPHATE METABOLISM IN RABBIT RETICULOCYTES

by

Alex Bruce MacDonald

A THESIS

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Dedicated
to
my wife Carole

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A. B. M.

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INTRODUCTION AND HISTORICAL

The series of reactions which lead to the eventual formation of protein from nucleic acid may be naively described as follows:

1. Deoxyribonucleic acid + nucleoside triphosphosphates

   DNA dependent
   RNA polymerase
   ⟶ Ribonucleic Acid

2. Amino Acids + soluble RNA

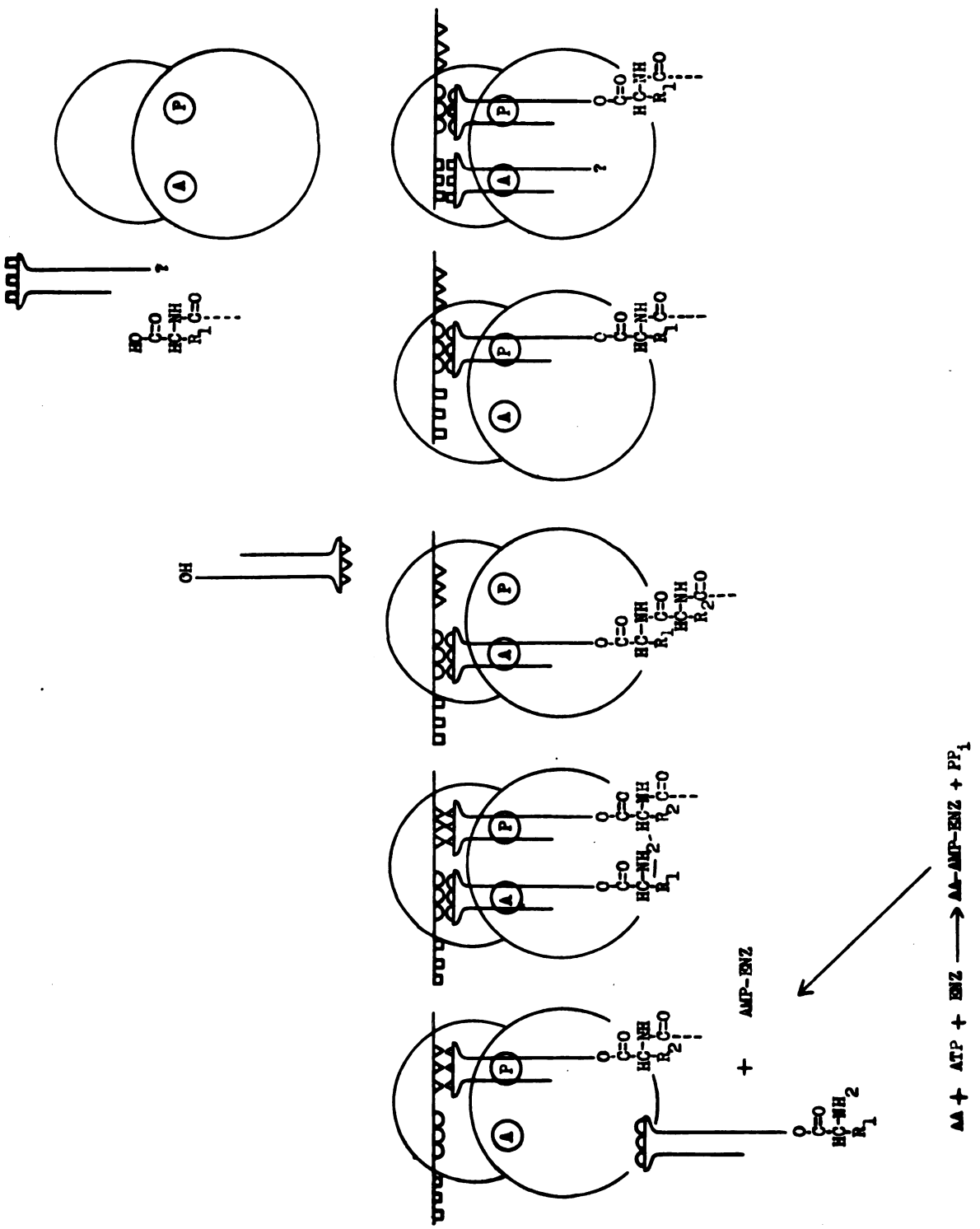
   Enzyme
   ATP
   ⟶ Aminoacyl-sRNA

3. Aminoacyl-sRNA + messenger RNA, Ribosomes
   ⟶ Polysomal Bound Protein

4. Polysomal Bound Protein
   ⟶ Ribosomes + Protein

Somewhere in reaction 3. or 4. or both, guanosine triphosphate is required as a cofactor. The initial observation by Keller and Zamecnik in 1956 (1) that GTP is required in the biosynthesis of protein has stimulated much research into the nature of this requirement. The elucidation of this requirement and the metabolism of GTP in an <u>in vitro</u> system of protein biosynthesis is the problem to which we have addressed ourselves in this thesis.

The latter two reactions mentioned above may best be described in a model after that proposed by Schweet (2).


AA + ATP + ENZ ⟶ AA-AMP-ENZ + PP$_i$
AMP-ENZ
P
A

The activation of amino acids through their acylation with sRNA has been known for some time (3,4,5,6,7). GTP is not required in these reactions so the necessity for this co-factor must occur after amino acid activation. The unit, comprising the amino acid and its specific sRNA is transferred to the ribosome. The specificity of the unit lies in the sRNA (8) which pairs with the complementary bases on the messenger RNA utilizing the ribosome as a support. This initial binding may be illustrated by considering the present status of binding sites on the ribosome. There are at least two sites for binding aminoacyl-sRNA to active ribosomes (9,10). Recently these have been designated the A site which binds aminoacyl-sRNA and the P site, occupied by peptidyl-sRNA (11,12). Considering only the A site, it has been shown that aminoacyl-sRNA binding is nonenzymatic and does not require GTP, either in E. coli (13,14,15,16) or reitculocyte ribosomes (17), when carried out in a medium containing high salt concentrations. This type of binding enabled Nirenberg and Leder (18) to make great advances in codeward determinations.

Perhaps the next occurrence in the reaction sequence is the formation of the peptide bond. GTP has been implicated in this event (19,20,21). It has been suggested that an exchange reaction occurs between aminoacyl-sRNA and GTP (19), resulting in the growing polypeptide being

phosphorylated at its carboxy group. The latter then reacts with the next incoming aminoacyl-sRNA forming a new peptide bond and splitting out sRNA. However, this involves cleavage of the peptide-sRNA complex which has been shown not to occur (22). Hawtrey (20) suggests that a ribosomal bound, high energy phosphate intermediate occurs through the formation of a phosphorylated orthoester of a hydroxyl group on the peptidyl-sRNA. The aminoacyl-ester link thus remains unbroken. Hydrolysis of the orthoester may occur in the presence of a transfer enzyme leading to formation of a new peptide bond and liberation of the sRNA. Utilization of purified factors from an E. coli system enabled Lipmann (21) to show a stoichiometry between peptide bond formation and the hydrolysis of GTP. However, the observation (23) that the ester bond by which the amino acid is fixed to the terminal of the sRNA contains sufficient energy required in the linking of the peptide bond indicates that there is no need for an additional energy supply in peptide bond formation. In addition to this high group potential argument, peptide bond formation has been observed in the absence of a GTP requirement (41) through the use of an sRNA analog, puromycin.

The completion of the formation of the peptide bond finds the P site occupied by deacylated sRNA. The removal of this deacylated sRNA has been reported as GTP depen-

dent (24).

The next event in the model is the transfer of the peptidyl-sRNA from the A site to the P site. The chronology of events now becomes important because this translocation (26) appears to be enzymatic and to require GTP. The cell-free systems utilized for this study, whether they be mammalian (27,28), bacterial (29,30,31,32) or from yeast (33), all seem to show a requirement of two soluble factors and GTP. One factor is apparently for peptide bond synthesis while the other catalyzes the translocation of the peptidyl-sRNA and presumably utilizes GTP. Thus GTP utilization would follow peptide bond formation. However, binding of N-formyl methionine to bacterial ribosomes, where N-formyl methionine initiates peptide formation as the N terminal component, shows a GTP requirement. This binding occurs before the formation of a peptide bond either with puromycin (34) or phenylalanine (32). These authors suggest that N-formyl methioninyl-sRNA actually binds to the P site in order to initiate peptide synthesis. The question of GTP utilization in this initial reaction has been amplified by the observation that a GTP analog, 5'-guanylyl methylene diphosphonate, which presumably cannot be hydrolyzed during protein synthesis reactions (35) is as effective as GTP in the binding of N-formyl methionine to ribosomes (Thack, R. E. or Clark, B. F. C., Unpublished Data). Thus it would

appear that GTP may also contain allosteric properties in addition to normal hydrolysis to GDP and inorganic phosphate (37,38). Allende (36) has recently presented preliminary evidence to indicate GTP binds to a component in a crude fraction which also appears to contain an initiation factor in E. coli.

The final synthetic step in the biosynthesis of proteins by ribosomes involves the release of the completed polypeptide chain from the ribosome. The exact mechanism for release is not well understood. The question mark for a specific releasing sRNA in the model is not to be taken as a hypothesis that such exists. It is included to clarify the types of releasing mechanisms which may operate in a cell-free system. On the other hand, the mode of action of the antibiotic puromycin as an inhibitor of normal synthesis appears to be that of releasing nascent protein as soluble peptidyl puromycin chains (39). The same type of reaction occurs with T1-ribonuclease digests of amino acyl-sRNA (40). This type of release is independent of GTP hydrolysis (41). The lack of an energy requirement may indicate that the puromycin or the sRNA fragment binds to the A site and are subsequently released after peptide bond formation due to the inability to "translocate" to the P site. A report of partial GTP utilization for puromycin release (26) indicates this type of mechanism. However, free protein chains have been shown to be the immediate product

of the normal release mechanism not peptidyl-sRNA (42) and the release of completed protein in the absence of incorporation has been shown to be GTP dependent (43). If release then is an extension of incorporation, GTP dependence may indicate a translocation onto the termination codon.

In addition to the above theories as to the site of action of GTP, it has been proposed that GTP may have a role which is regulatory in nature and to function by antagonizing the effect of an inhibitor of protein synthesis at the ribosomal level (44).

A requirement for GTP in protein synthesis seems ubiquitous but its role is unknown in all systems investigated to date. We made the following assumptions in initiating our studies:

A. GTP may act allosterically or as an energy requirement or both.

B. In studying a specific role, one must include the entire metabolism of the compound in the system studied.

C. The relationship of GTP to other cellular functions must await the purification of the enzymes involved and a detailed analysis of the reactions catalyzed.

MATERIALS AND METHODS

Compounds

Uniformly labeled L-(^{14}C)-valine, guanosine 5'-triphosphate-8-(^{14}C), guanosine 5'-diphosphate-8-(^{14}C), guanosine 5'-monophosphate-8-(^{14}C) were purchased from Schwartz Bio-Research Inc., Orangeburg, New York. (^{32}P) Carrier free inorganic phosphate was obtained from Tracerlab, Waltham, Massachusetts. Nucleoside triphosphates were obtained from P-L Laboratories, Milwaukee, Wisconsin. Nembutal was from Abbott Laboratories, North Chicago, Illinois. Heparin sodium and analytical reagent grade toluene from Fischer Scientific Company, Chicago, Illinois. The acrylamide and compounds for polymerization of the gels were purchased from Canal Industrial Corporation, Rockville, Maryland. The scintillators and thixotropic gel powder were acquired from Packard Instrument Company, Inc., Downers Grove, Illinois. Cotton cellulose was from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, New York. Nitrocellulose filters were obtained from Carol Schleicher and Schuell Co. Keene, New Hampshire. Polyethyleneimine was acquired from Chemirad Corp., East Brunswick, New Jersey. Reduced glutathione was purchased from Mann Research Labora-

tories, Inc. New York, New York. Phenylhydrazine hydrochloride, dioxane and naphthalene were from Distillation Products Industries, Rochester, New York. Puromycin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Aureomycin hydrochloride (chlortetracycline) was obtained from Lederle Laboratories, New York, New York. Gougerotin was a gift from Dr. J. M. Clark, Jr., Biochemistry Division, University of Illinois, Urbana, Illinois and Dr. A. Miyaka of Takeda Chemical Industries, Ltd., Osaka, Japan. Dithiothreotol (Cleland's Reagent) was from Worthington Biochemical Corporation, Freehold, New Jersey. Streptomycin sulfate was purchased from General Biochemicals, Chagrin Falls, Ohio. Sephadex gels and Sephadex columns were acquired from Pharmacia Fine Chemicals, Inc. Piscataway, New Jersey. Bio-Gel P-100 was obtained from Bio-Rad Laboratories, Richmond, California. All other chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri.

Biological Materials

Preparation of Rabbit Reticulocytes

Male New Zealand rabbits of approximately 7 pounds were made reticulocytic by 4 daily injections of 0.175 ml of 2.5% neutralized phenylhydrazine (45). Injections were administered subcutaneously. On the sixth day after the beginning of the injections the animals received a solution containing 2,000 I. U. of heparin and 100 mg of Nembutal by intravenous injection. Blood was collected immediately by heart puncture. The red blood cells were separated from the plasma by centrifugation for 20 minutes at 2,000 x g in a Servall centrifuge. The plasma was decanted and its volume recorded. The cells were suspended in NKM solution (a solution containing 0.13 M NaCl, 0.005 M KCl and 0.0075 M $MgCl_2$), using a volume equal to that of the plasma. The suspension was filtered through glass wool. The filtrate, containing the cells, was centrifuged (20 min- x 2,000 g). The layer of white cells, was removed by aspiration after the washing and centrifugation of the cells. The cells, which sedimented, were resuspended in NKM solution and the suspension was centrifuged once more. The supernatant fluid was removed. The packed cells were lysed by adding 4 volumes of a 0.0025 M $MgCl_2$ solution and stirring gently for 10 minutes. After centrifugation at

15,000 x g for 10 minutes, the supernatant liquid was decanted and the pelleted materials discarded. After sedimentation of the reticulocyte ribosomes by centrifugation of the supernatant solution from the previous step at 78,000 x g for 90 minutes the ribosomal pellets which were obtained were suspended in a small volume of cold 0.25 M sucrose by gentle homogenization with a Porter homogenizer. The high speed supernatant fraction so obtained, was then used as the starting material from which the GTPase was isolated. The ribosomal suspension was treated in either one of three ways:

1. The ribosomal suspension was centrifuged at 15,000 x g for 20 minutes. The supernatant liquid containing the ribosomes was frozen and stored at -196°. This suspension is referred to as 1X ribosomes.
2. The ribosomal suspension was diluted with Medium B (0.25 M sucrose, 0.0175 M $KHCO_3$ and 0.002 M $MgCl_2$) and again centrifuged at 78,000 x g for 90 minutes. The ribosomal pellet was resuspended in cold 0.25 M sucrose, centrifuged at 15,000 x g for 20 minutes, frozen and stored at -196°. This suspension is referred to as 2X ribosomes.
3. A 1X ribosomal suspension was made up to a final concentration of 1% in deoxycholate (DOC) by the

addition of 10% DOC (deoxycholic acid neutralized to pH 7.0 with NaOH) at 4°. The mixture was incubated for 2 minutes at 4°, diluted with Medium B and treated in the same manner as in the preparation of 2X ribosomes. If the ribosomes were to be treated a second time with DOC, the process would be repeated. All biological materials were prepared at 0 to 4°. High speed centrifugation was carried out in a Spinco model L-2 preparative ultracentrifuge.

Cell-Free System Enzyme Fraction

The enzyme fraction used in the cell-free system was prepared by the addition of powdered ammonium sulfate to the high speed supernatant solution to yield the protein fraction that was precipitated between 40 and 70% saturation at 0°. This precipitate was dissolved in 0.1 M Tris-HCl-buffer, (pH 7.5 at 25°), containing GSH (1 mM) and reprecipitated by the addition of ammonium sulfate to 70% saturation. The final protein precipitate, which was relatively free of hemoglobin, was dissolved in a small volume of a solution containing 0.02 M Tris-HCl buffer (pH 7.5 at 25°), containing EDTA (1 mM), $MgCl_2$ (1 mM), GSH (1 mM) and dialyzed overnight against 100 volumes of the same solution. The dialyzed enzyme preparation was stored at -196°, in the presence of 0.02 M GSH.

Prelabeling of Ribosomes with (^{14}C) Valine

For the prelabeling of ribosomes with (^{14}C) valine in the whole cell system to produce labeled polysomes, intact washed reticulocytes were preincubated by a modification of the method of Borsook, Fischer and Keighley (45) in a solution that contained 0.30 ml of packed cells per ml, ferrous ammonium sulfate (0.1 mM), Tris-HCl buffer (pH 7.5 at 25°, 0.01 mM), rabbit plasma (0.05 ml/ml), NaCl (0.077 M), KCl (2.9 mM), $MgCl_2$ (4.1 mM), an amino acid mixture from which valine had been omitted (47) (0.15 ml/ml) and (^{14}C) valine specific activity 20 uc/umole (0.025 mM). The solution was incubated for 5 minutes at 37°, and the reaction stopped by the addition of a cold solution containing NaCl (0.13 M), KCl (5 mM) and $MgCl_2$ (7.5 mM). Following sedimentation as before, the cells were washed twice more by resuspension and centrifugation and the ribosomes were isolated by the usual procedure.

For the cell-free system (44) the preincubation of ribosomes with (^{14}C) valine to produce ^{14}C labeled monosomes was carried out in a medium containing mM ATP, 2.5 mM phosphoenol pyruvate, 10 ug/ml pyruvate kinase, 0.05 M Tris-HCl buffer, (pH 7.5 at 25°), 4 mM $MgCl_2$, 0.05 M KCl, 0.2 M glutathione, 0.05 mM in each of the 19 amino acids (except valine), 5 mg/ml ribosomes, 4 mg/ml supernatant

enzymes, and 0.05 mM in (^{14}C) valine (specific activity 10 uc/um). The solution was incubated for 60 minutes at 37° and the reaction was stopped by addition of 10-12 volumes of Medium B containing a 100 fold excess in unlabeled valine. The ribosomes were isolated by centrifugation at 78,000 x g for 90 minutes. The ribosomal pellets were resuspended in a small volume of 0.25 M sucrose and stored at -196° until used.

Synthesis of (^{32}P) Guanosine Triphosphate Labelled in the Gamma Position by Spinach Chloroplasts

The petioles were removed from 50 g of fresh spinach leaves (48). The leaves were rinsed, blotted and added to a chilled mortar. 5 ml of a solution containing 0.05 M Tris-HCl (pH 7.4), 0.1 mM GSH and 0.3 M sucrose were added and the leaves were ground for 10 minutes. The resultant slurry was strained through 4 layers of cheesecloth previously soaked in cold 0.05 M Tris-HCl-(pH 7.4). The slurry was centrifuged for 2 minutes at 200 x g to remove whole cells and debris. The supernatant solution was carefully decanted and centrifuged at 1,500 x g for 5 minutes. The pellet, containing the chloroplasts, was mixed very gently with a small painting brush and resuspended in 10 ml of the sucrose-Tris-GSH solution. Chloroplasts prepared in this manner were used within 30 minutes. As received, the (^{32}P) inorganic phosphate (10 mc)

is carrier free and contains no pyrophosphates. The (^{32}P) inorganic phosphate solution of 0.02 N HCl was neutralized with 0.5 N NaOH, followed by the addition of 200 umoles of Tris (pH 7.8), 25 umoles of $MgCl_2$, 5 umoles of potassium phosphate buffer (pH 7.8), 8 umoles of GDP. The solution was mixed, transferred to a photosynthesis reaction vessel. The original container was rinsed with enough water to bring the total volume up to 2.7 ml. 0.1 umoles of phenazine methosulfate and 0.2 ml of the chloroplast suspension were then added, the suspension was stirred, flushed with nitrogen for 3 minutes, and placed in a glass water bath at 18°. The suspension was illuminated for 15 minutes by placing the bath between 2 Ken-Rad 300 watt flood lamps insulated by heat shields. Termination of the reaction was effected by the addition of 0.33 ml of 50% trichloroacetic acid. The mixture was transferred to a thick walled centrifuge tube and allowed to stand in an ice bath for 20 minutes, centrifuged at 5,000 x g, and the supernatant transferred to a 30 ml round bottom centrifuge tube fitted with a ground glass stopper. Three 10 ml volumes of ether were used to extract the TCA. Any remaining ether was removed by flushing with N_2 until no ether odor was apparent. (^{32}P) GTP was isolated by chromatography over polyethyleneimine. Lyophization of the (^{32}P) GTP eluted in this manner usually resulted in a 40% yield (3-3.5 umoles) with a specific

activity of approximately 120 uc/umole. (^{32}P) GTP prepared in this manner was 96-98% pure with no detectable radioactive label in the beta position.

Analytical Procedures

Separation of Guanosine Nucleotides by Polyethyleneimine Treated Cellulose Column Chromatography

PEI was dissolved in H_2O, neutralized to pH 7.6 with concentrated HCl and diluted to a 20% solution. Cotton cellulose was added (15 g/100 ml of PEI solution) and stirred 12 hours at 28°. The cellulose was washed twice with H_2O and stored in a 15% slurry at 4°. The PEI-cellulose was stable under these conditions for approximately 2 months.

Column preparation was carried out as follows: The PEI cellulose was washed twice with buffer (0.1 M NH_4HCO_3, 0.01 M HCOOH, 0.001 M Tris pH 7.0 at 4°). The column was packed and washed overnight with the buffer. The sample was layered on the column and rinsed into the column with a small amount of buffer. The flow rate was 0.4 ml/minute with a fraction volume of 2.5 ml. The nucleotides were eluted using a step gradient. Inorganic phosphate was eluted prior to guanosine monophosphate. The mononucleotides were eluted with approximately 75 ml of the buffer. When the concentration was increased to 0.3 M NH_4HCO_3, 0.03 M HCOOH, 0.001 M Tris-HCl (pH 7.5 at 4°),

the dinucleotides were eluted in approximately 50 ml. Increasing the concentration to 0.75 M NH_4HCO_3, 0.075 M HCOOH and 0.001 M Tris-HCl (pH 7.5 at 4°) eluted the trinucleotides. The elution pattern of the nucleotides was determined by cochromatography. If the nucleotides were to be isolated, in order to determine the radioactivity in each fraction, 1 mumole of each of the reference standards was cochromatographed to establish the location of the respective guanosine derivatives in the eluate solution.

Determination of GTP Dependent Release of Polypeptides from Ribosomes

Studies of the GTP dependent release of polypeptides from ribosomes were done by incubation of the prelabeled ribosomes in a solution containing 50 mM Tris-HCl, (pH 7.5 at 25°), 20 mM glutathione, 50 mM KCl, 4 mM $MgCl_2$, and GTP as indicated in a total volume of 1.0 ml. No incorporation of amino acids occurs under these conditions, however finished alpha and beta hemoglobin chains are released from the ribosomes. The amount of GTP dependent release was calculated by subtracting the amount of protein non-specifically released in a similar assay in which no GTP had been added. The value of this nonspecific release was usually about 10% that of the total radioactivity in the assay. Following incubation for 40 minutes at 37°, the solutions were placed in 4 ml cellulose centrifuge tubes

and centrifuged at 105,000 x g for 60 minutes. Each supernatant was then analyzed for radioactive protein (44).

Assay for Hydrolysis of (^{14}C) GTP and (^{32}P) GTP

The assay solution for the hydrolysis of (^{14}C) GTP in the presence of ribosomes and the corresponding conversion to (^{14}C) GDP was carried out in a normal release assay solution consisting of 0.05 M Tris-HCl (pH 7.5 at 25°), GSH 0.02 M, KCl 0.05 M, $MgCl_2$ (4 mM) in a total volume of 1 ml. After incubating at 37°, 1 ml of 9% TCA (cold) was added followed by 1 ml of a solution containing GMP, GDP and GTP (1 uM in each). The sample was centrifuged at 1,500 x g for 5 minutes. The precipitate was washed twice with 1 ml of 1% TCA. The supernatants were pooled and the TCA extracted 3 times with 10 ml volumes of ether. The samples were chromatographed over PEI-cellulose as described. Each peak was pooled, lyophilized and redissolved in a small volume of a solution containing 7.5 mg/ml of ATP as carrier. The solutions were plated on stainless steel planchets and dried over P_2O_5 in vacuo. The planchets were weighed and counted in a low-background Nuclear-Chicago Automatic Geiger Counter. All values have been corrected for self-absorption of radiation and background radiation and have been calculated as total counts per minute at infinite thinness.

Binding Assay

The binding of (^{14}C) GTP or (^{32}P) GTP to ribosomes was carried out in a normal release assay as described above for the hydrolysis of radioactive GTP. Ribosomal concentration was usually kept at 0.5 mg/assay. After incubating at 37° the reaction was terminated by the addition of 5 ml of cold buffer-salt solution (Tris-HCl 0.05 M (pH 7.5 at 25°), GSH 0.001 M, KCl 0.050 M, $MgCl_2$ 0.004 M). The suspension was filtered through a nitrocellulose filter of 45 micron pore size (18). The filter was rinsed 3 times with 5 ml volumes of the buffer-salt solution. The filter was placed in a glass counting vial and dried at 100° for 30 minutes. After drying, 15 ml of scintillation fluid was added (0.5% PPO, 0.01% POPOP in toluene) and the samples were counted in a Packard Model 3003 liquid scintillation spectrometer.

Dissociation of Ribosomes into Subunits

Ribosomes were dissociated into 40S and 60S subunits by dialysis against a solution of 0.1 mM EDTA and 0.01 M Tris-HCl (pH 7.5 at 25°). The dialysis tubing (Visking) containing the ribosome sample was placed in a Buchler rocking device equipped with a constant flow dialysis attachment. Dialysis was carried out for 36 hours with a dialysate flow rate adjusted to 5 ml/minute.

Sucrose Gradient Analysis

Linear sucrose gradients of from 15 to 30% sucrose containing 0.01 M Tris-HCl (pH 7.5 at 25°) were used to fractionate ribosomal subunits. Linear sucrose gradients of from 15 to 30% sucrose containing 0.01 M Tris-HCl (pH 7.5 at 25°), 0.0015 M MgCl, 0.001 M KCl were used to fractionate polysomes from monosomes (41). Materials to be analyzed were adjusted to a total volume of 1.0 ml and layered onto a gradient of 30 ml. Centrifugation was carried out at 4° in a Beckman SW 25.1 rotor at 63,581 x g for 14 hours in order to fractionate the ribosomal subunits or 3.5 hours for the fractionation of ribosome-polysome mixture. Contents of each tube were then analyzed for materials absorbing at 260 mu by pumping through a Gilford spectrophotometer equipped with a flow cell (0.5 cm path length). Results were plated automatically by a Sargent Model SR recorder. Flow rate through the cell was maintained at 5 ml/minute using a Buchler polystaltic pump. Effluent from the flow cell was collected in 1.0 ml portions with a Packard Model 231 fraction collector.

Phenol Extraction

The assays for (^{32}P) protein formation were incubated using conditions described for the GTP dependent release of protein from ribosomes described earlier. In addition

to 1X ribosomes or enzyme and (^{32}P) GTP, 0.6 mg of a yeast RNA hydrolysate was added to reduce nonspecific binding of nucleotides. The reaction mixture was incubated for 10 minutes at 37° and terminated by the addition of 4 ml of phenol previously adjusted to pH 8.5 with concentrated NH_4OH (49). The sample was stirred for 30 minutes then washed 7 times with a 30 ml volume of a solution containing 0.1 mg/ml of RNA hydrolysate, 0.01 M PO_4 (pH 7.8), 0.05 M pyrophosphate, 0.01 M EDTA and 15% phenol. After each rinse the aqueous phase was removed and the interface was dissolved with 0.5 ml of concentrated NH_4OH. Finally 1 ml of bovine serum albumin (15 mg/ml) was added followed by the rapid addition of 30 ml of acetone under vigorous stirring. This produced a flocculent precipitate which was centrifuged and washed by resuspension and centrifugation with 30 ml of acetone followed by a similar washing with 30 ml of ether. After drying in air, the precipitate was transferred to a counting vial. Each tube was rinsed with 1 ml of 0.5 N NaOH to dissolve any residual protein (sometimes heating to 60° for 30 minutes was required). The dried precipitate was dampened with 0.2 ml of p-dioxane before addition of the NaOH. When the precipitate had dissolved, 15 ml of a counting mixture was added and the contents of the vial were shaken vigorously. The mixture contained 7 g of 2,5 diphenyloxazale, 150 mg of 1,4-bis-(5-phenyl-

oxazolyl)-benzene, 50 g of naptholene and 36 g of thixotropic gel powder dissolved in 200 ml of toluene, 30 ml of absolute ethanol and 800 ml of p-dioxane.

Assay for GTPase or ATPase

The levels of activity for GTPase or ATPase were determined by incubating the enzyme fraction in the presence of GTP or ATP, terminating the reaction and analyzing the amount of inorganic phosphate formed. The enzyme fractions were incubated in a solution containing 0.05 M Tris-HCl (pH 9.0 at 37°), $MgCl_2$ (4 mM), KCl (3 mM), dithiothreotol (0.5 mM) GTP or ATP (0.4 mM) in a total volume of 1 ml. The reaction was carried out for 20 minutes at 37°, 0.1 ml of 50% TCA was added to stop the reaction. The sample was centrifuged and the supernatant was analyzed for inorganic phosphate by the Berenblum and Chain method (52) as modified by Martin and Doty (53). The supernatant was added to a biphasic mixture containing 0.5 ml of 1 M H_2SO_4, 0.5 ml of 10% ammonium molybdate and 5 ml of isobutanol-benzene (50:50). The vessel was shaken for 15 seconds. After separation of the 2 layers, 4 ml of the organic phase was removed and added to 6 ml of ethanol containing 3.2% H_2SO_4. 0.5 ml of a freshly diluted solution of $SnCl_2$ (10% $SnCl_2 \cdot H_2O$ in concentrated HCl diluted 1:200 with 0.5 M H_2SO_4) was added. The solutions were mixed and the color which developed was measured at 680 mu on a Coleman Jr.

spectrophotometer. The inorganic phosphate formed was calculated from a standard curve included with each series of assays. 50 to 400 mumoles of phosphate as KH_2PO_4 (0.001 M in 0.5 M H_2SO_4) was added to the buffer-salt mix above and diluted to 1 ml. 0.1 ml of TCA was added. The inorganic phosphate standards were extracted in the same manner as the sample assays.

Protein Determination

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (50) as modified by Oyama and Eagle (51). Ribonuclear protein was determined by the method of Ts'O and Vinograd (69).

Streptomycin Precipitation of Nucleic Acids

Removal of nucleic acids from the enzyme fractions was accomplished by precipitation with streptomycin sulfate according to the method of Stuart and Lehman (56). The absorbance of the solution was determined at A^{260} and A^{280}. The nucleic acid concentration was determined by the method of Warburg and Christian (57). The amount of streptomycin sulfate to be added was calculated to provide a ratio of 5:1 nucleic acid to streptomycin on a w/w basis. A 5% solution of streptomycin sulfate, freshly prepared, was added to a cold high speed supernatant solution which was then stirred for 30 minutes, centrifuged at 15,000 x g and dialyzed against 7 liters of a solution containing

0.01 M glycine (pH 9.0), 0.01 M $MgCl_2$, 0.001 M KCl and 0.0001 M dithiothreotol.

Sephadex Fractionation

Fractionation over Sephadex G-100 was usually carried out in a 2.5 x 100 cm column. The gel was prepared by adding an appropriate amount to a solution containing 1 M KCl, 0.01 M glycine (pH 9.0), 0.01 M $MgCl_2$, 0.0001 M DTT. After stirring overnight in the cold, the gel was allowed to settle, decanted and rinsed 3 times with a solution similar to that mentioned above but with 0.001 M KCl. The column was poured in approximately a 5% slurry with an effective hydrostatic head of 20 cm. The column was washed overnight with the buffer. Samples were added to the column by layering the sample directly onto the column bed by the use of a syringe fitted with a thin polyethylene tube. In those cases where the protein concentration was low, the sample was made up to 10% in sucrose in order to increase its density above that of the buffer which covered the top of the column bed. The flow rate was usually 0.5 ml per minute with a fraction volume of 3 ml. The protein profile of the eluate was determined by reading the absorbence at 280 mu of each fraction.

Disc Electrophoresis

Disc electrophoresis was carried out by a modification of the method of Ornstein and Davis (58). All materials were freshly prepared just prior to use. The acrylamide was recrystalized from ethyl acetate followed by recrystalization from chloroform and was stored at 4°. Polyacrylamide was prepared as follows: One part solution A (HCl N, 4.8 ml; Tris; 3.7 g, N,N,N^{1}N^1-tetramethylethylinediamine ("TEMED") 0.023 ml; $MgCl_2$ 1 M, 0.1 ml; KCl 0.1 M, 0.1 ml; dithiothreotol (DDT) 0.05 M 0.1 ml; H_2O to 10 ml) was mixed with 2 parts solution B (Acrylamide, 2.8 g; N,N^1-methylene bisacrylamide ("BIS"), 0.074 g; $MgCl_2$ 1 M, 0.1 ml; KCl 0.1 M, 0.1 ml; DTT 0.05 M, 0.1 ml H_2O to 10 ml) and 1 part H_2O. The polymerization reaction was catalyzed with 4 parts catalyst (ammonium persulfate 0.070 g; $MgCl_2$ 1 M 0.5 ml; H_2O to 50 ml). The polymerization was carried out at room temperature at pH 8.9 in 12 x 1 glass cylinder filled to a height of 6 cm and covered with a small volume of H_2O. The cylinders containing the gel beds were then fitted with a condenser and cooled to 4°. The gel beds were subjected to a current of approximately 2.5 ma/cylinder (using a buffer consisting of a 1 to 8 dilution of solution A with TEMED omitted) prior to additions of the sample. The buffer was then removed and the sample was layered on the top of the gel bed. The buffer concentration of the sample was adjusted to that of

the running buffer (see below) by the addition of an appropriate volume of a 10 times concentrated running buffer. Enough polyacrylamide P-100 was then added to the sample to absorb all of the sample solution. Running buffer (.005 M Tris, .038 M glycine, 1×10^{-4} 1 M $MgCl_2$, 1×10^{-5} M KCl, 1×10^{-5} M DTT was then carefully placed over the P-100 layer. Electrophoresis was conducted at 2.5 ma per tube for 8 hours. The gel beds were removed from the cylinders. Those gel beds which were to be stained were placed for 20 minutes in buffalo black stain dissolved in 7% acetic acid at 10 ma of current per gel bed. Those gel beds which were to be analyzed for enzyme activity were cut as indicated. Each fraction was placed in a plastic centrifuge tube and pulverized into a mash. The protein was extracted from the acrylamide with 3 ml of 0.01 M glycine (pH 9.0), 0.01 M $MgCl_2$, 0.001 M KCl and 0.0001 M DTT. The suspension was centrifuged at 15,000 x g for 10 minutes and the supernatant removed by filtration through glass wool. The mashed acrylamide was resuspended and centrifuged twice more and the supernatant solutions were pooled.

Nucleoside Diphosphokinase Assay

Enzyme fractions were assayed for nucleoside diphosphokinase activity by the method of Mourad and Parks (54) which is similar to the one employed by Berg and Jaklik (55).

The reaction mixture contained per 0.5 ml: glucose 10 mM; -TPN, 0.1 mM; yeast hexokinase, 0.25 ug; glucose-6-P dehydrogenase, 1 ug; $MgCl_2$, 25 mM; KCl, 10 mM; Tris-HCl, (pH 7.5), 0.15 M; ADP, 0.025 mM; GTP, 0.050 mM; and enzyme 100 to 300 ug. The reaction was started by the addition of TPN. Measurements were made by following the increase in absorbance at 340 mu at 37° on a Gilford spectrophotometer. Results were plotted automatically by a Sargent SR recorder.

RNAase Treatment of Ribosomes

Ribosomes were treated with ribonuclease A at a concentration of 1 ug/mg of ribosome for 30 minutes at room temperature. The precipitate which formed was removed by centrifugation and washed with 3 volumes of buffer (0.01 M glycine (pH 9.0), 0.01 M $MgCl_2$, 1 mM KCl, 0.1 mM DTT). The pooled supernatants were then dialyzed against the same buffer.

Chromatography on PEI Paper

Whatman No. 1 papers were cut and impregnated with a 2.5% solution of PEI as described by Verachtert et.al. (59). GTP as a reference compound was applied about 3 inches from the base of the paper together with the samples obtained from incubation with (^{32}P) inorganic phosphate. Development was achieved by descending chromatography at 20°,

using a mixture of aqueous solutions of 2 N LiCl, 1.5 N formic acid (1:1). Ultraviolet absorbtion was detected with a Mineralight (SL 2537) and radioactivity was measured with a Packard Model 7200 Radiochromatogram Scanner.

RESULTS

Hydrolysis of GTP as a Function of Ribosomal Integrity

It would appear axiomatic that in order to study the role of GTP in a metabolic process one must use an experimental system which is GTP dependent. The reticulocyte ribosomal cell-free system, however, when engaged in amino acid incorporation and the release of protein, shows no GTP dependence if an ATP generating system and soluble enzymes are present (60). Separation of the 2 main ribosomal events of protein biosynthesis, that is, the separation of processes of amino acid incorporation into protein from those of the release of completed proteins from the ribosome (43), lead to a system which is dependent upon GTP for protein release. The products obtained from GTP, when that molecule was incubated under the conditions prescribed for the release of protein (see Methods), has been shown here to be GDP and inorganic phosphate. Determination of the reaction products formed from GTP involved the use of both (^{14}C) GTP and (^{32}P) GTP. The purity of (^{14}C) GTP and (^{32}P) GTP was verified by PEI-cellulose chromatography (Figures 1 and 2). (^{14}C) GTP contained 3.6% (^{14}C) GDP and and 0.5% (^{14}C) GMP as contaminants. (^{32}P) GTP contained

Figure 1: Distribution of radioactivity in guanosine-5'-triphosphate -8-(^{14}C) by chromatography over the polyethylene-imine-cellulose column. Conditions are given in Methods. Peak 1 is GMP, Peak 2 is GDP and Peak 3 is GTP. Shaded area indicates radioactivity. See Methods for details of separation.

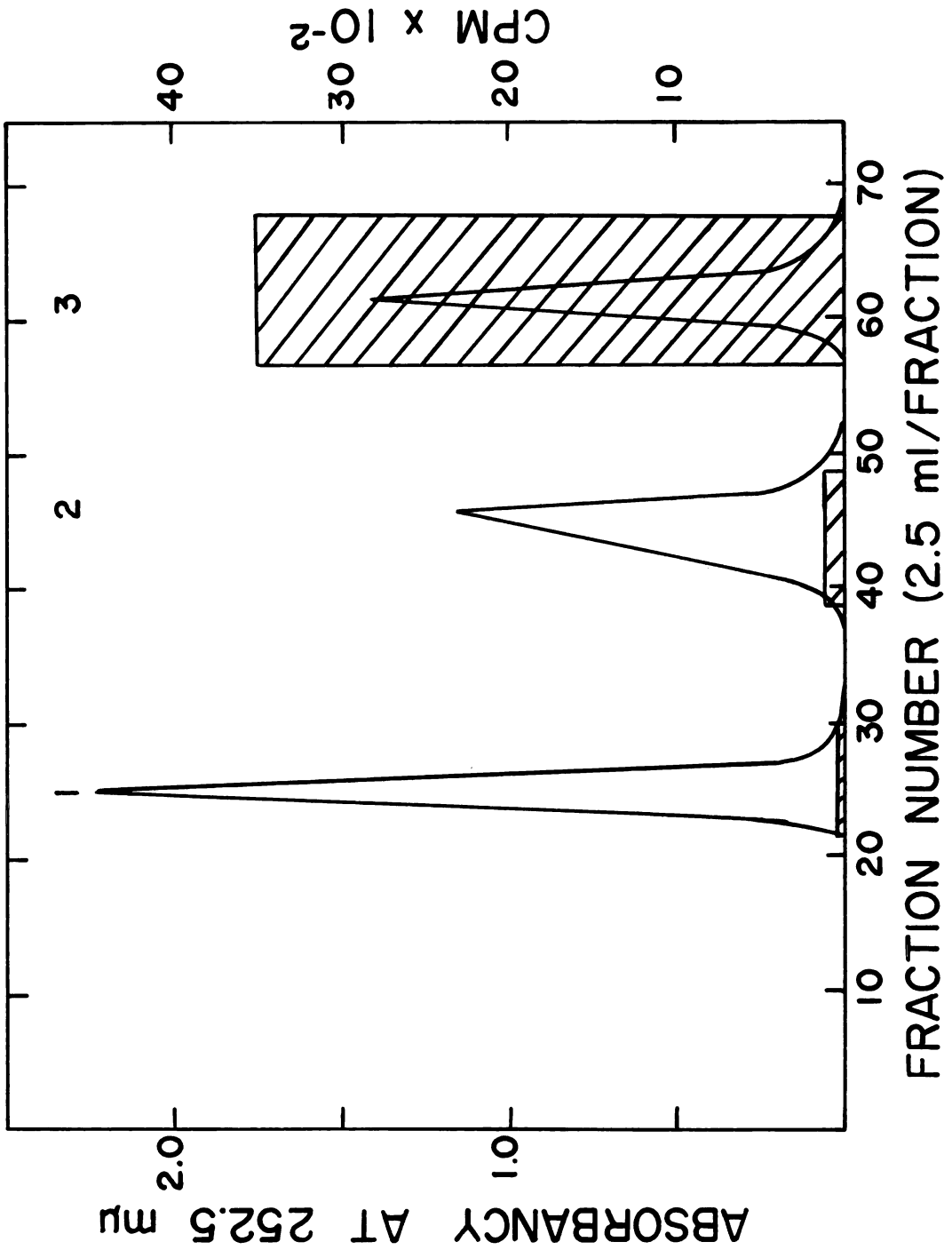
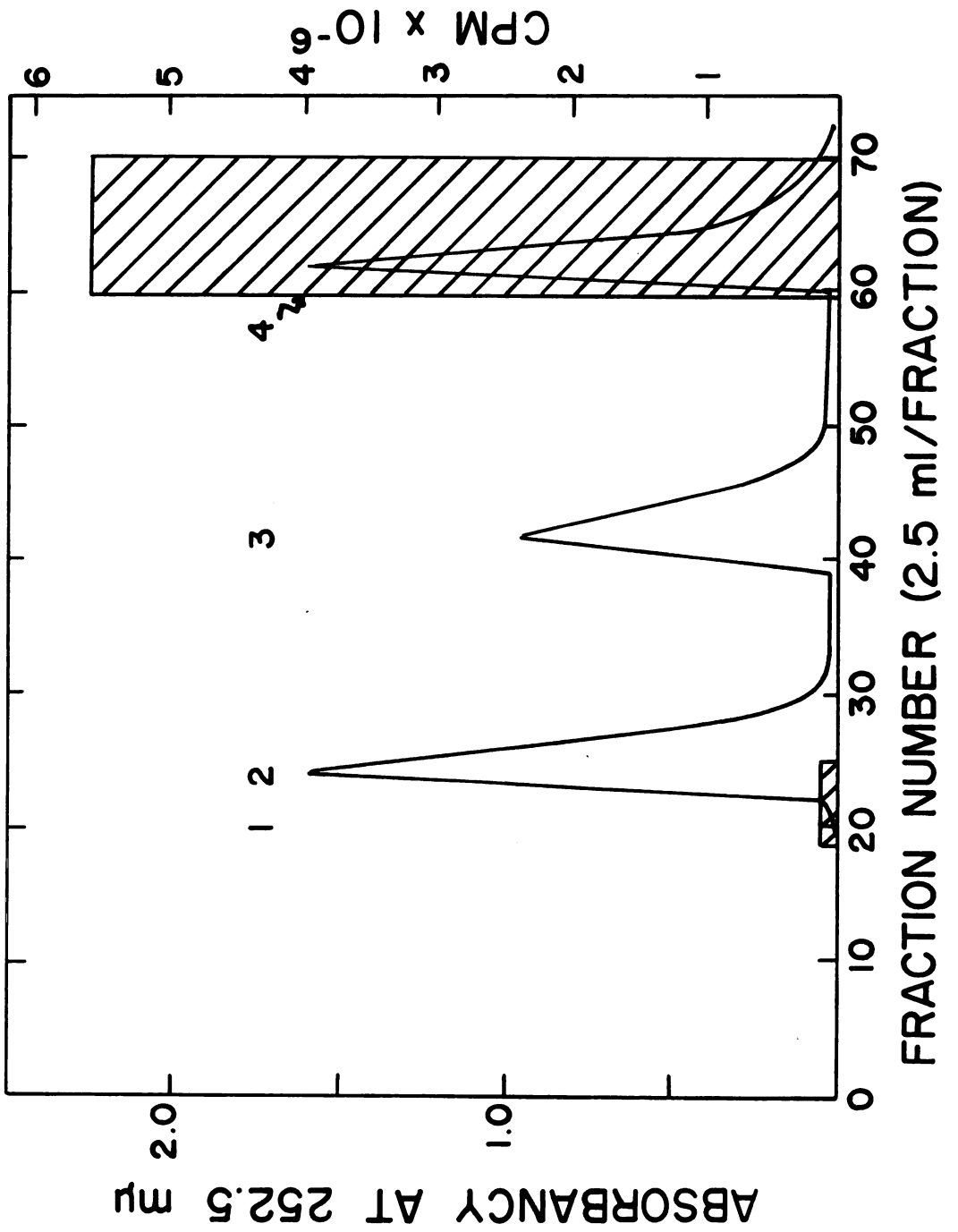

ABSORBANCY AT 252.5 mμ
2.0
1.0
CPM x 10-2
40
30
20
10
1
2
3
10
20
30
40
50
60
70
FRACTION NUMBER (2.5 ml/FRACTION)

Figure 2: Distribution of radioactivity in guanosine-5'-triphosphate labeled in the gamma position with (^{32}P) inorganic phosphate. Method is given in Methods. 1 is volume at which inorganic phosphate is eluted from the column, 2 is GMP, 3 is GDP and 4 is GTP (see Methods for details of separation). Shaded area indicates radioactivity.


ABSORBANCY AT 252.5 mμ
2.0
1.0
0
10
20
30
40
50
60
70
FRACTION NUMBER (2.5 ml/FRACTION)
CPM x 10-6
6
5
4
3
2
1
1 2
3
4 4a

4.41% (^{32}Pi) and 0.8% (^{32}P) GDP contamination. Incubation of (^{14}C) GTP in the release reaction mixture under conditions that hydrolyzed one-half of the nucleoside triphosphate added resulted in the nucleotide profile observed in Figure 3. The major reaction product observed was GDP (fractions 39-50) and unreacted GTP. Only small amounts of GMP (fractions 22-32) were detected. A similar experiment using (^{32}P) GTP is illustrated in Figure 4. The only labeled material recovered (other than unreacted (^{32}P) GTP) was eluted in the region of the eluate diagram where inorganic phosphate had been shown to emerge. From these data, the conclusion has been drawn that the end products of GTP metabolism ribosomes engaged in the protein release reaction are GDP and inorganic phosphate.

Protein synthesis has been shown to occur on the polysomes of cells (61). Because of the GTP dependence of the system, the protein releasing system is ideal for studying any differences in GTP hydrolysis which may be related to polysomal integerity. Experiments were thus designed to study GTP utilization and the release of protein by polysomes as contrasted to that of monosomes. These studies were carried out using polysomes obtained from intact reticulocytes labeled with (^{14}C) valine to produce monosomes bearing labeled nascent protein. The amount of released polypeptide was then compared with the percentage of (^{32}P)

Figure 3: Distribution of radioactivity in (^{14}C) GTP after 50% hydrolysis by ribosomes. Peak 1 is front material, peak 2 is GMP, peak 3 is GDP and peak 4 is GTP (see Methods for details of the separation). Radioactivity is indicated by shaded area. Three mg of ribosomes were reacted with 60 mumoles (^{14}C) GTP (specific activity of 1.5 uc/umole) for 10 minutes at 37°.

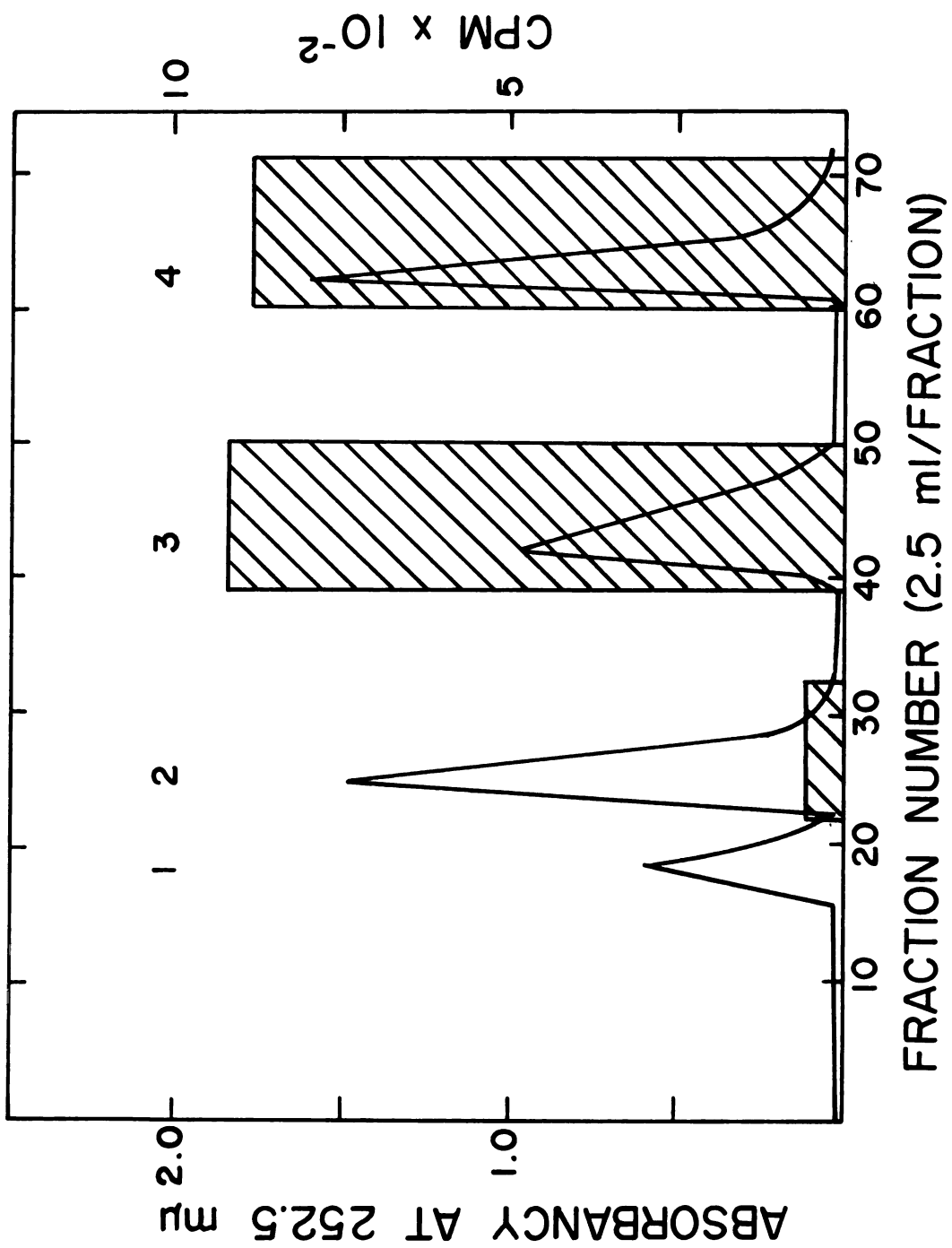
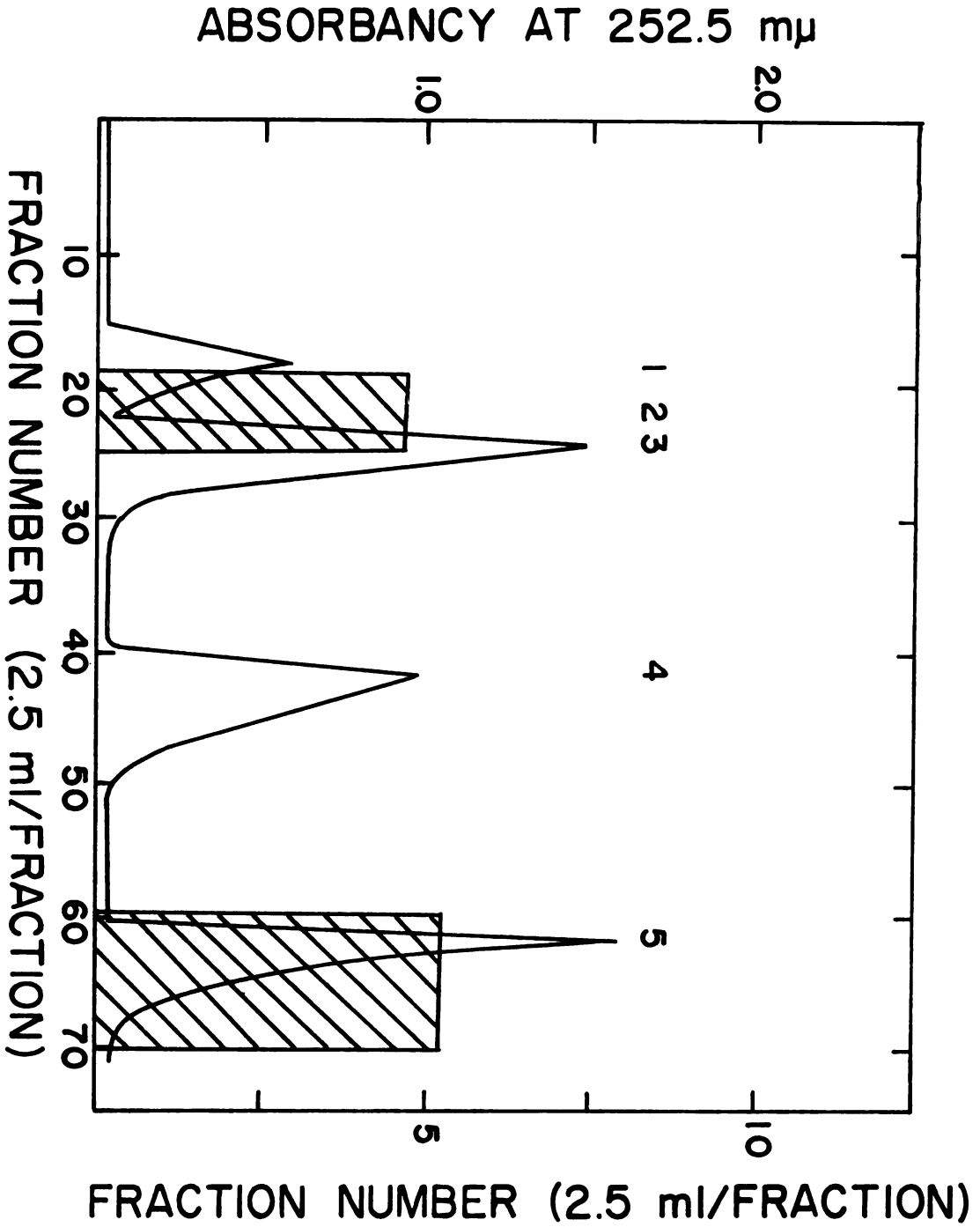

ABSORBANCY AT 252.5 mμ
2.0
1.0
CPM x 10⁻²
10
5
1
2
3
4
10
20
30
40
50
60
70
FRACTION NUMBER (2.5 ml/FRACTION)

Figure 4: Distribution of radioactivity from (^{32}P) GTP after 50% hydrolysis by ribosomes. Three mg of ribosomes were reacted with 60 mumoles (^{32}P) GTP (labeled in gamma position). Specific activity of approximately 1 uc/umole. 1 is front material, 2 is volume of inorganic phosphate elution, 3 is GMP, 4 is GDP and 5 is GTP. Shaded area indicates radioactivity.


ABSORBANCY AT 252.5 mμ
1.0
2.0
FRACTION NUMBER (2.5 ml/FRACTION)
10
20
30
40
50
60
70
1
2
3
4
5
5
10
FRACTION NUMBER (2.5 ml/FRACTION)

GTP hydrolyzed during various times of incubation at 37°.

The sucrose density gradient profile obtained using 0.5 mg of the prelabeled polysomal material (Figure 5a) indicates the presence of a large proportion of reticulocyte polysomes. Incubation of the labeled polysomes resulted in significant amounts of release of labeled protein (Figure 5b). After 40 minutes, 26.6% of the (^{32}P) GTP added was hydrolyzed by 1 mg of polysomes (Figure 5b). The sucrose density profile of the monosome preparation is shown in Figure 6a. One mg of monosomes, although releasing less peptide (Figure 6b) hydrolyzed 25.8% of the (^{32}P) GTP in the same period of time. Thus it is evident that polysomes are not required for maximum GTP hydrolysis as reported by others (31, 62).

In order to examine the association of the GTP hydrolyzing factor with the ribosomal particles an attempt was made to remove the factor by repeated resuspension and sedimentation from Medium B (see Methods). The ribonucleoprotein concentration was adjusted to 3 mg/ml and the GTPase activity of 3 mg of ribosomes was determined. The GTPase activity of 2X ribosomes was also compared to ribosomes which had been treated twice with a 1% solution of sodium deoxycholate (see Methods). Table I indicates that approximately one half the activity is retained on the ribosome after deoxycholate treatment. Table I also indicates

Figure 5: Effect of polysomes on GTP hydrolysis. Sixty mumoles of GTP added to 1 mg of ribosomes as polysomes per assay. Reaction terminated at times indicated. Parallel experiments were performed with (^{14}C) valine labeled polysomes and unlabeled GTP and unlabeled polysomes with (^{32}P) GTP. ●—● , (^{14}C) peptide released from polysomes + GTP, ○—○ (^{14}C) peptide released — GTP, □—□ , per cent of (^{32}P) GTP hydrolyzed. Labeling and gradient technique are given in Methods.

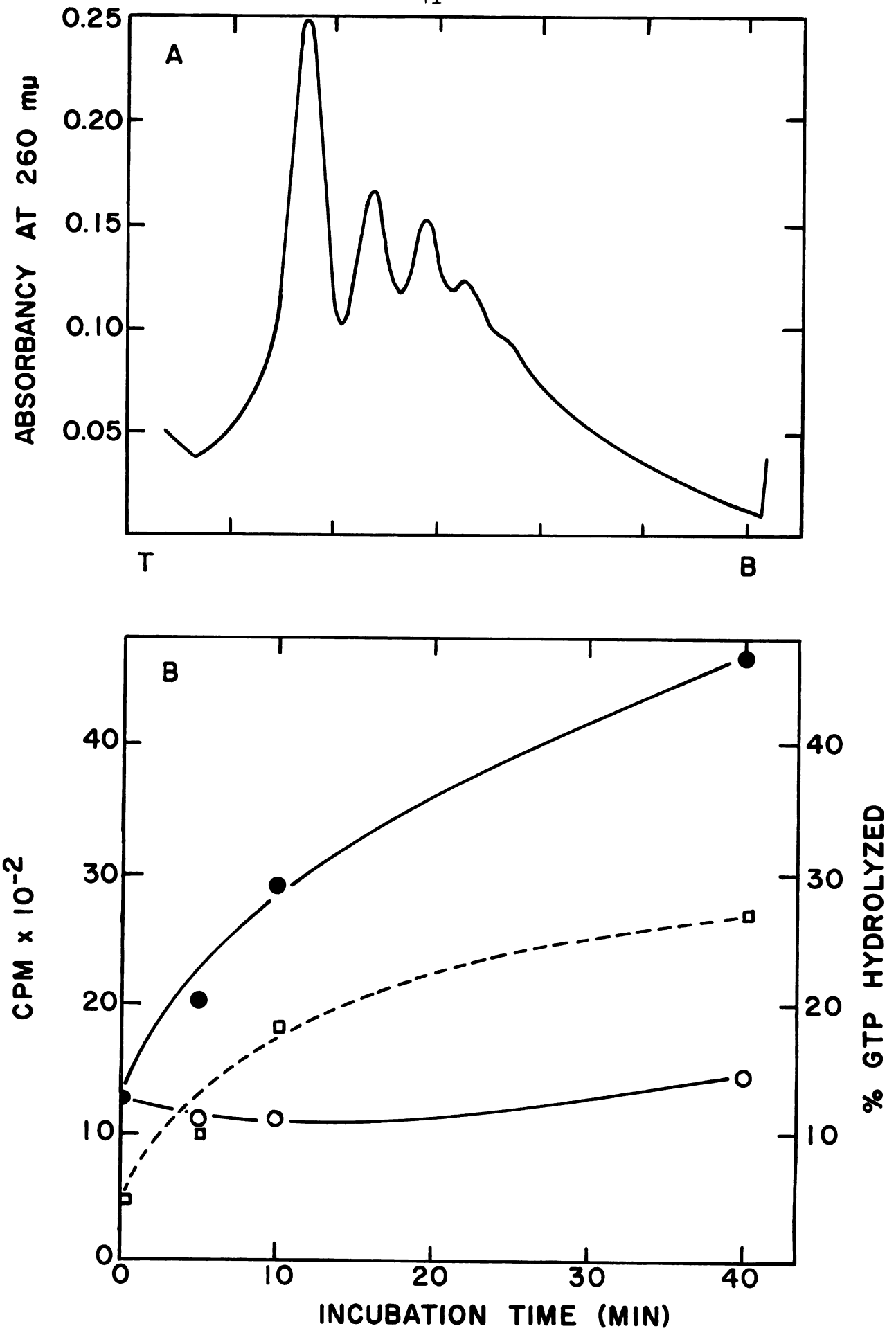
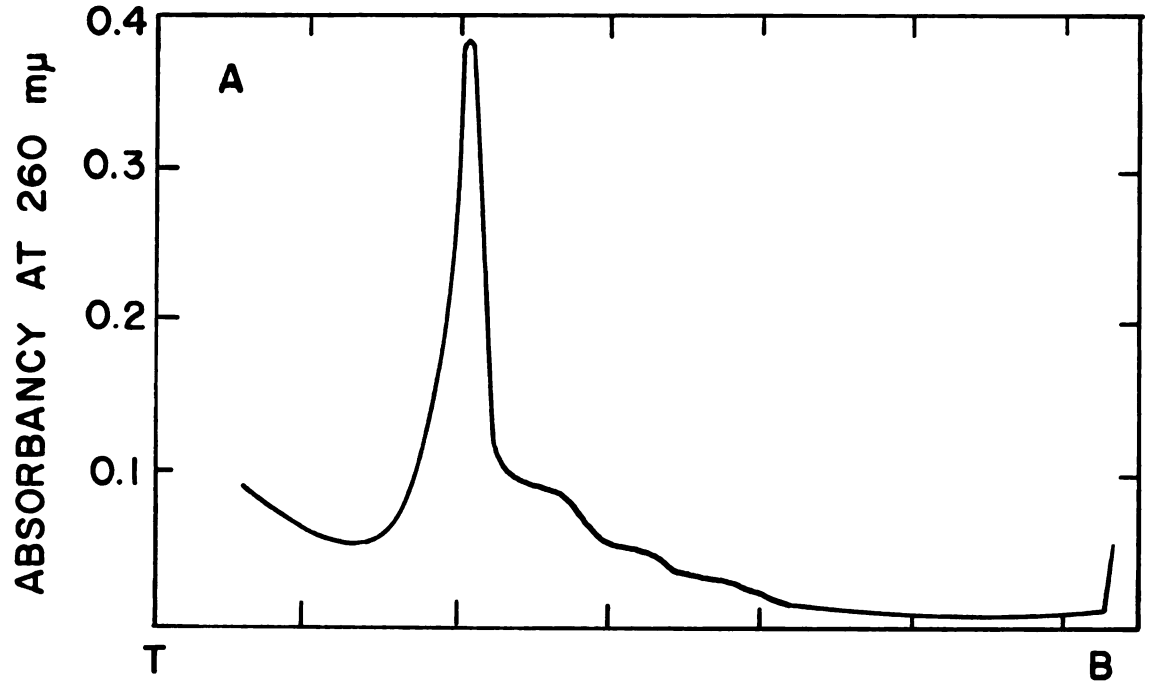

A
ABSORBANCY AT 260 mμ
0.25
0.20
0.15
0.10
0.05
T
B
B
CPM x 10⁻²
% GTP HYDROLYZED
40
30
20
10
0
0
10
20
30
40
INCUBATION TIME (MIN)

Figure 6: Effect of monosomes on GTP hydrolysis. Sixty mumoles of GTP added to 1 mg of monosomes per assay. Reactions were carried out as indicated in Figure 5. ●—● , (^{14}C) peptide released from ribosome + GTP, ○—○ (^{14}C) peptide released — GTP, □—□ per cent (^{32}P) GTP hydrolyzed.


A
ABSORBANCY AT 260 mμ
0.4
0.3
0.2
0.1
T
B

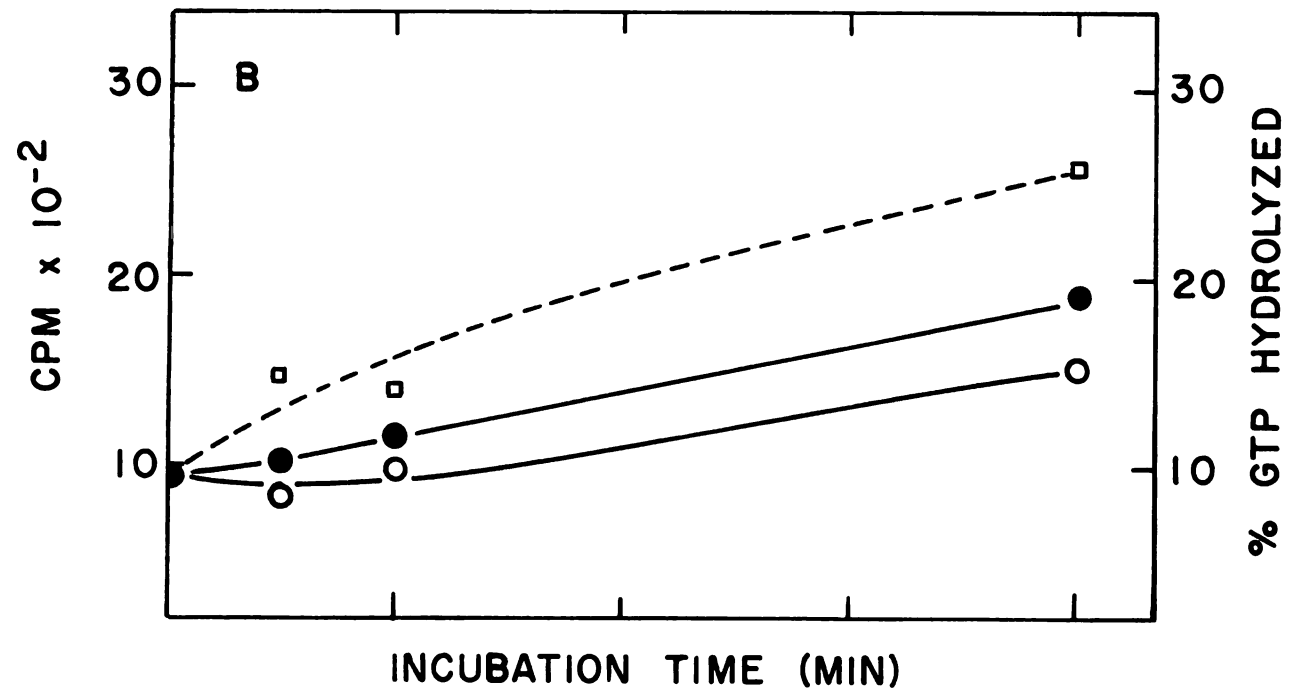

B
CPM x 10-2
30
20
10
% GTP HYDROLYZED
INCUBATION TIME (MIN)

Table I: Effects of Washing and Deoxycholate Treatment on Ribosomal Bound GTPase

	Products of Hydrolysis of (^{14}C) GTP (%)		
	(^{14}C) GTP	(^{14}C) GDP	(^{14}C) GMP
Pooled Ribosomes washed six and seven times	69.2	27.8	2.8
Ribosomes treated twice	65.0	34.6	0.4
Control 2X Ribosomes	41.4	55.7	2.8

Three mg ribosomes incubated with 60 mumoles (^{14}C) GTP (specific activity of 3 uc/umole) for 10 minutes at 37°. Conditions given in Methods.

that the GTPase is bound quite strongly to the ribosomes in reticulocytes since the GTPase hydrolyzing factor remains even after 6-7 washings.

Table II lists the effects of various other agents on the (^{14}C) GTP hydrolysis. The most startling result was the total lack of effect of pancreatic ribonuclease on the hydrolysis of GTP. The concentration of RNase used (5 ug/3 mg ribosomes) was sufficient to completely degrade the ribosomal particles. Similarly, parachloromercuribenzoate showed no effect upon the GTPase activity. Diisopropylfluorophosphate, a specific reagent for serine, residues in proteins, decreased the hydrolysis by 50%. Gougerotin, an antibiotic whose mode of action in the inhibition of peptide bond formation, is similar to that of puromycin, except that no peptide bond formation occurs (41), had no effect upon the ribosomal bound GTPase.

Binding of GTP to Ribosomes

As suggested in the introduction to this work, the mode of action of GTP may be an allosteric effect in addition to hydrolysis or it may be utilized in both types of reactions. An allosteric type of reaction would necessitate binding to the enzyme in order to effect a conformation change. One must keep in mind, however, that binding of GTP, in itself, may not be associated with any function

Table II: Effect of Various Agents on Ribosomal Bound GTPase

Agent	Products of Hydrolysis of (^{14}C) GTP (%)		
	(^{14}C) GTP	(^{14}C) GDP	(^{14}C) GMP
RNAase (5 ug/ml)	43.4	52.8	3.7
PCMB* (0.1 umole)	41.4	57.5	1.4
DIFP (30 umoles)	69.4	30.0	0.7
Gougerotin (1 umole)	47.3	50.3	2.5
Control	47.3	49.3	3.3

*GSH was omitted from the reaction mixture.

PCMB is parachloromercuribenzoate and DIFP is diisopropyl-fluorophosphate.

of the nucleoside triphosphate or it may be associated with an enzyme not directly involved in protein synthesis as, for example, nucleoside diphosphokinase.

Results of studies designed to search for such binding of GTP to ribosomes are presented below. Data presented in Figure 7 demonstrates that a binding of ^{14}C labeled GTP to ribosomes is present under the conditions of the assay. The extent of binding is linearly dependent upon the ribosome concentration in the assay and is largely absent in reaction mixtures containing ribosomes which had been previously heated to 65-70° for 5 minutes. Figure 8 presents the inactivation profile of the binding reaction as a function of the heating of ribosomes for 5 minutes at the temperature indicated prior to analysis for GTP binding. The temperature inactivation profile obtained is highly suggestive of thermal denaturation of an enzymic component.

The binding of ^{14}C labeled GTP to ribosomes as a function of GTP concentration is depicted in Figure 9. Maximum saturation of binding is obtained at a GTP concentration of 25 mumoles in a 1.0 ml reaction mixture containing 0.5 mg of ribonucleoprotein. The time course of the binding reaction (Figure 10) indicates a rapid initial reaction which reaches completion at 20 minutes of incubation. Zero time values indicate that the binding reaction also proceeds at

Figure 7: Dependence of (^{14}C) GTP binding on ribosome concentration. Twenty-five mumoles (^{14}C) GTP, (specific activity of 25 uc/umoles, was added to ribosomes in 1 ml of reaction mixture. ●—● radioactivity found. ○—○ radioactivity bound from controls preheated to 65-70° for 5 minutes. Reactions were carried out for 2 minutes at 37°.

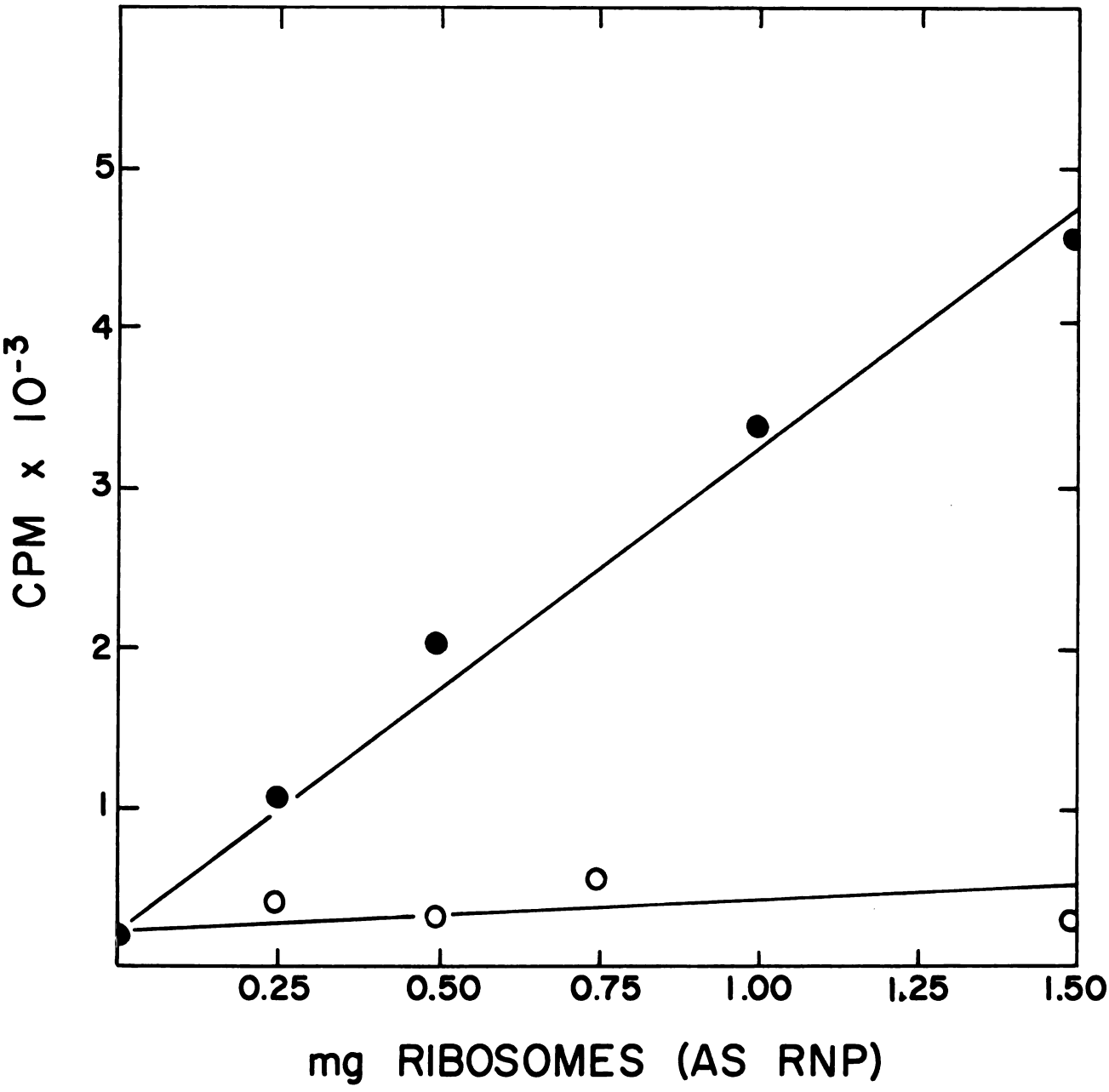

CPM x 10-3
5
4
3
2
1
0.25
0.50
0.75
1.00
1.25
1.50
mg RIBOSOMES (AS RNP)

Figure 8: Effect of preheating in binding of (^{14}C) GTP to ribosomes. 0.5 mg 2X ribosomes were pre-heated at the temperature indicated for 5 minutes. The reaction mixture was then cooled to 4°. Twenty-five mumoles of (^{14}C) GTP (specific activity of 25 uc/umoles was added. Samples were then incubated 2 minutes at 37°.

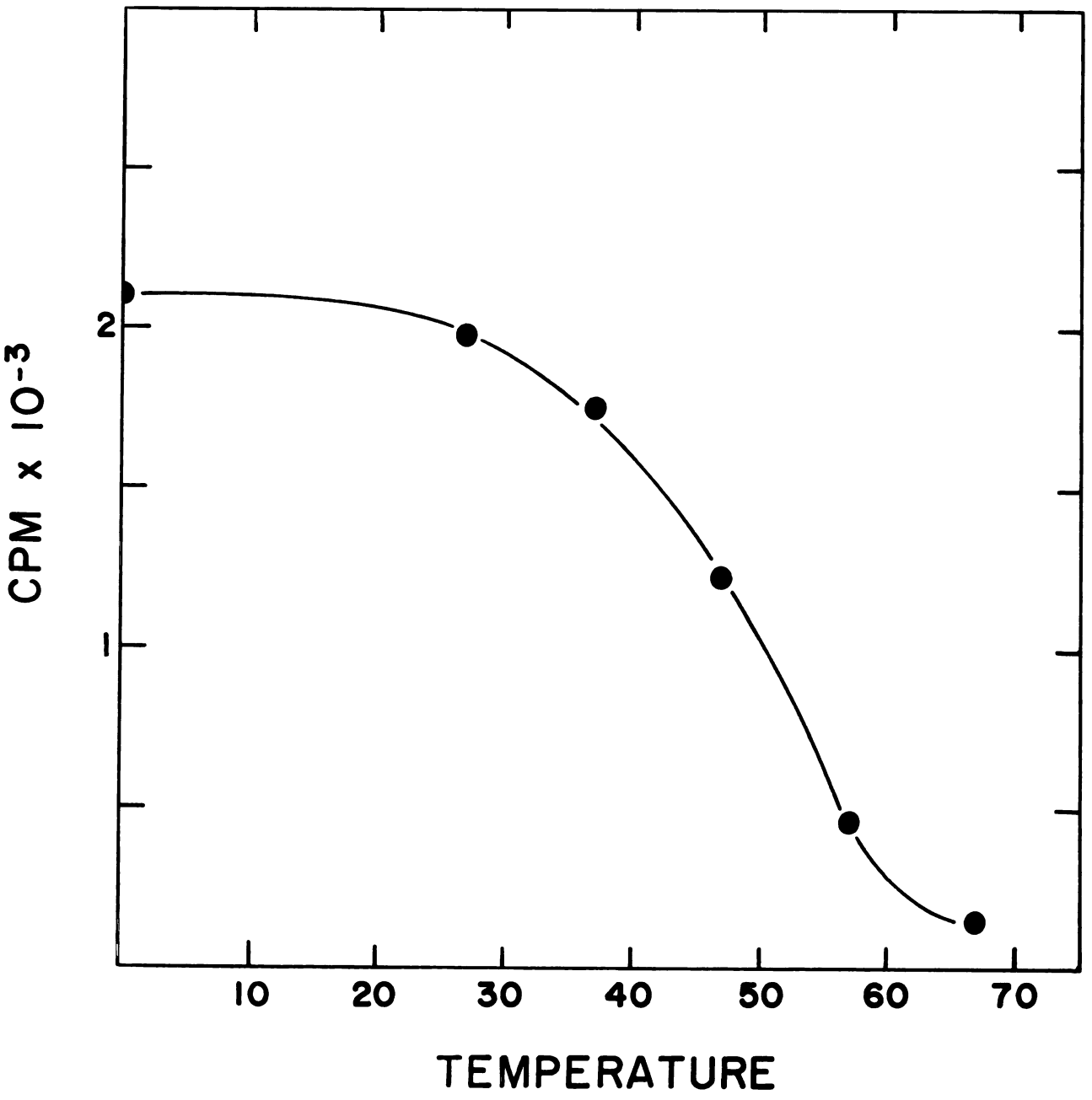

CPM x 10-3
2
1
10
20
30
40
50
60
70
TEMPERATURE

Figure 9: Binding of (^{14}C) GTP to ribosomes. Each assay contained 0.5 mg of ribonucleoprotein. ●—● , nontreated ribosomes, ○—○ , pre-heated ribosomes.

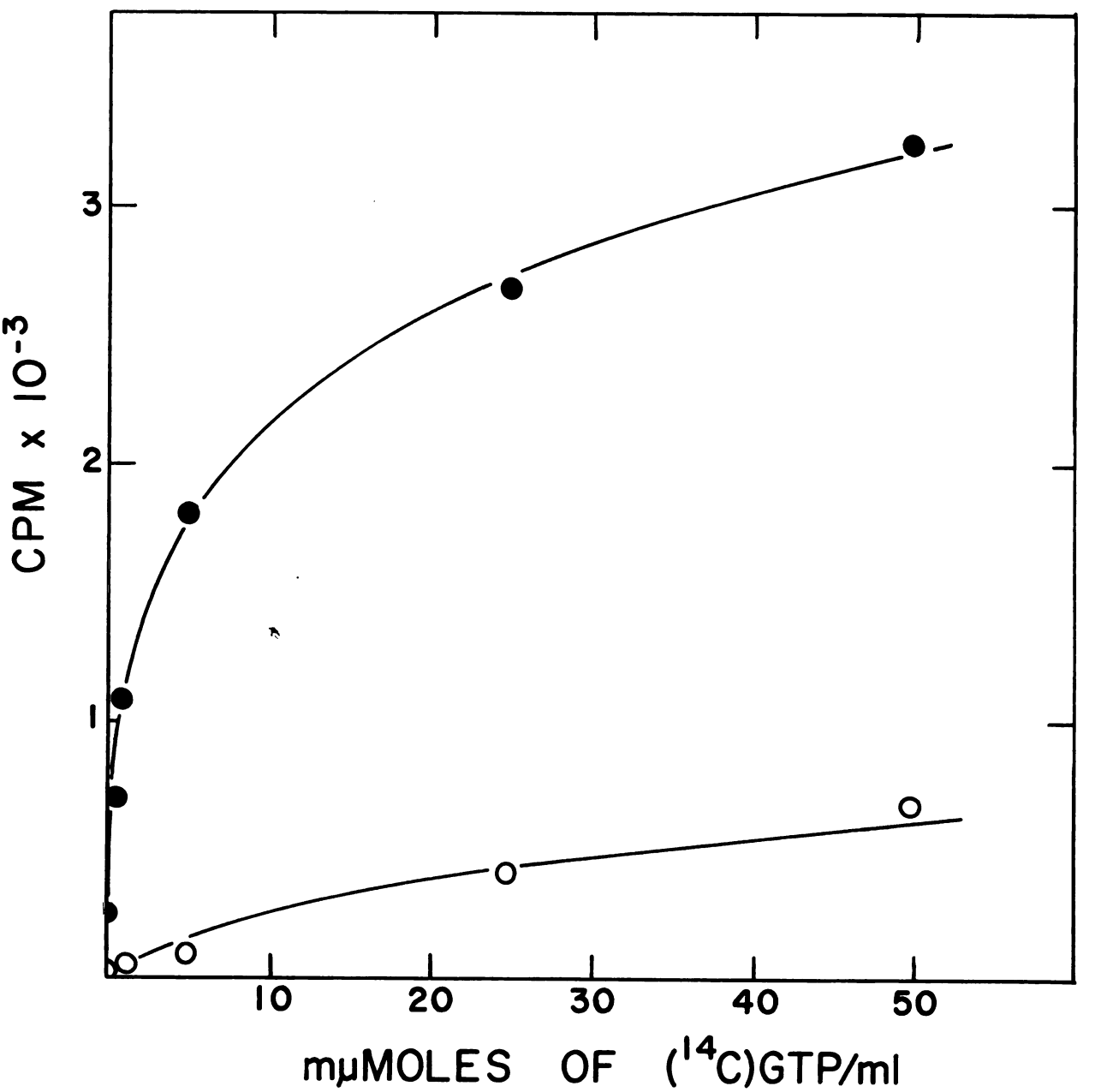
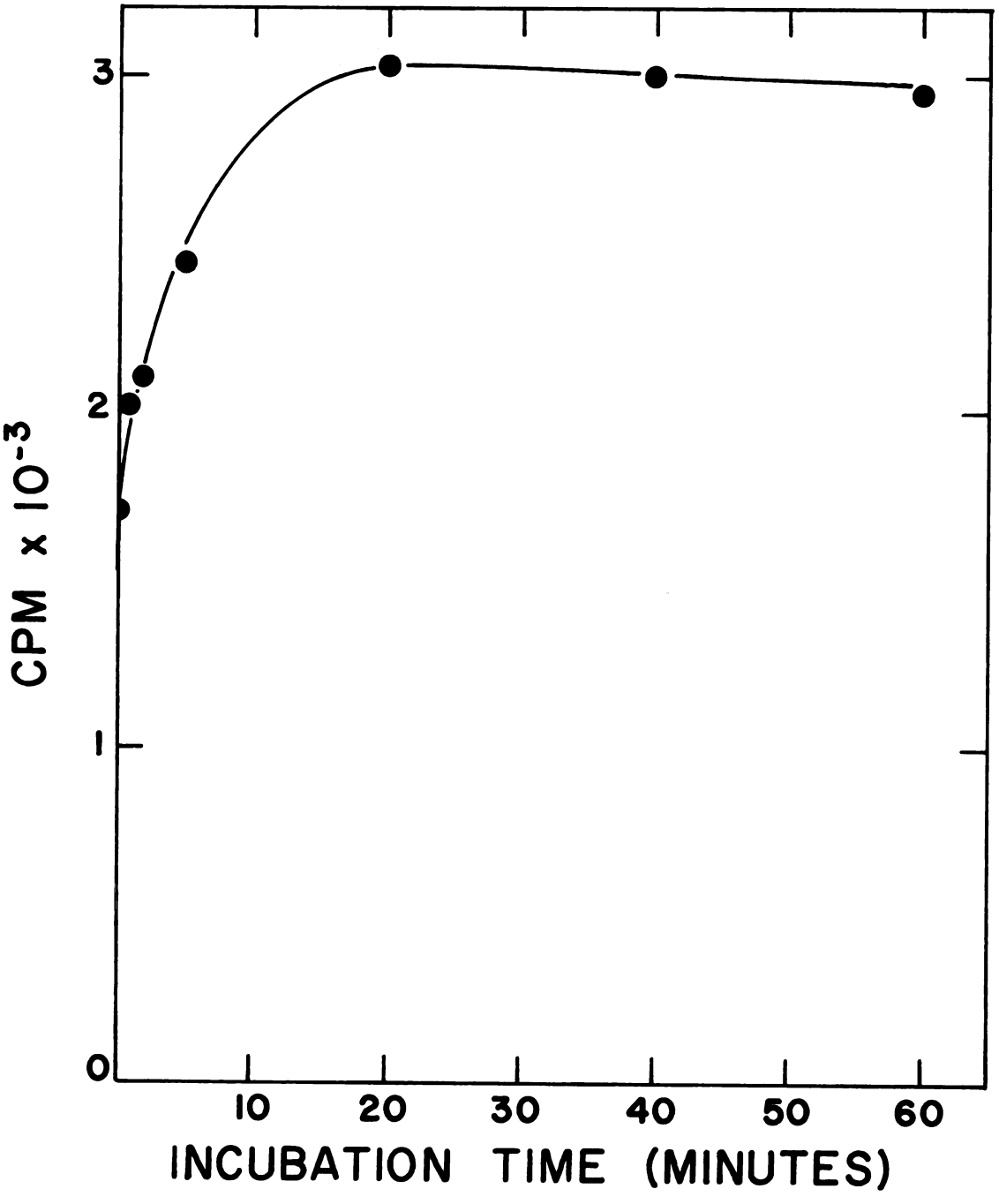

CPM x 10-3
3
2
1
10
20
30
40
50
mμMOLES OF (14C)GTP/ml

Figure 10: Effect of time on (^{14}C) GTP binding to ribosomes and 25 mumoles (^{14}C) GTP were incubated at 37° for the time indicated. Values have been corrected for the radioactivity present in the preheated controls.


CPM x 10-3
0
1
2
3
10
20
30
40
50
60
INCUBATION TIME (MINUTES)

reduced temperatures as during the preparation of the assays in an ice water bath. The ratio of moles of GTP bound per mole of ribosome was 1:4 at 2 minutes and 1:2 at 20 minutes. (^{14}C) GDP was also found to bind to ribosomes to a similar extent to that observed for (^{14}C) GTP (Figure 11). No binding of (^{14}C) labeled GMP could be demonstrated (Table III). Further, binding of ^{14}C labeled ATP to ribosomes was also detected but to a lesser extent than that of GTP or GDP. One mole of (^{14}C) ATP was bound per 8 moles of ribosomes after 20 minutes of incubation. Adding GTP at twice the concentration of the ATP in the assay reduces the (^{14}C) ATP binding by one half. The reverse experiment, that is, the reduction of labeled GTP binding by unlabeled ATP shows similar properties. Pretreating with ATP does not reduce the amount of (^{14}C) ATP bound beyond that observed when the ATP is added after the (^{14}C) GTP. In other experiments the binding of (^{32}P) GTP (Table IV) was reduced by 30% by the simultaneous addition of a ten fold excess of ATP. A twenty fold excess of ATP reduced the binding to 56% of the control. Preincubation with pancreatic RNase has a small effect, but protease pretreatment reduces the GTP binding to that observed in the heat inactivated controls indicating the protein nature of substances involved in the binding reaction.

Figure 11: Binding of (^{14}C) GDP to ribosomes. Twenty-five mumoles of (^{14}C) GDP (specific activity of 25 uc/umole) and 0.5 mg of ribosomes were incubated for 2 minutes at 37°. Values have been corrected for the binding observed with preheated ribosomes.

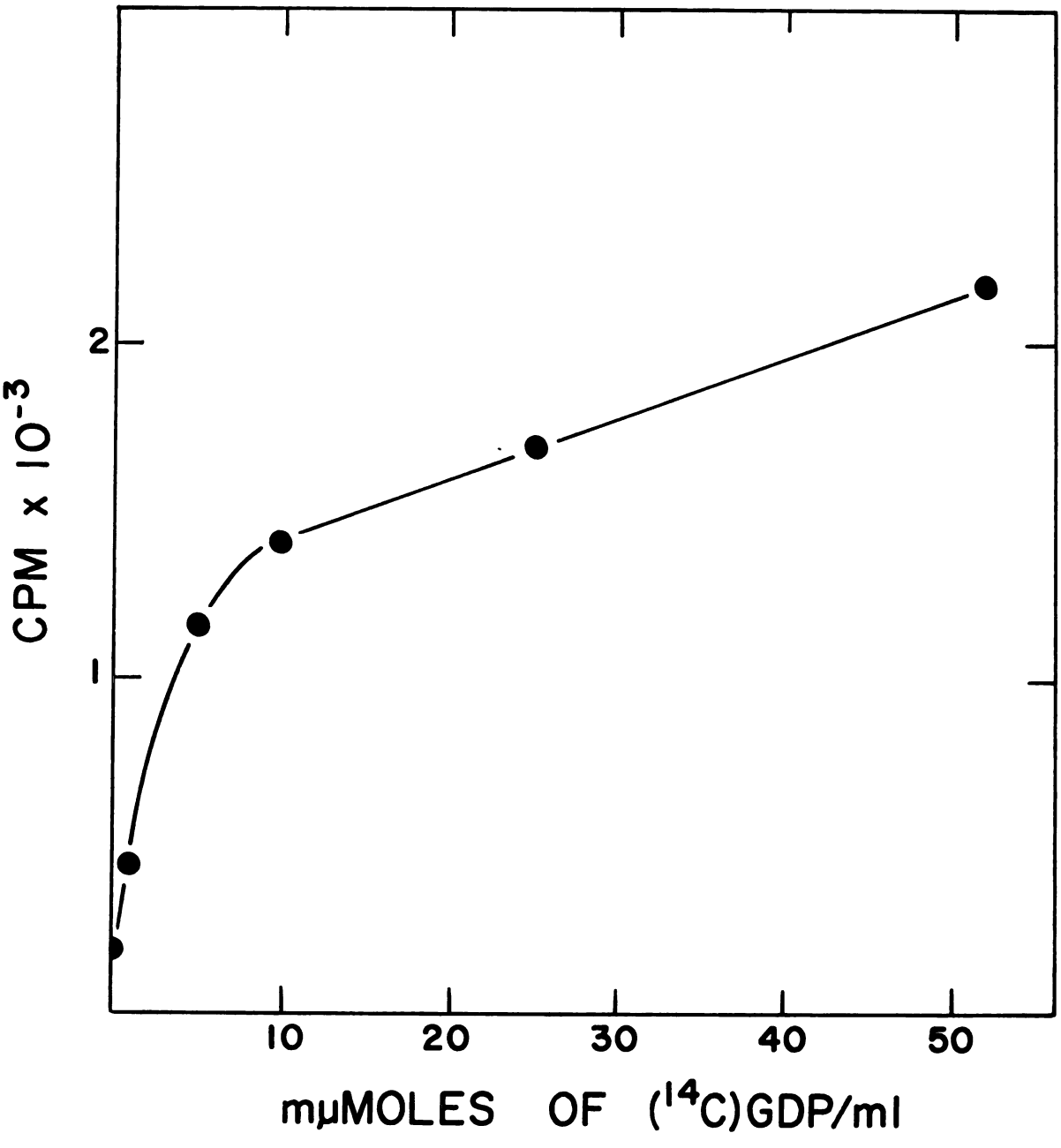

CPM x 10-3
2
1
10
20
30
40
50
mμMOLES OF (14C)GDP/ml

Table III: Binding Effects of ^{14}C Nucleotides

Nucleotide	Ribosomal Bound Radioactivity cpm
(^{14}C) GTP	3486
(^{14}C) GMP	81
(^{14}C) ATP	835
(^{14}C) ATP pretreated with 50 mumoles GTP	442
(^{14}C) GTP pretreated with 50 mumoles ATP	1771
(^{14}C) GTP added before 50 mumoles ATP	1855

Binding was carried out using 25 mumoles of nucleotide. The specific activity of (^{14}C) GMP and (^{14}C) GTP were 25 uc/mole, (^{14}C) ATP was of specific activity 36 uc/umole. Values for (^{14}C) ATP have been corrected for the specific activity difference. 0.5 mg of 1X ribosomes were used in each case. Assays were carried out as described in Methods.

Table IV: Binding of (^{32}P) GTP to Ribosomes

Nucleotide	Ribosomal Bound Radioactivity cpm
(^{32}P) GTP	1358
(^{32}P) GTP + 100 mumoles ATP	1151
(^{32}P) GTP + 250 mumoles ATP	957
(^{32}P) GTP + 500 mumoles ATP	645
(^{32}P) GTP Ribosomes pretreated with 10 ug of RNAase	978
(^{32}P) GTP Ribosomes pretreated with 10 ug of protease	167

25 mumoles (^{32}P) GTP of low specific activity (3 uc/umoles), 0.3 mg of yeast RNA hydrolysate was added to each assay to reduce nonspecific binding of nucleotides. 0.5 mg 1X ribosomes was added to each assay.

Washing of the ribosomes 4 times by resuspension and centrifugation (Figure 12) was found to reduce the capacity of ribosomes to bind GTP to approximately 30% of the original value observed with once sedimented ribosomes. The amount of GTP which could be bound to the ribosomal particles GTP then remained constant with washing up to and including the seventh wash. The reduction in GTPase activity with successive washings of the ribosomes correlates reasonably well with the reduction of GTP binding capacity following similar treatment of the ribosomes (see Table I).

The effect of various enzyme and protein synthesis inhibitors upon GTP binding to ribosomes is listed in Table V. Note that PCMB produces a slight reduction (24%) in GTP binding ; to ribosomes.

Finally, if binding is indeed indicative of the site of action of GTP in protein synthesis, we should see a variation in GTP binding patterns using ribosomes actively engaged in peptide synthesis as compared to those which are not. Warner (61) has shown increased labeling of amino acids in the polysomal fraction due to peptide synthesis. A comparison was therefore made between the GTP binding properties of ribosomal monomers as compared to polysomes. Six mg of 1X ribosomes were layered on a sucrose density gradient. Following centrifugation as described in Methods 1 ml fractions were collected from the gradient and assayed for

Figure 12: Retention of binding of (^{14}C) GTP capacity of ribosomes after repeated washing by resuspension and centrifugation. The concentration of ribosomes was adjusted to 0.5 mg/assay.

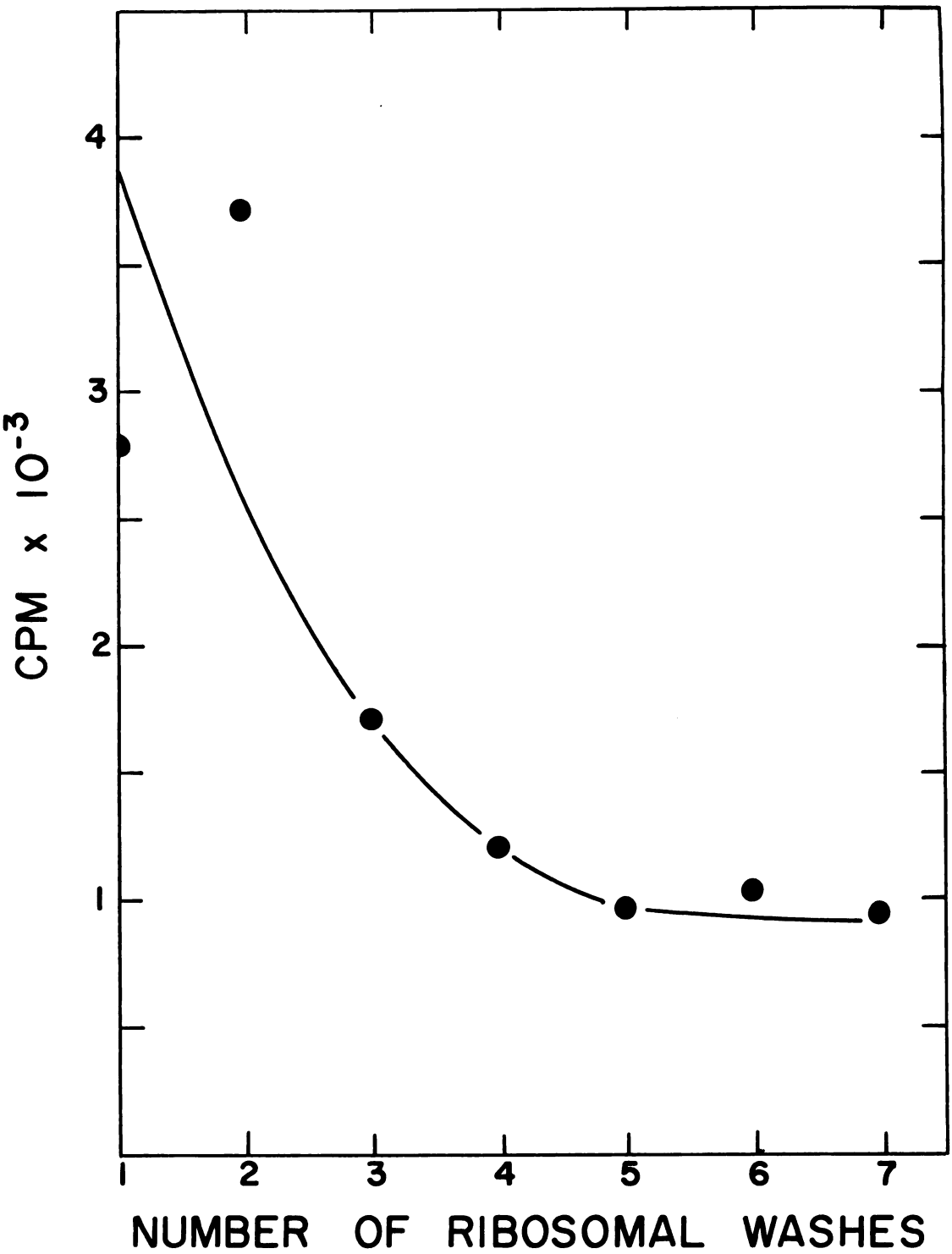

CPM x 10-3
4
3
2
1
1
2
3
4
5
6
7
NUMBER OF RIBOSOMAL WASHES

Table V: Effect of Various Agents on GTP Binding to Ribosomes

		Bound Radioactivity	
		Treated Ribosomes cpm	Untreated Ribosomes cpm
PCMB	1 umole	1420	1863
DIFP	5 mumoles	1740	1782
DOC	0.5 mg	1968	2222
Puromycin	1 mumole	2032	2312
Streptomycin	60 mumoles	1999	2312
Chlortetrachline	20 mumoles	1997	1804
Gougerotin	1 mumole	2094	1942

(^{14}C) GTP had a specific activity of 25 uc/umole, 0.5 mg of 2X ribosomes were added to each assay. Untreated controls are included for comparison as the assays were performed using several different preparations of ribosomes.

(^{14}C) GTP binding. Figure 13 illustrated distribution of radioactivity through the polysomal fraction. The binding coincides with the optical density profile. The distribution is ordered, that is, no area possesses a greater ratio of optical density to radioactivity than another. It can be concluded therefore that the binding does not show an increase in the area of higher protein synthesis, that is, the polysomal region of the gradient.

Since polysomes do not show any differences in GTP binding when compared to monosomes, an examination was made of binding by ribosomal subunits in order to determine if differences existed in the binding ability of 1 subunit as compared to the other. Ribosomes were dissociated into their respective subunits (see Methods), layered on a sucrose gradient and treated in a manner similar to the polysomes and monosomes in the preceding experiment. Figure 14 shows no great differences in distribution of GTP binding between the 40S and 60 subunits. Thus, GTP binding gives us no clue of GTP action in relation to the A or P site (see model) since according to the model presented only the 60S fraction should contain these sites.

Figure 13: Distribution of radioactivity from binding of (^{14}C) GTP to polysome and monosomes. Bars indicate radioactivity. Spaces between the bars were utilized for preheated controls which were used as blank values.

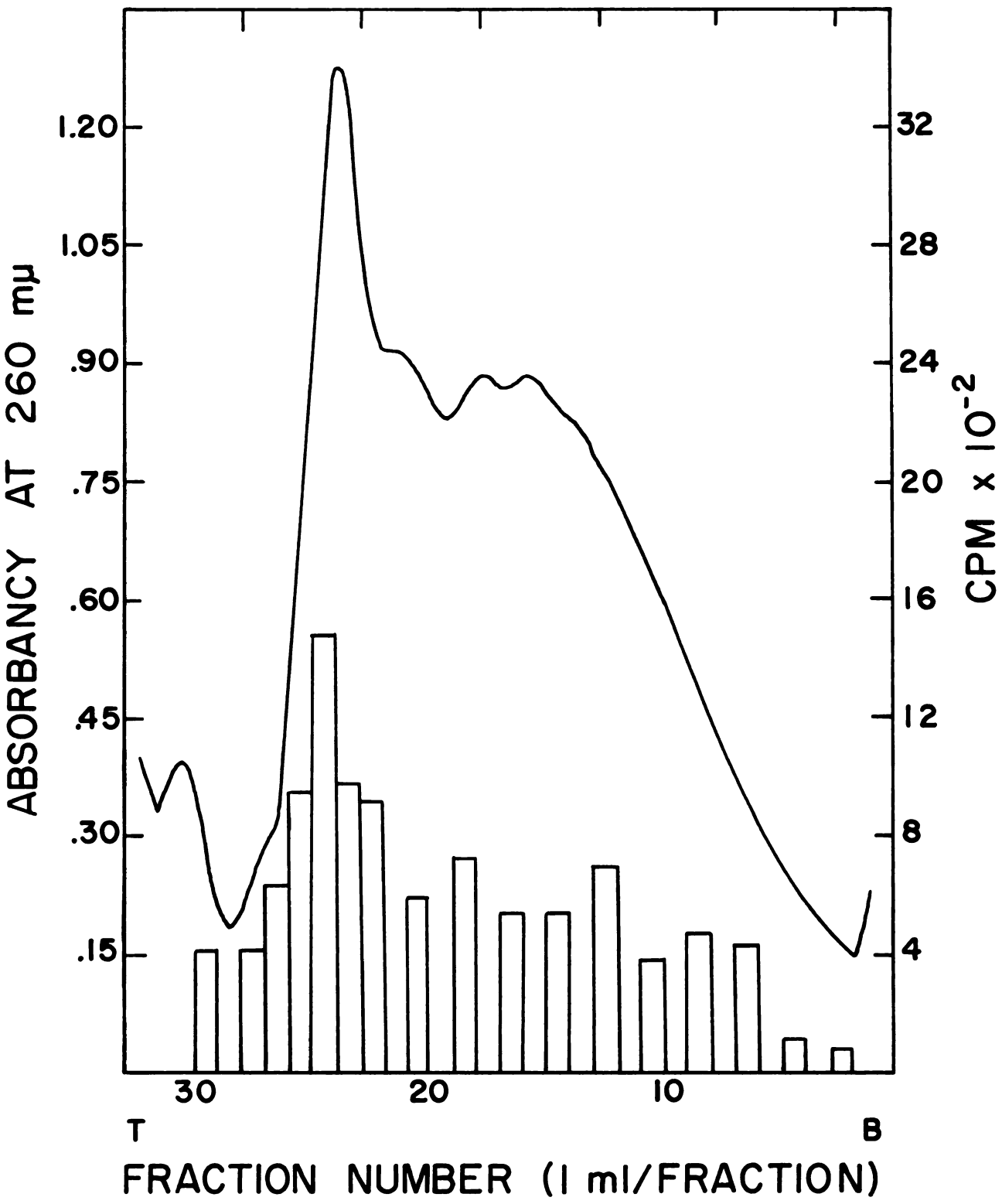
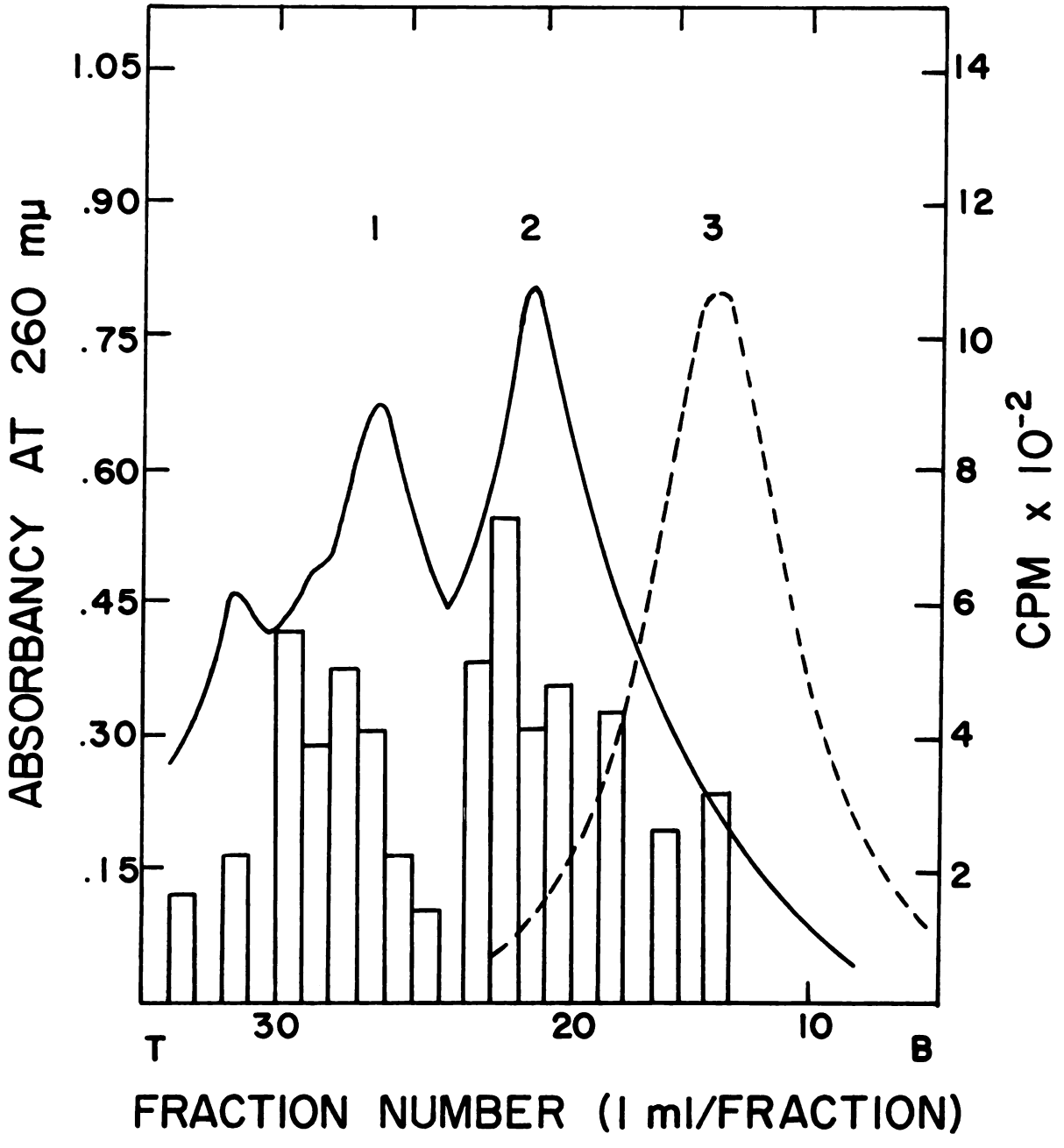

ABSORBANCY AT 260 mμ
1.20
1.05
.90
.75
.60
.45
.30
.15
CPM x 10⁻²
32
28
24
20
16
12
8
4
30
20
10
T
B
FRACTION NUMBER (1 ml/FRACTION)

Figure 14: Binding of (^{14}C) GTP to subunits of ribosomes. 1 is 40S subunit, 2 is 60S subunit and 3 is 78S ribosomes run simultaneously but on a separate gradient and included in the graph as a marker. S values are inferred from their relative positions in the gradient.

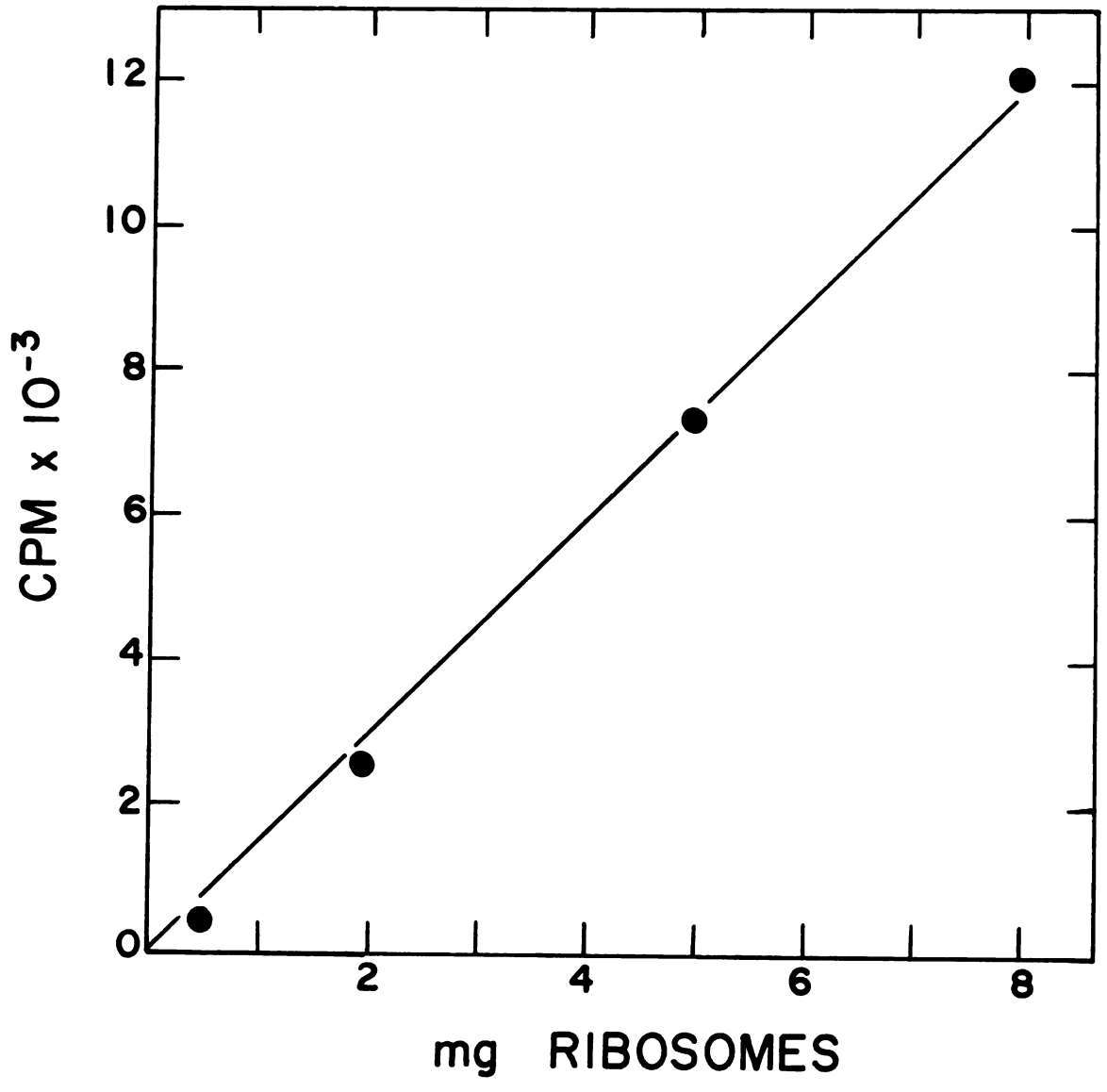

1.05
.90
.75
.60
.45
.30
.15
ABSORBANCY AT 260 mμ
14
12
10
8
6
4
2
CPM x 10-2
1
2
3
T
30
20
10
B
FRACTION NUMBER (1 ml/FRACTION)

Ribosomal Phosphate Intermediate

The observation that both (^{32}P) GTP and (^{14}C) GTP could be shown to bind to ribosomes suggested that GTPase might function through a high energy intermediate involving transfer of the gamma phosphate of GTP. The major problem confronting an investigator in this type of study is the nonspecific binding of ^{32}P leading to extraordinarily high blank values. The method developed by Bieber (49) to determine the presence of phosphoproteins in particles from bovine heart mitochondria produced blank values which were well within a workable range (see Methods). The purity of the (^{32}P) GTP was such that any phosphoprotein complex formed could arise only from the gamma position of (^{32}P) GTP (Figure 2). The contamination by ^{32}Pi was negligible. No label appears in the GDP portion of the chromatogram either before or after hydrolysis by ribosomes indicating no exchange of labeled phosphate was occurring between the beta and gamma positions of the molecule.

The dependence of the phenol extractable radioactive material on ribosomal concentration can be seen in Figure 15. The linearity of the values on the graph is indicative of the sensitivity of the method since 4×10^7 cpm of (^{32}P) GTP were added to each assay. The values shown in

Figure 15: Influence of ribosomal concentration on phenol extractable ^{32}P. Ribosomes were incubated with 5 mumoles of (^{32}P) GTP for 30 seconds at 37°.


CPM x 10-3
12
10
8
6
4
2
0
2
4
6
8
mg RIBOSOMES

the graph are corrected for those counts present in the pre-heated controls. Figure 16 shows the effect of increasing the concentration of (^{32}P) GTP in the assay. Phenol extractable radioactivity increased throughout the range of GTP concentrations studied. The time course of the formation of the extractable material proved most interesting in that the curve was linear through 5 minutes (Figure 17) but a change in kinetics was then experienced. Thus one of the parameters of the assay had become limiting. Such a curve is difficult to explain on the basis of a true phosphate intermediate since one would expect the intermediate to become saturated early in the reaction and the values would then be expected to level off. However, if a phosphoryl acceptor of a transitory type is present, that is, an intermediate which passes on the phosphate but which does exist for a finite period of time, such kinetics could be obtained. The other possibility, of course, is that the results reflect the presence of a multiple phosphoenzyme system. Figure 18 shows the time course obtained by trichloroacetic acid precipitation. The precipitation and sample preparation are given in Methods. The intermediate is stable to TCA treatment and the sodium hydroxide step in the sample preparation which is sufficient to cleave the aminoacyl-sRNA bond. The phospho compound thus seems to be quite stable to acid which would appear to

Figure 16: The effect of (^{32}P) GTP concentration on phenol extractable radioactivity. One mg of ribosomes was incubate 10 minutes at 37° with labeled GTPase indicated. Phenol extraction and counting procedures are described in Methods. ●—● , with ribosomes, ○—○ , with preheated ribosomes.

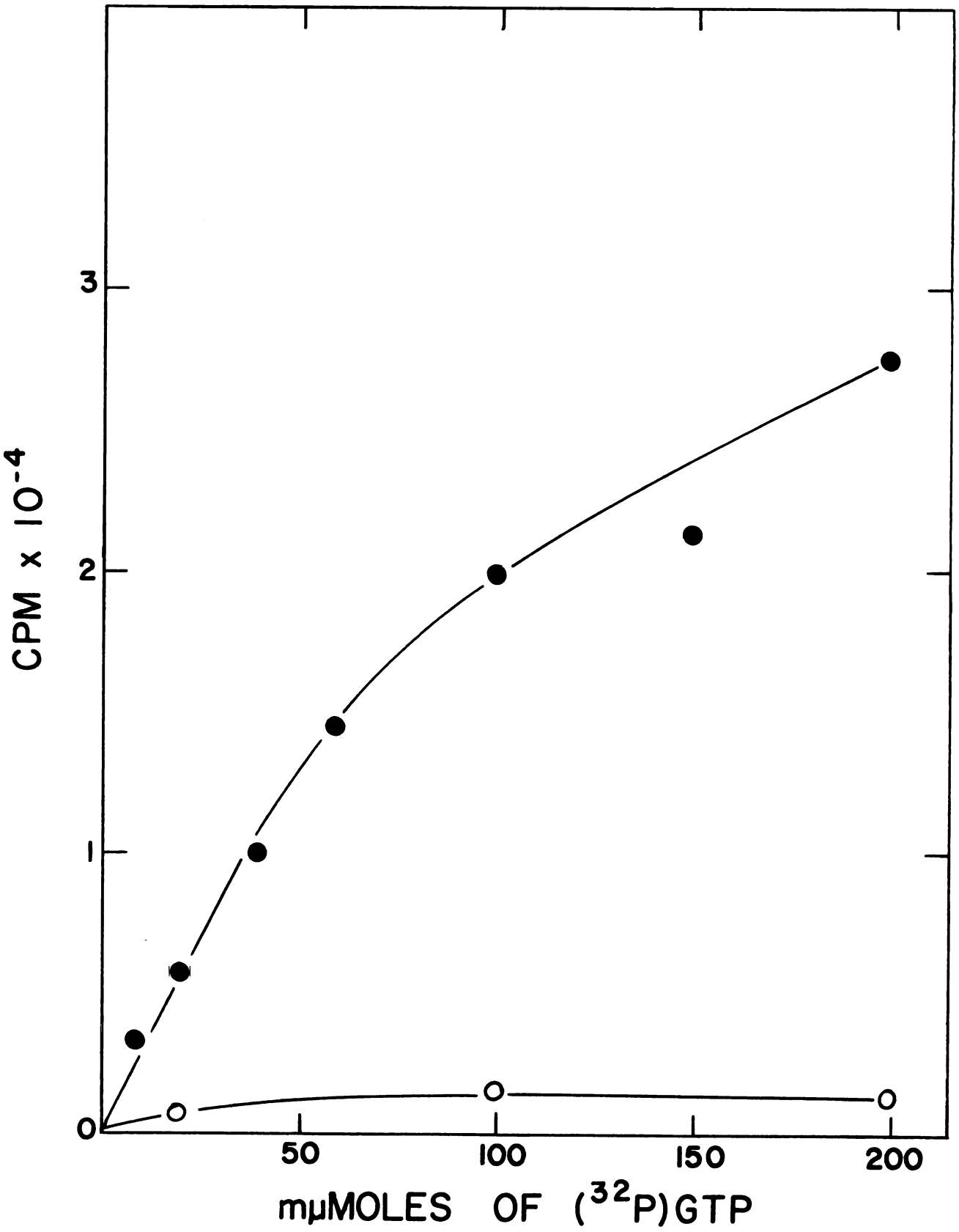

CPM x 10⁻⁴
3
2
1
0
50
100
150
200
mμMOLES OF (³²P)GTP

Figure 17: The effect of time of incubation upon the formation of a phenol extractable labeled material. One mg ribosomes was incubated with 20 mumoles of (^{32}P) GTP at 37° for the indicated times. ●—● ribosomes. O—O , preheated ribosomes.

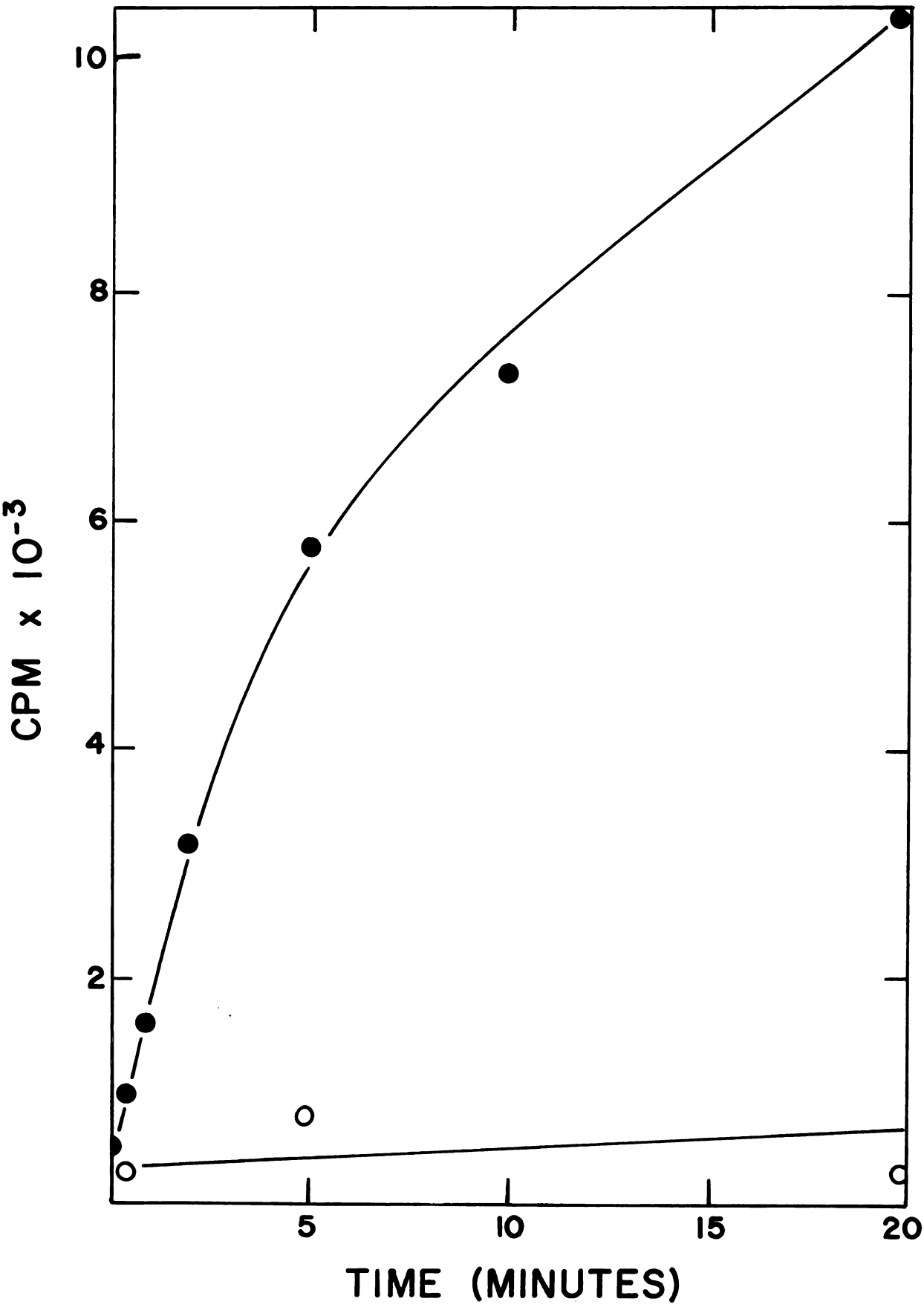
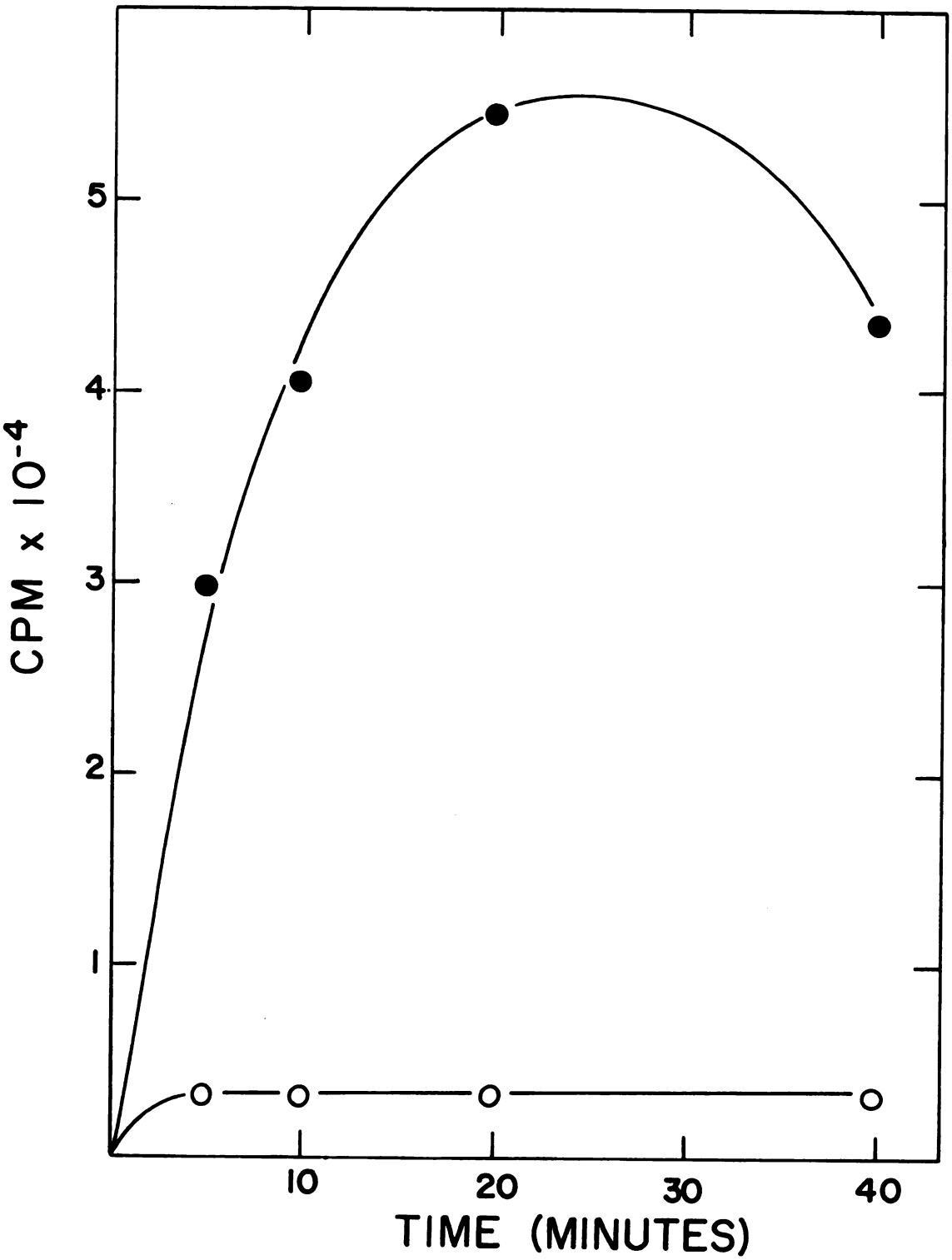

10
8
6
4
2
CPM x 10-3
5
10
15
20
TIME (MINUTES)

Figure 18: The effect of time on the formation of trichloroacetic acid precipitable, ribosomal bound radioactivity from (^{32}P) GTP. Twenty mumoles (^{32}P) GTP were incubated with 1 mg of ribosomes for the indicated period of time. ●—● ribosomes. ○—○, preheated ribosomes.

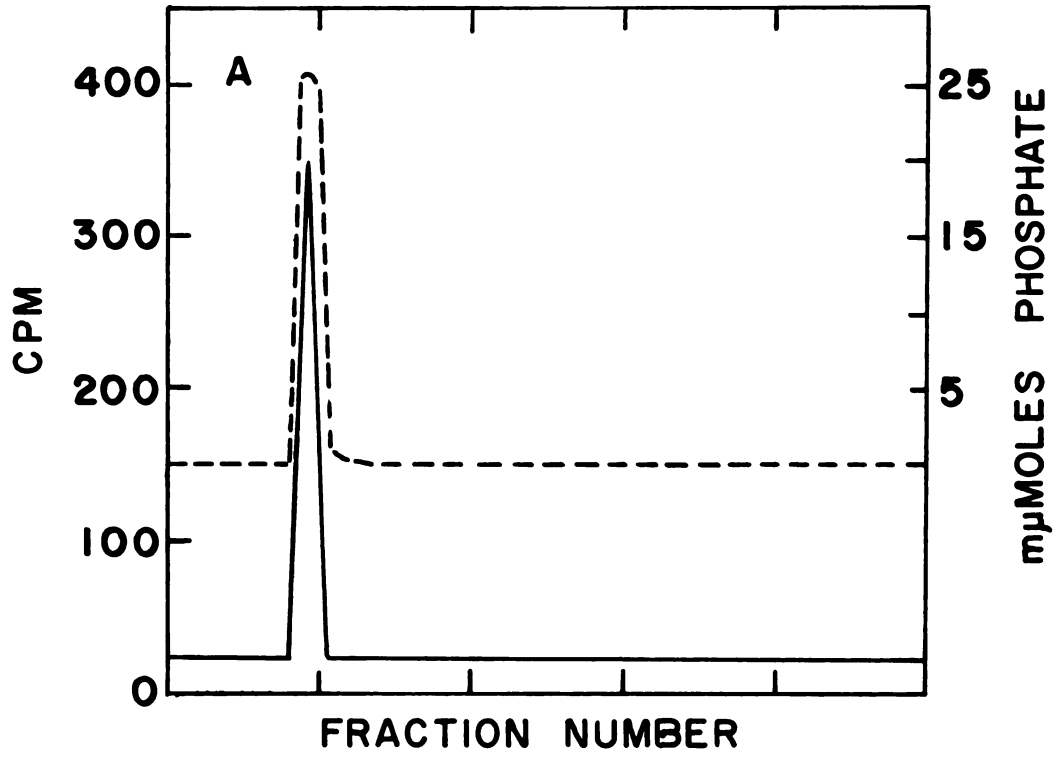

CPM x 10-4
5
4
3
2
1
10
20
30
40
TIME (MINUTES)

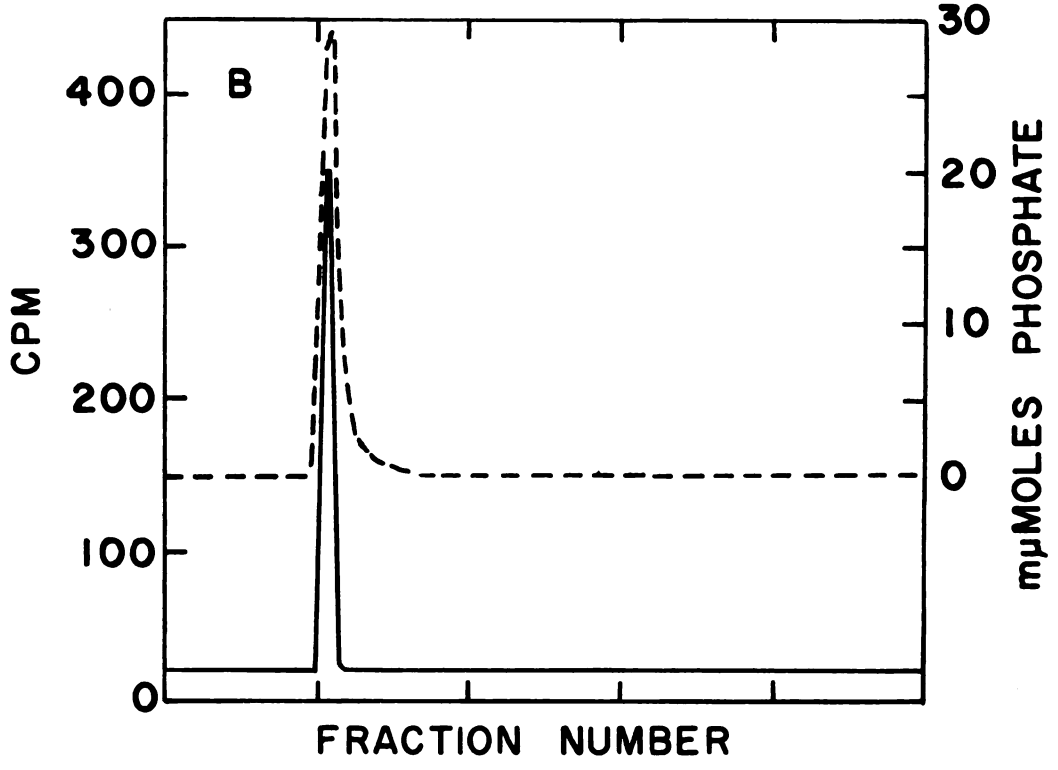
exclude a phosphorus to nitrogen bond. Pretreatment of the ribosomes with pronase reduces the phenol extractable radioactivity values comparable to those found in assays using preheated ribosomes. Susceptibility to pronase indicates that the phenol extractable material is indeed a phosphoprotein. The phosphoprotein obtained from a phenol extract was also found to be unstable to either extensive acid or base hydrolysis (Figure 19a and b). However, the denatured protein obtained from the phenol extraction procedure was solubilized by trypsin digestion for 6 hours at 37°. An aliquot of the trypsin digest was added to the phosphate assay mix (see Methods). Only 10% of the radioactivity (1,000 cpm) were found in the organic (inorganic phosphate) phase of the phosphate assay. One may conclude, therefore, that the intermediate is not phosphohistadine (stable to TCA) nor a nucleotide (unstable to acid or base hydrolysis) but probably is a protein bound form of phosphate.

Isolation of a Reticulocyte GTPase

The previous studies have indicated a GTPase is associated with ribosomes which is largely solubilized by repeated washing of the ribosome. 1X ribosomes contain approximately 10% of the total of GTPase present in the lysate supernatant fraction. The high speed supernatant

Figure 19: Chromatography of the phenol extractable phosphoprotein following acid or base hydrolysis. Precipitate from phenol extract dried, hydrolyzed in 1.5 ml of 3N KOH in sealed tube or 100° for 4 hours or hydrolyzed in 1.5 ml 6N HCl in sealed tube at 100°for 14 hours. Samples were diluted, neutralized and passed over a Dowex-1 Cl^- column (2 x 15 cm). Elution was achieved by means of a linear gradient containing 0.01 to 1 M NH_4HCO_3 buffer at pH 8.5. Radioactivity (solid line) was determined by scintillation counting in Bray's solution. Phosphate was determined as in Methods (dotted line).

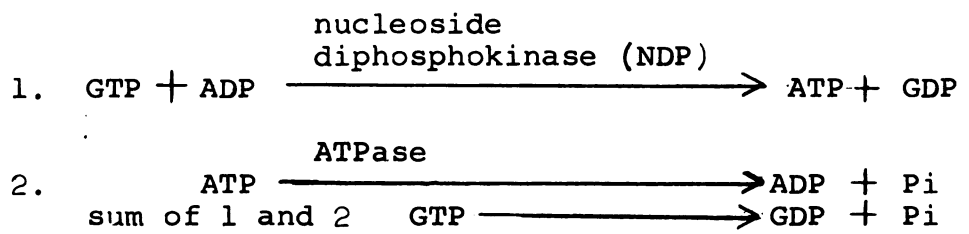

A
400
300
200
100
0
CPM
25
15
5
mμMOLES PHOSPHATE
FRACTION NUMBER


B
400
300
200
100
0
CPM
30
20
10
0
mμMOLES PHOSPHATE
FRACTION NUMBER

fraction therefore, would be the obvious starting point for the isolation of a reticulocyte GTPase. In addition, the pretreatment of ribosomes with RNAase has shown that the GTPase can be "uncoupled", that is, catalyze the hydrolysis of GTP in the absence of ribosomes. In the beginning of this study two decisions were made:

1. Analyses would rely on GTPase activity only, not as a function of aminoacyl-sRNA binding, etc.
2. An attempt would be made to separate a GTPase from other activities which may give rise to the same products.

This second decision made it necessary to determine ATPase and nucleoside diphosphokinase activity at each step in the purification. It is entirely likely the following sequence could take place which would result in an apparent GTPase activity:



ADP would need be present in only calalytic amounts to serve in this series of reactions. In addition to the argument of GTP specificity, any studies of phosphoprotein intermediates become suspect if NDP kinase is present in the reaction mixture since this enzyme may form a phosphate

intermediate (63).

The assay used for GTPase and ATPase is given under Methods. Initial studies indicated that glutathione normally present in the release assay, interfered with the development of color in the phosphate assay. The need for a sulfhydryl reagent which was found using more purified fractions was not apparent with crude enzyme preparations since addition of PCMB to an ammonium sulfate fraction resulted in retention of more than 50% of the GTPase activity (Table VI). Magnesium ion was shown to be required but could be replaced to a certain extent by manganous ion (Figure 20). Kinases which hydrolyze nucleoside triphosphates forming nucleoside diphosphates and inorganic phosphate generally require magnesium or manganous ion in a stoichiometric relationship to the nucleoside triphosphate substrate (64). Other requirements, such as the pH at which to study the reaction (7.2 at 37°) and potassium ion concentration of the assay were initially assumed to be similar to the release system. Further studies, however, showed these conditions to be far from optimal for the GTPase activity.

The initial step in the fractionation of the high speed supernatant involved the removal of RNA by a streptomycin precipitation. There was no large increase in specific activity (Table VII) but the step removed much in-

Table VI: Effect of PCMB on GTPase and ATPase Activities

Enzyme Source	Activity (Units)	% Decrease in activity
30-60% Ammonium sulfate fraction + GTP	79	
+PCMB + GTP	47	41
+ ATP	104	
+PCMB + ATP	61	41

Reaction was carried out at 37° with 3 mg of enzyme fraction per assay. Assay conditions are given under Methods. 0.1 uM PCMB was added where indicated. A unit of activity for GTPase or ATPase is defined as 1 mole of GTP (or ATP) hydrolyzed in 20 minutes at 37° per mg of enzyme.

Figure 20: Effect of magnesium and manganous ions on GTPase activity of the 30-60% ammonium sulfate fraction. Three mg of protein were incubated with 400 mumoles of GTP for 20 minutes at 37°. Inorganic phosphate was assayed as in Methods. values have been corrected by assaying heat denatured controls. ●—● , magnesium ion, ○—○, manganous ion. EDTA pretreatment consisted of the addition of EDTA to 50 mM to the reaction mixture.

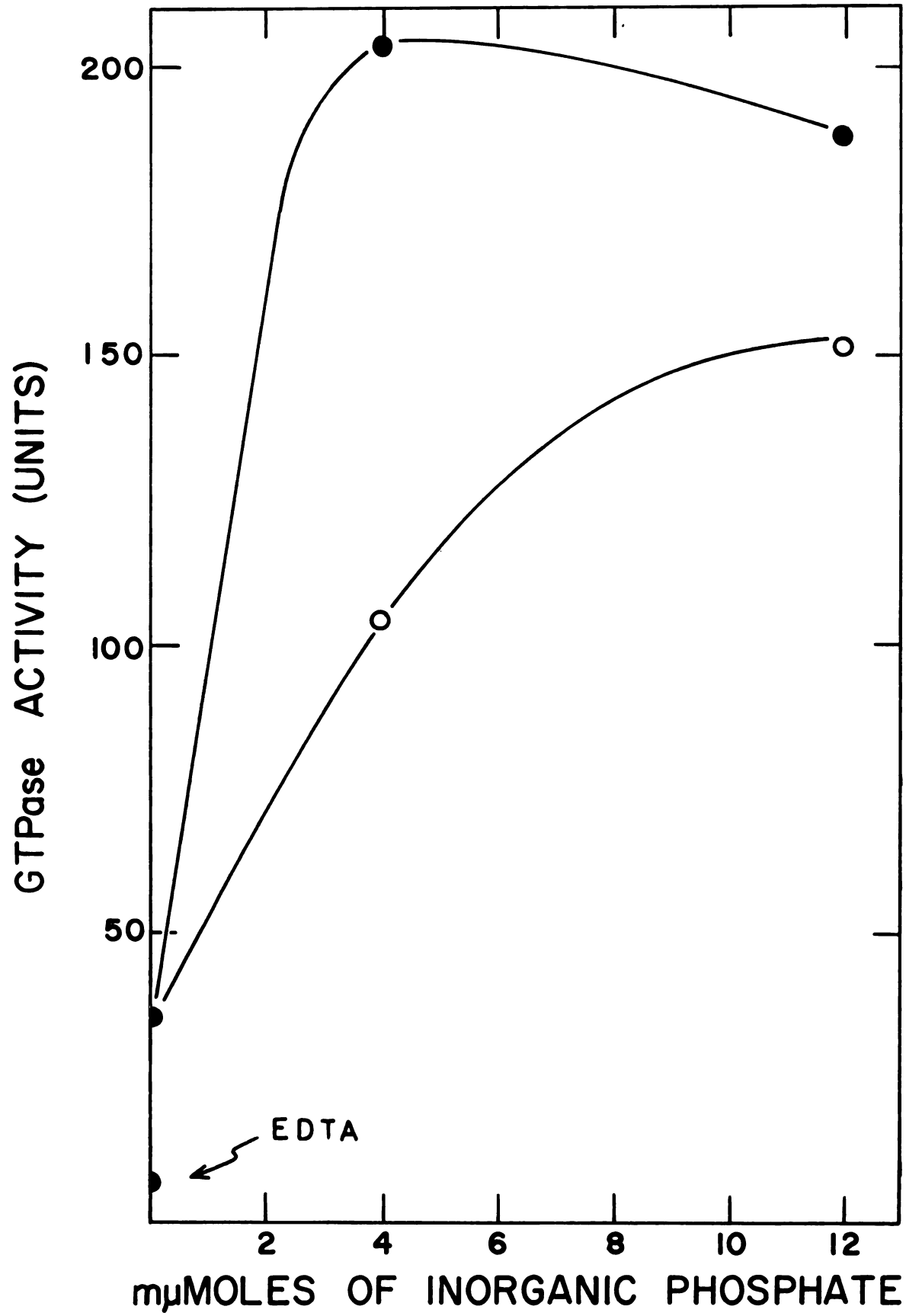

200
150
100
50
GTPase ACTIVITY (UNITS)
EDTA
2
4
6
8
10
12
mμMOLES OF INORGANIC PHOSPHATE

Table VII: Effect of Streptomycin Sulfate Treatment on High Speed Supernatant Fraction

	High Speed Supernant Fraction	Streptomycin Treated Fraction
Volume (ml)	188	220
Protein (mg)	9,400	8,800
GTPase (units)	54,500	58,000
Specific Activity (units per mg)	5.8	6.6
ATPase (units)	30,000	66,000
NDP kinase (units)	2.5×10^4	2.4×10^4

Protein was determined as in Methods. A unit of NDP kinase has been defined as the number of mumoles of ATP produced (via TPN reduction) in 20 minutes at 37°/mg of enzyme.

organic phosphate or "free phosphate" (65). Preliminary ammonium sulfate fractionation (Figure 21) revealed that ATPase activity was higher than GTPase activity in all fractions except the 50-60% and 60-70% of saturation fractions. The 30-60% fraction was initially chosen for further study since the 60-70% of saturation fraction was grossly contaminated with hemoglobin. Using the fraction obtained between 30-60% of saturation, the addition of ADP to an equal number of moles of GTP was found to cause a 28% increase in GTPase activity. This was an excellent indication the NDP kinase system coupled to an ATPase worked well under these conditions.

The following list indicates the methods which were tried in order to further purify the enzyme. All methods were studies in depth and resulted in either a reduced specific activity or no recoverable activity. These were DEAE Sephadex, DEAE cellulose, carboxymethyl Sephadex, carboxymethyl cellulose, isoelectric precipitation, ethanol fractionation, alumina C gamma adsorbtion and $CaPO_4$ gel adsorbtion.

Fractionation by Sephadex G-100 fractionated the 30-60% ammonium sulfate fraction into at least 2 peaks (Figure 22). Each peak was pooled as indicated and the protein concentrated by precipitation with ammonium sulfate at 90% of saturation. The GTPase activity was found to be approxi-

Figure 21: Ammonium sulfate fractionation of streptomycin treated high speed supernatant fraction. Precipitation with ammonium sulfate was carried out as described in Methods. The pH of the solutions was constant at pH 7.5 by increasing the Tris-HCl to 0.1 M. Fractionation was carried out at 4°. Dotted line indicates GTPase, solid line indicates ATPase and shaded area indicates total protein.

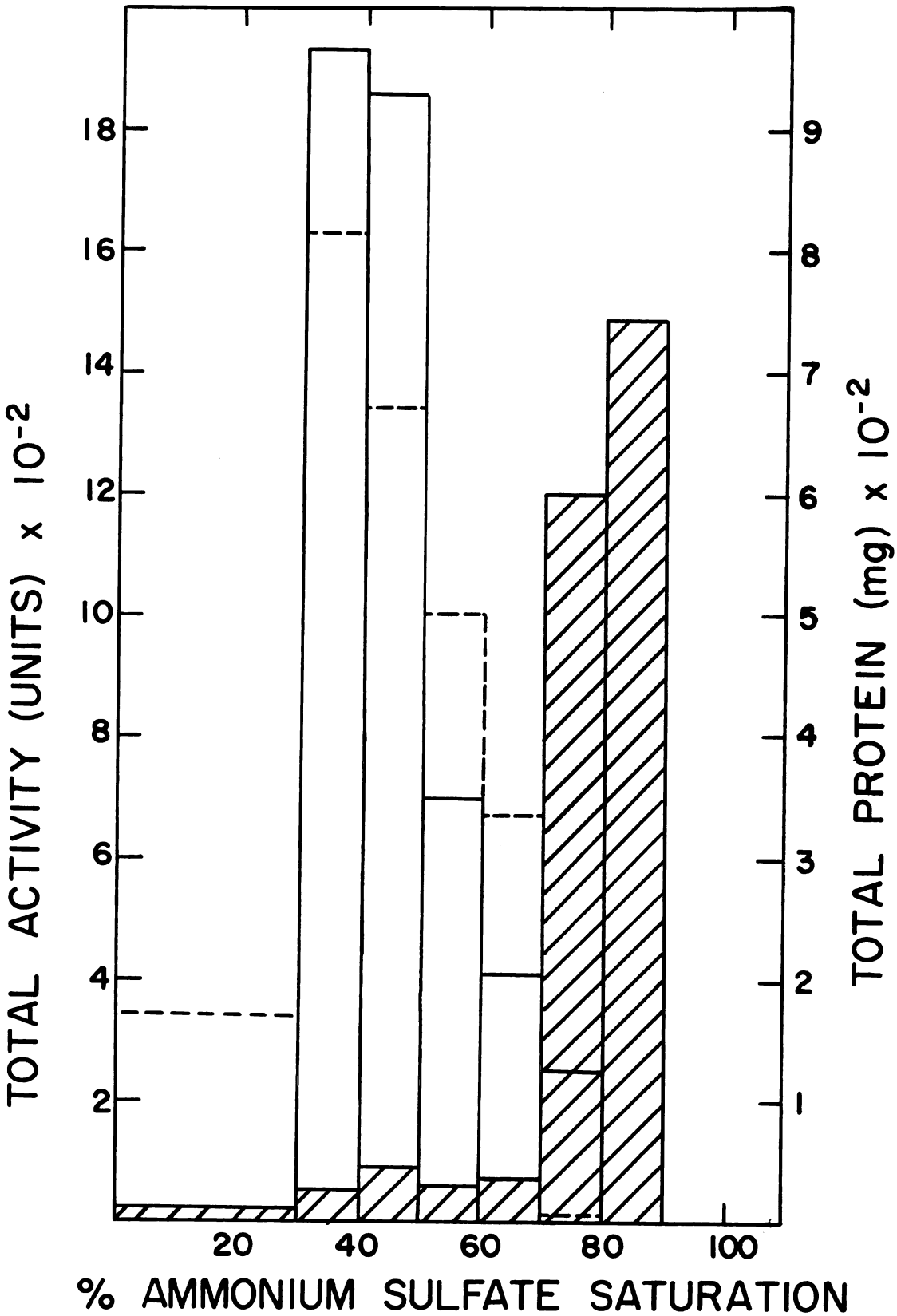
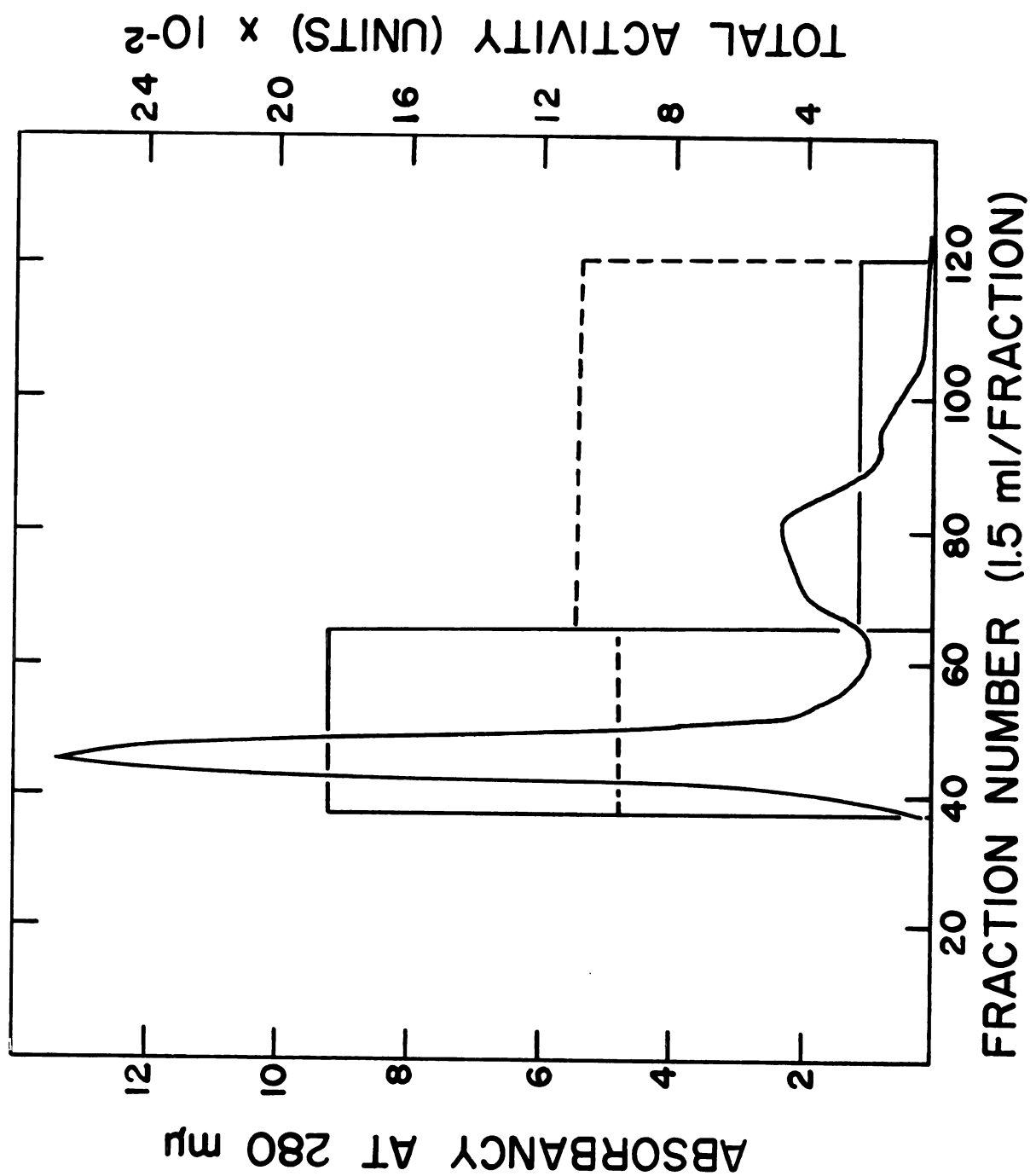

TOTAL ACTIVITY (UNITS) x 10^{-2}
TOTAL PROTEIN (mg) x 10^{-2}
% AMMONIUM SULFATE SATURATION
18
16
14
12
10
8
6
4
2
9
8
7
6
5
4
3
2
1
20
40
60
80
100

Figure 22: Fractionation of 30-60% enzyme fraction over Sephadex G-100. The procedure is given in Methods. The peaks were pooled as shown and concentrated by ammonium sulfate precipitation. GTPase activity is shown by dotted line and ATPase by solid line.


TOTAL ACTIVITY (UNITS) x 10-2
24
20
16
12
8
4
ABSORBANCY AT 280 mμ
12
10
8
6
4
2
FRACTION NUMBER (1.5 ml/FRACTION)
20
40
60
80
100
120

mately equally distributed between the 2 major peaks of 280 mu absorbing materials eluted from the column. Especially encouraging was the fact that the second peak of GTPase activity (Table VIII). The NDP kinase was distributed about equally between the 2 peaks. Chromatography over Sephadex C-75, G-200 or Bio-Gel P-100 did not fractionate the NDP kinase any more efficiently from the GTPase activity. Rechromatography of the second peak, now referred to as G-100-2 (Figure 23), did not accomplish any further purification. The major peak of this rechromatography was bimodol but separation of the components by this method has not been achieved. Both major protein peaks obtained by gel filtration contain GTPase activity (Figure 24a) but analysis of the amount of protein in each peak indicates a higher specific activity in the second peak to be eluted (24b).

The specific activity of the GTPase was quite low considering the stage of purification we had attained. Adding back ribosomes to the assays gave only an additive effect as did the addition of preheated high speed supernatant solution. In reviewing other parameters, an extensive stimulation in GTPase activity was experienced with increases in pH of the assay medium. By comparison the release assay shows a slight inhibition with increase of pH. Nevertheless, the pH curve of GTPase activity (Figure 25)

Table VIII: The Effect of Gel Filtration Through Sephadex G-100 on the 30-60% Ammonium Sulfate Fraction

	30-60% Ammonium Sulfate	G-100-1	G-100-2
Protein			
mg/ml	15	6.9	6.3
Total (mg)	135	48.3	43.5
GTPase			
Specific Activity (units/mg)	21	20	25
Total Activity	2,800	970	1,090
ATPase			
Specific Activity (units/mg)	21	38	5
Total Activity	2,800	1,800	241
NDP Kinase			
Specific Activity (units/mg)	11	8	12
Total Activity	1,500	386	522

G-100-1 is the first peak eluted from the Sephadex G-100 column while G-100-2 is the second peak eluted.

Figure 23: Second Fractionation of second peak (G-100-2) Sephadex G-100

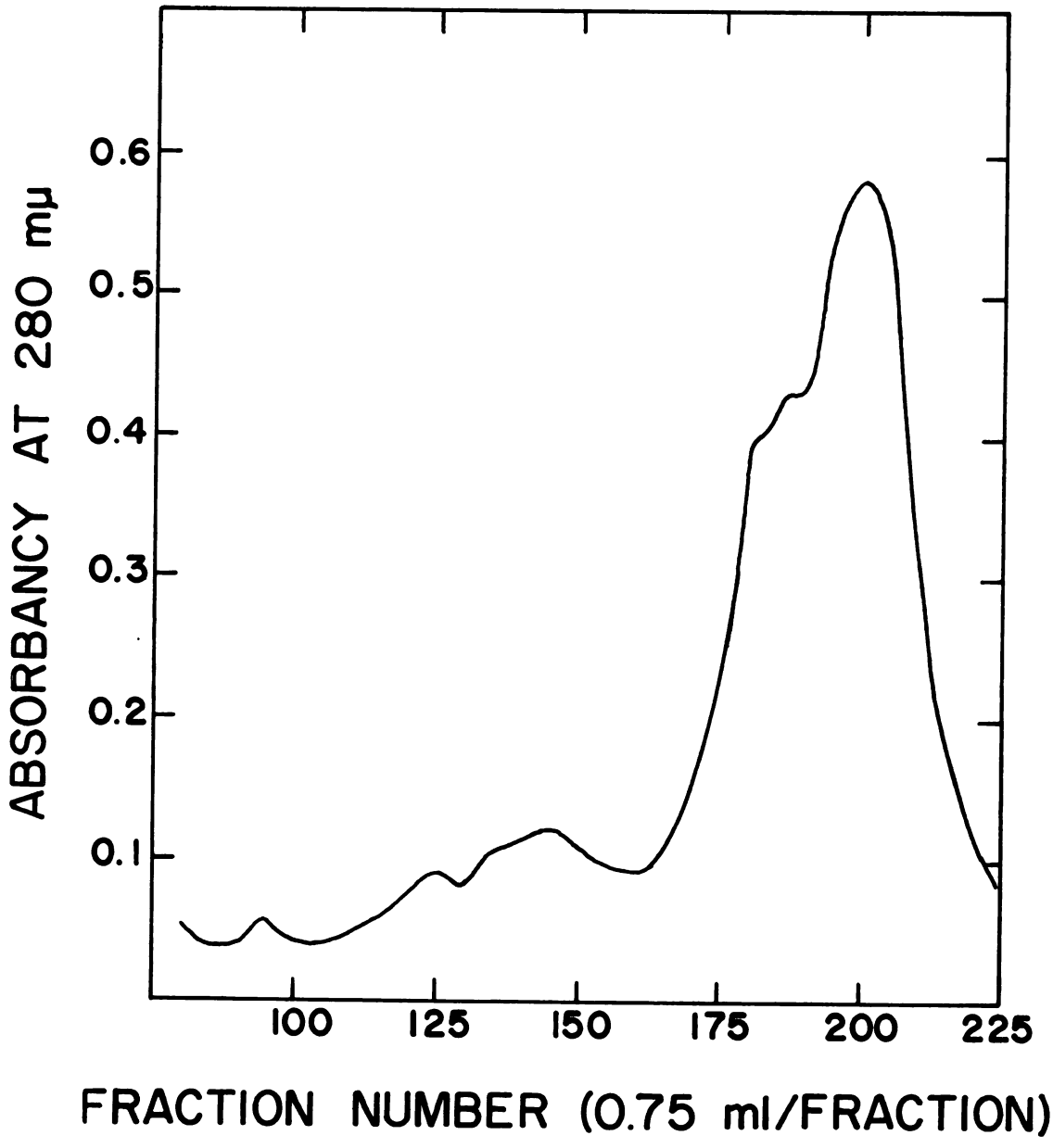
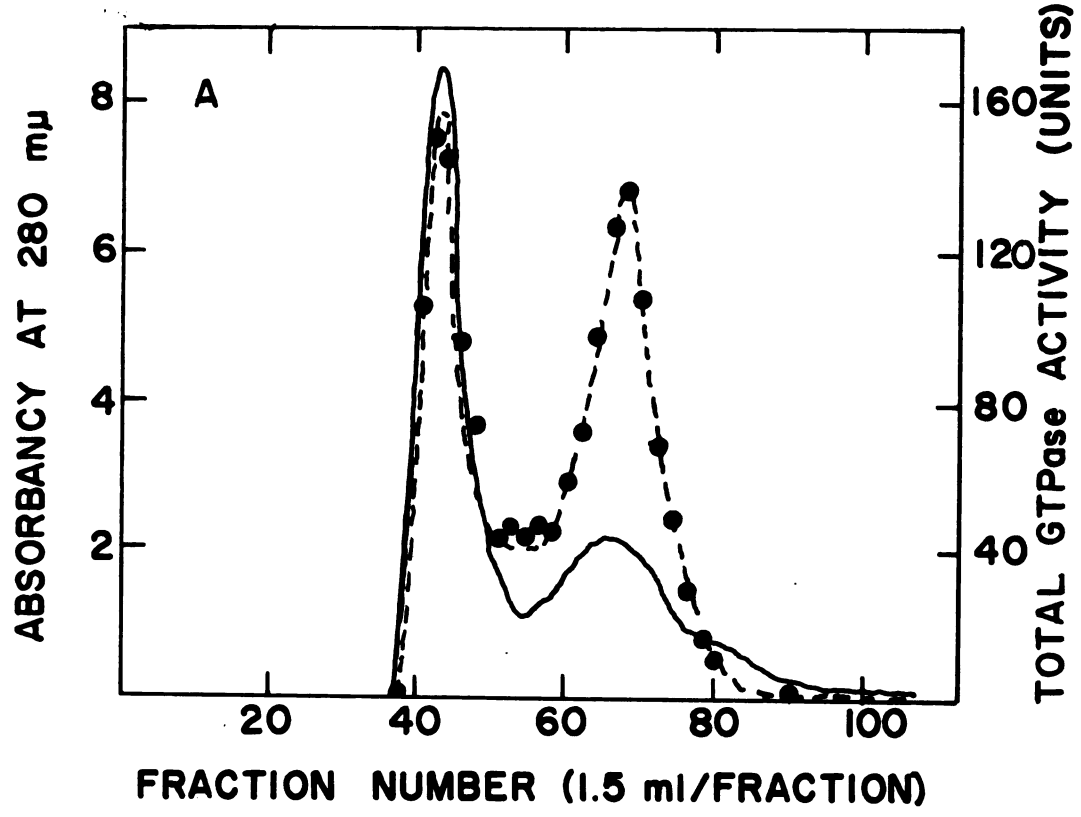

ABSORBANCY AT 280 mμ
0.6
0.5
0.4
0.3
0.2
0.1
100
125
150
175
200
225
FRACTION NUMBER (0.75 ml/FRACTION)

Figure 24: a. Distribution of GTPase activity after Sephadex G-100 fractionation. Solid line is optical density at 280 mu, dotted line is GTPase activity.

b. Distribution of specific activity on Sephadex G-100 fractionation. Solid line is protein concentration, dotted line is GTPase specific activity.


A
ABSORBANCY AT 280 mμ
TOTAL GTPase ACTIVITY (UNITS)
FRACTION NUMBER (1.5 ml/FRACTION)
2
4
6
8
40
80
120
160
20
40
60
80
100

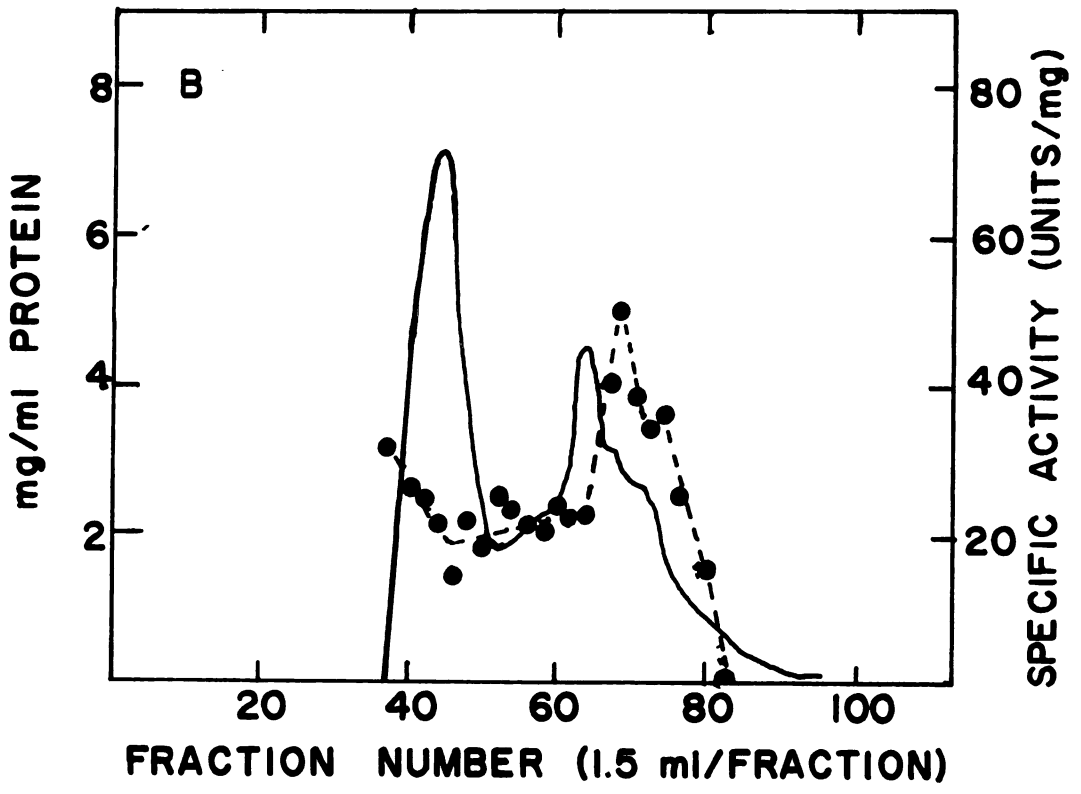
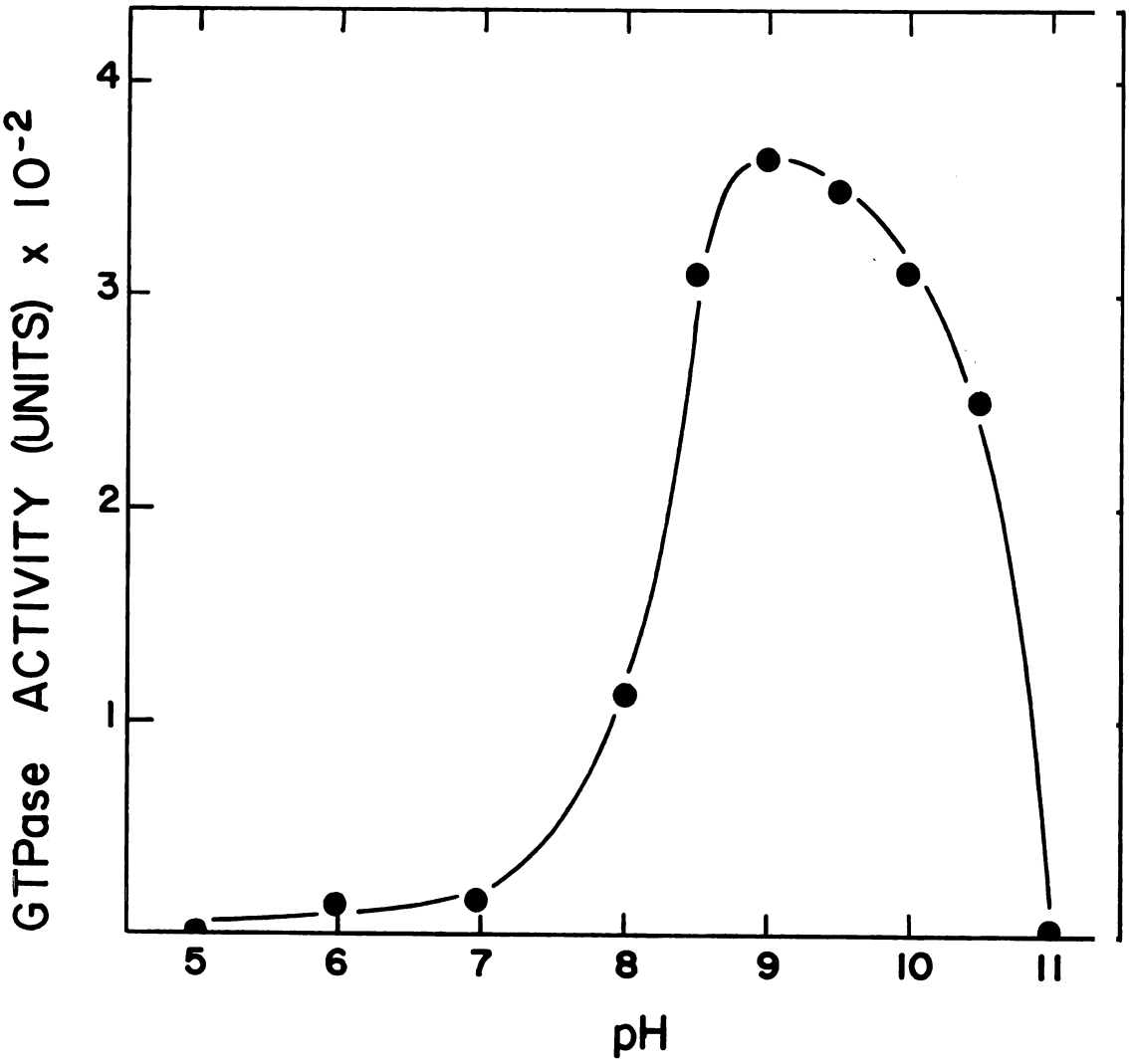

B
mg/ml PROTEIN
SPECIFIC ACTIVITY (UNITS/mg)
FRACTION NUMBER (1.5 ml/FRACTION)
2
4
6
8
20
40
60
80
20
40
60
80
100

Figure 25: Effect of pH on GTPase activity at 37°. The incubation was carried out for 20 minutes with 400 mumoles GTP and 2 mg of ribosomes. Tris-HCl buffer (50 mM) at the pH indicated was added to assay except in the case of the pH 11.0 value which was carried out in glycine-KOH buffer.


GTPase ACTIVITY (UNITS) x 10-2
4
3
2
1
5
6
7
8
9
10
11
pH

indicated a maximum near pH 9.0. The difference in pH from pH 7.2 to pH 9.0 is reflected in an increase of the GTPase activity of nearly 25 fold. The other parameter reviewed was the requirement for a sulfhydryl reagent. The observation had been made that dilution inhibited activity (Table IX). The GTPase activity was restored, and even enhanced nearly three fold, by the addition of dithiothreotol to the diluted protein solution. There was no detecta ble effect on the phosphate determination due to the addition of DTT in the range utilized in the assay (Figure 26). The lack of sulfhydryl requirement in the more crude fractions, mentioned earlier, was probably due to reduction by a substance which was subsequently removed. The ATPase and NDP kinase did not respond in nearly so dramatic a fashion. The ATPase showed little or no increase in specific activity over a similar pH range.

The fractionation by ammonium sulfate was again attempted. Figure 27 indicates the dramatic effect of the pH and DTT on the GTPase. If we compare this graph to Figure 18 it is evident that a 50-70% fractionation has some advantages over the 30-60% fraction previously used. The 50-70% fraction contains approximately one third of the ATPase activity present in the high speed supernatant fraction. Chromatography over Sephadex G-100 produced the profile shown in Figure 28. There is no detectable ATPase

Table IX: Effect of Dilution on G-100-2 Fraction

	Agent Added	Total Units of Activity
0.5 mg	---	126
0.5 mg	0.76 ml H_2O	28
0.5 mg	0.76 ml Ethylene glycol	109
0.5 mg	0.76 ml H_2O + 0.5 mM DTT	292
0.5 mg	0.76 ml H_2O + 15 mg Bovine serum albumin	0

The enzyme solution was allowed to stand overnight at 4^o in 0.5 M Tris-HCL (pH 9.0 at 37^o), $MgCl_2$ (4 mM) KCL (3 mM). The volume of enzyme used was 0.163 ml. The control enzyme solution was kept at 4^o overnight and was brought up to assay volume immediately before the addition of GTP.

Figure 26: The effect of dithiothreotol upon the assay for inorganic phosphate. Solid line indicates no DTT added, dotted line indicates assays containing 0.5 mM DTT.

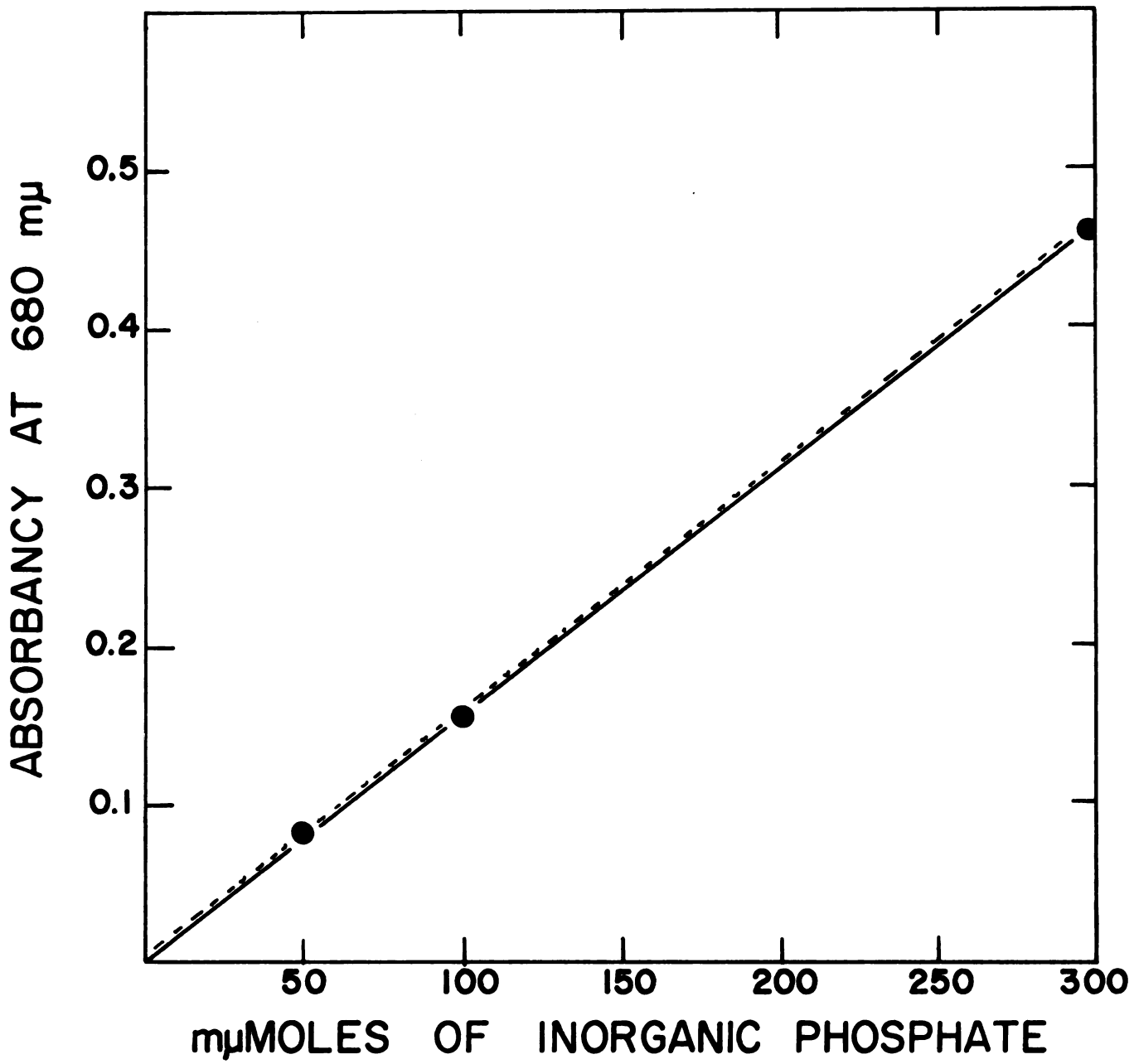

ABSORBANCY AT 680 mμ
0.5
0.4
0.3
0.2
0.1
50
100
150
200
250
300
mμMOLES OF INORGANIC PHOSPHATE

Figure 27: Ammonium sulfate fractionation in the presence of DTT and assayed at pH 9.0 at 37°. 0.5 mg of each fraction was incubated under the usual conditions (see Methods). Dotted line is GTPase activity, solid line is ATPase and shaded area is total protein.

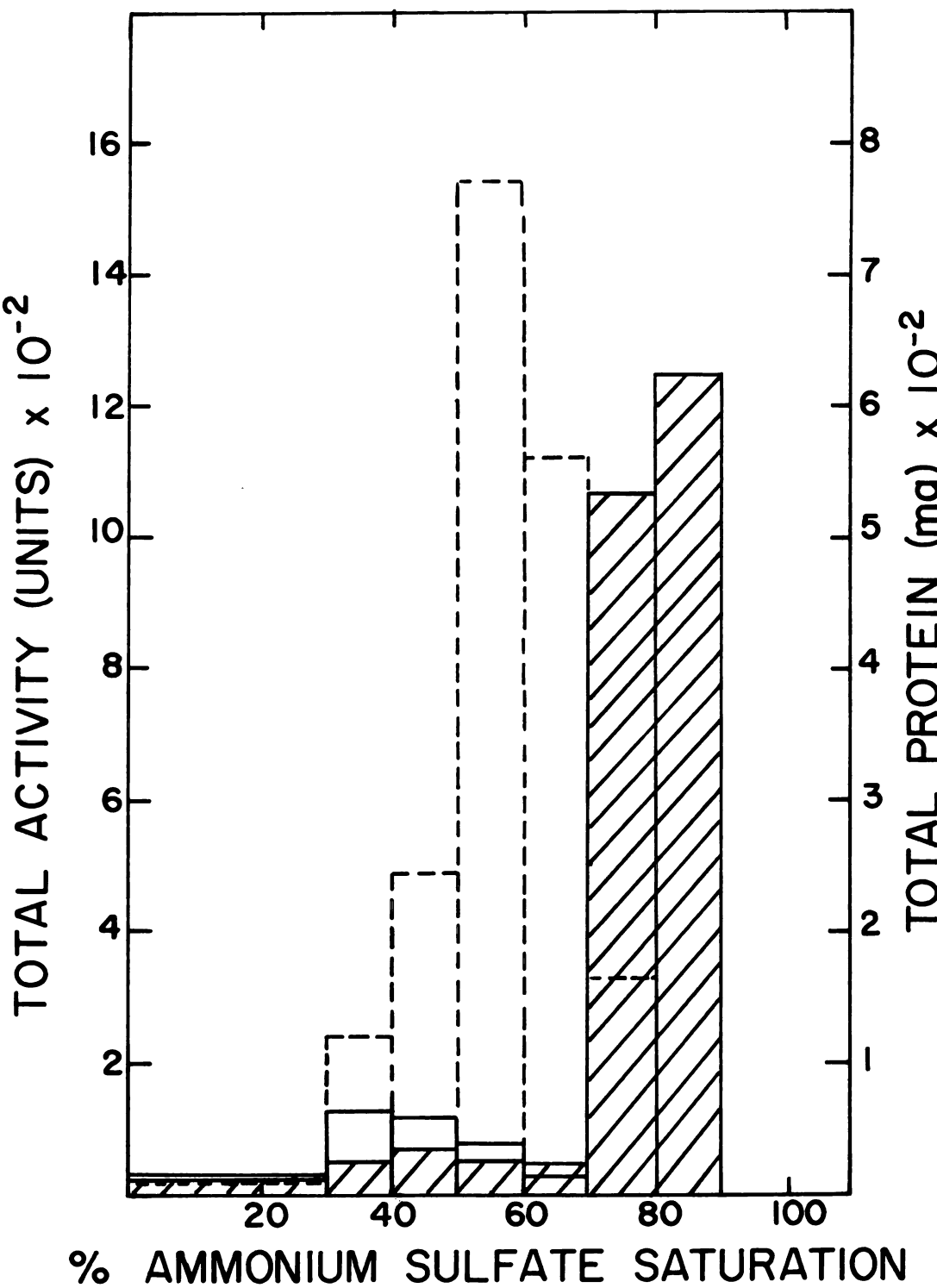
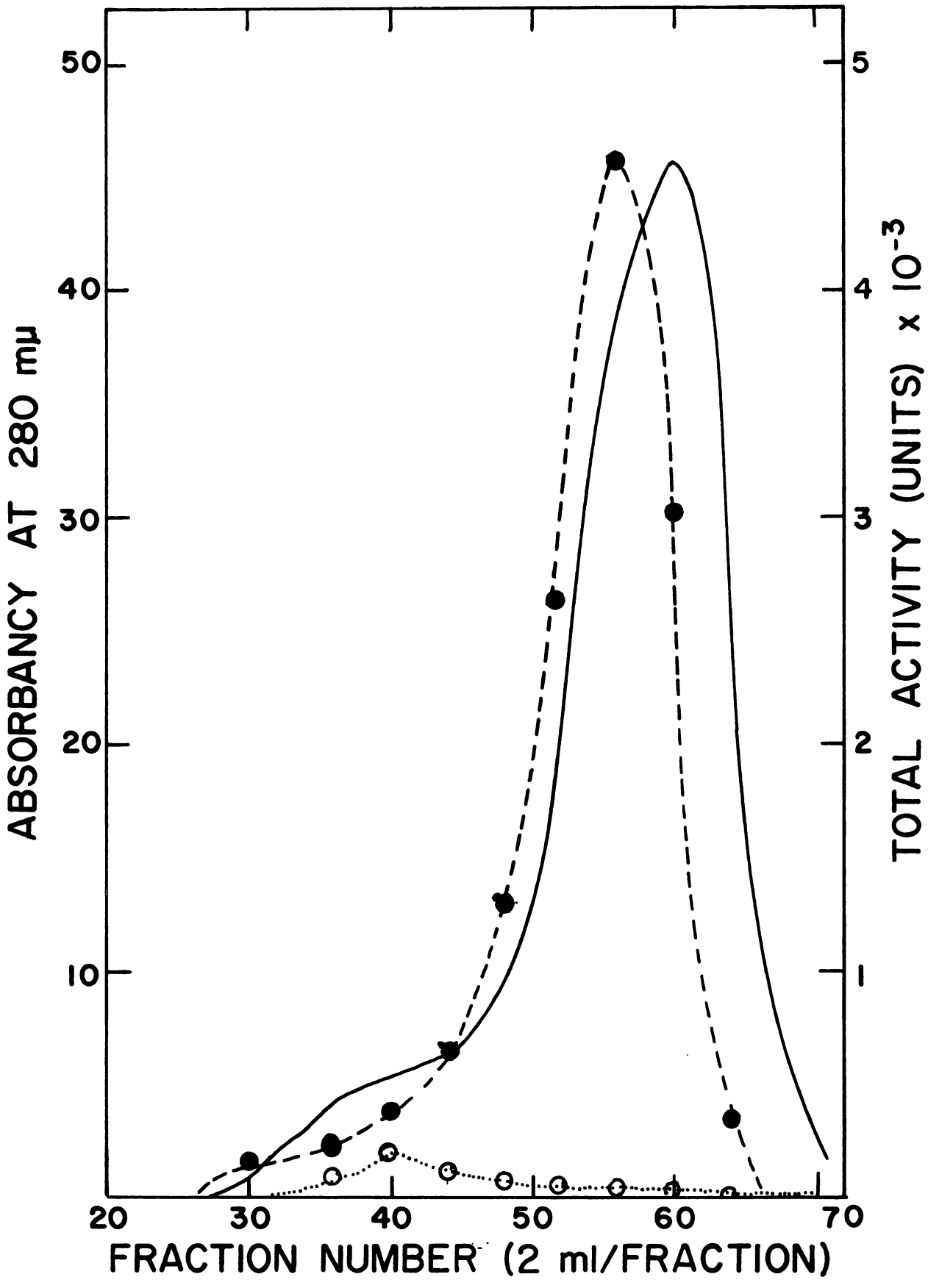

TOTAL ACTIVITY (UNITS) x 10^{-2}
TOTAL PROTEIN (mg) x 10^{-2}
16
14
12
10
8
6
4
2
8
7
6
5
4
3
2
1
20
40
60
80
100
% AMMONIUM SULFATE SATURATION

Figure 28: Fractionation of 50-70% ammonium sulfate fraction on Sephadex G-100. GTPase activity is represented by the dashed line. ATPase activity is represented by the dotted line. The absorbancy at 280 mu is shown by the solid lines.


ABSORBANCY AT 280 mμ
TOTAL ACTIVITY (UNITS) x 10-3
FRACTION NUMBER (2 ml/FRACTION)
50
40
30
20
10
5
4
3
2
1
20
30
40
50
60
70

activity in the second peak eluted from the column. Notice that the GTPase activity previously shown to elute near the column front (see Figure 25a) is absent. Concentration of the second peak by ammonium sulfate precipitation accomplished an additional purification (Table X). Reprecipitation of the G-100-2 fraction with 70% ammonium sulfate achieved a quantitative recovery of the GTPase activity while reducing the total protein by 86%. A summary of the purification achieved up to and including this step appears in Table XI. The nucleoside diphosphokinase activity is still present but the fraction is entirely free of detectible ATPase activity. A nearly 50 fold overall purification has been achieved in the procedures mentioned. In addition, it has been demonstrated that a specific GTPase does exist in reticulocytes which is not catalyzed by way of the series of reactions involving nucleoside diphosphokinase and an ATPase mentioned earlier.

Using the reprecipitated G-100-2 fraction, the GTPase activity is linear up through approximately 250 units of activity per assay (Figure 29). The activity curve may be seen to pass through the origin and is linear up to 250 ug of enzyme added with the amount of substrate used. The time course of the reaction (Figure 30) indicates a linearity up to 1 hour. The GTPase activity is heat labile (Figure 31). The heat denaturation of GTPase activity

Table X: Ammonium Sulfate Fractionation of G-100-2

% Saturation	Protein (mg)	Total Units Recovered
0-70	103	121,000
70-80	419	0
80-90	199	0

Table XI: Summary of Enzyme Purification Through Sephadex G-100 Fractionation

	High Speed Supernatant Fraction	Streptomycin Treated	0-50% Ammonium Sulfate	50-70% Ammonium Sulfate	G-100-2
Protein (mg)	17,000	14,000	388	1,683	103
GTPase (units)	433,000	555,000	57,000	242,000	121,000
Specific Activity	25	40	146	144	1,170
Per cent Recovery		128		56	28
Purification		1.6X		5.8X	46.8X
ATPase (units)	201,000	125,000	30,000	17,000	0
Kinase (units)	2.5×10^4	2.5×10^4		9,200	2,200

Figure 29: **Effect of enzyme concentration on the GTPase assay.**

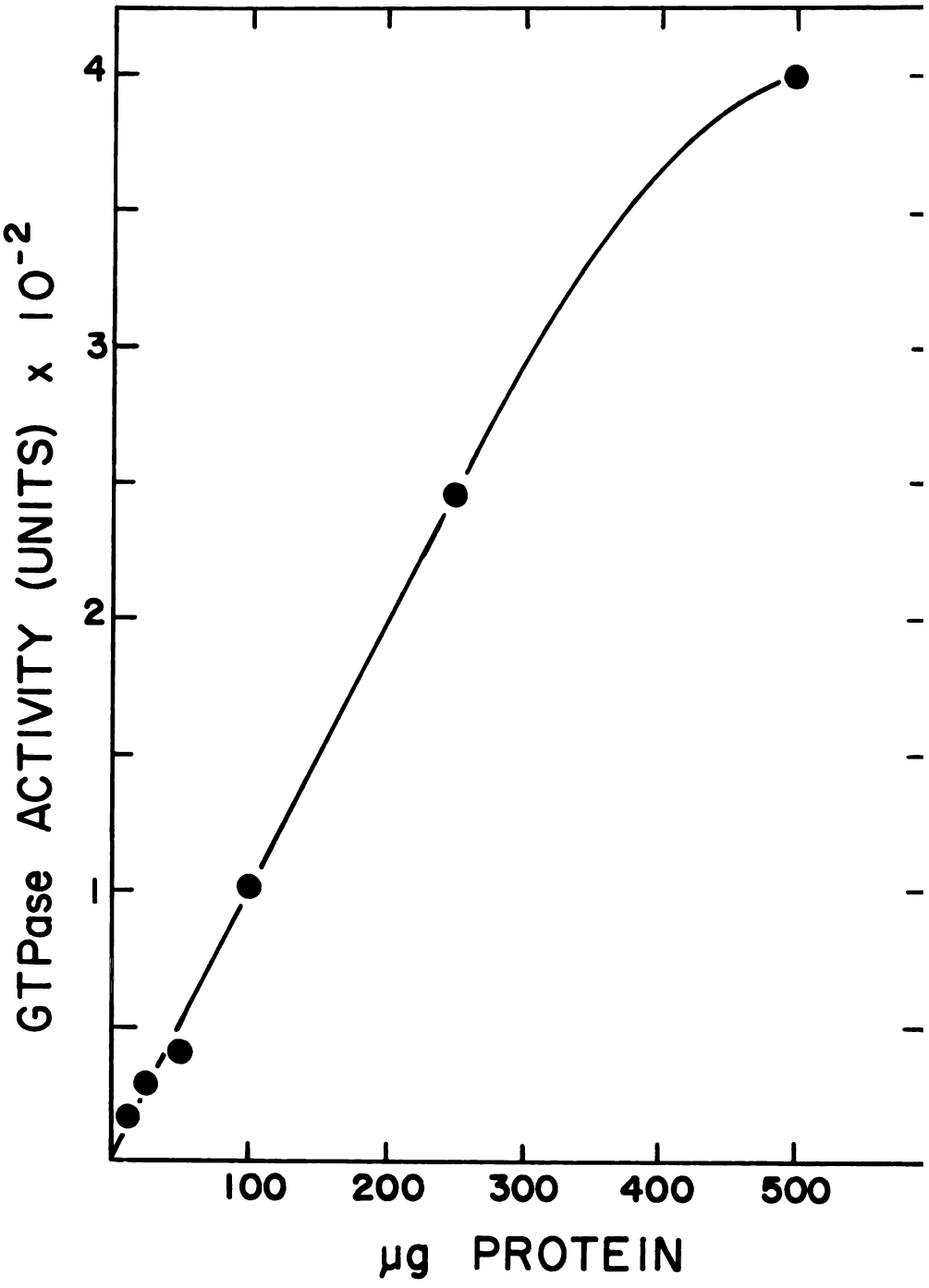

GTPase ACTIVITY (UNITS) x 10^{-2}
4
3
2
1
100
200
300
400
500
µg PROTEIN

Figure 30: Time course of the GTPase reaction using the G-100-2 fraction. 100 ug of protein was incubated with 400 mumoles of GTP (see Methods).

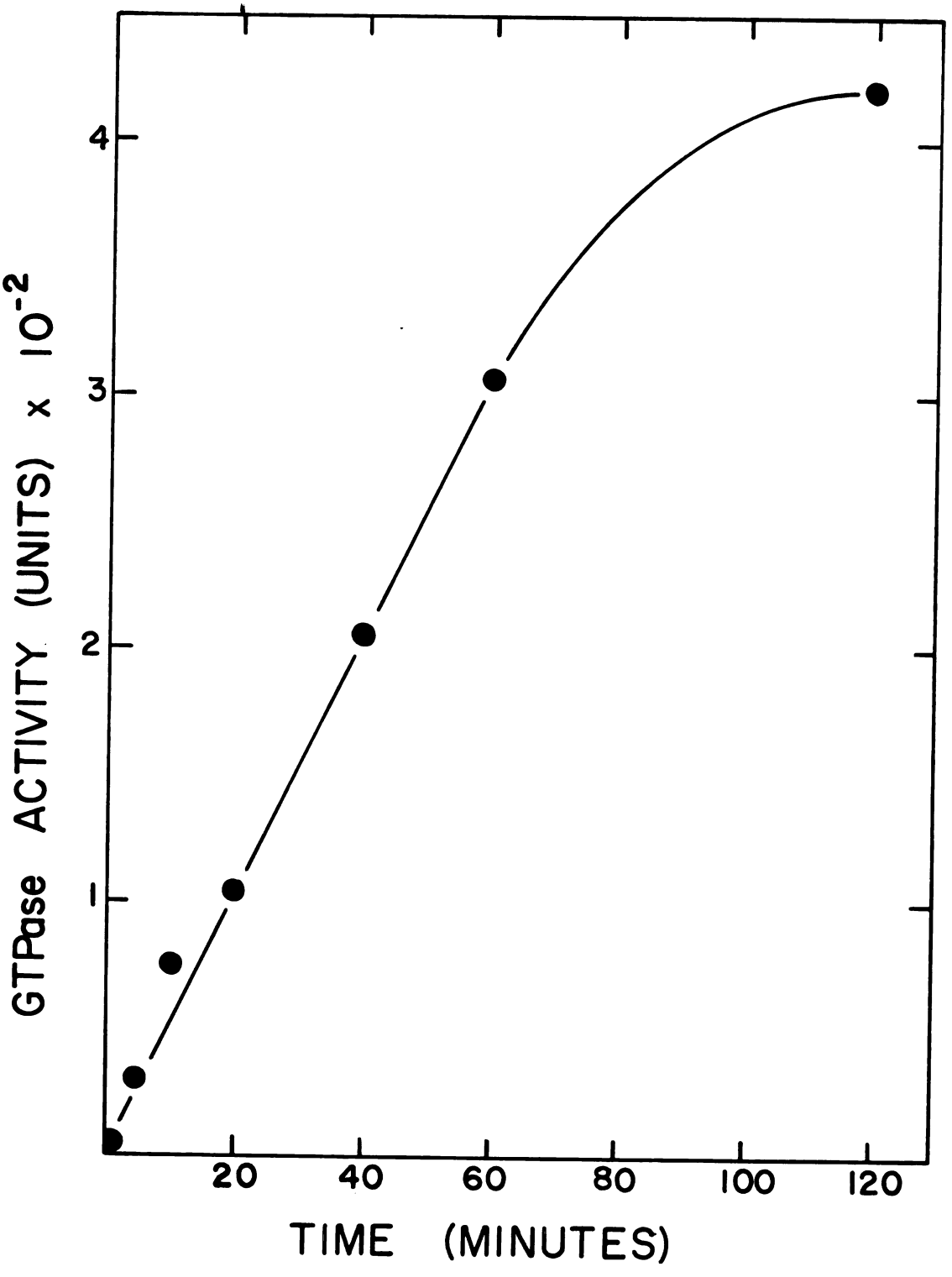
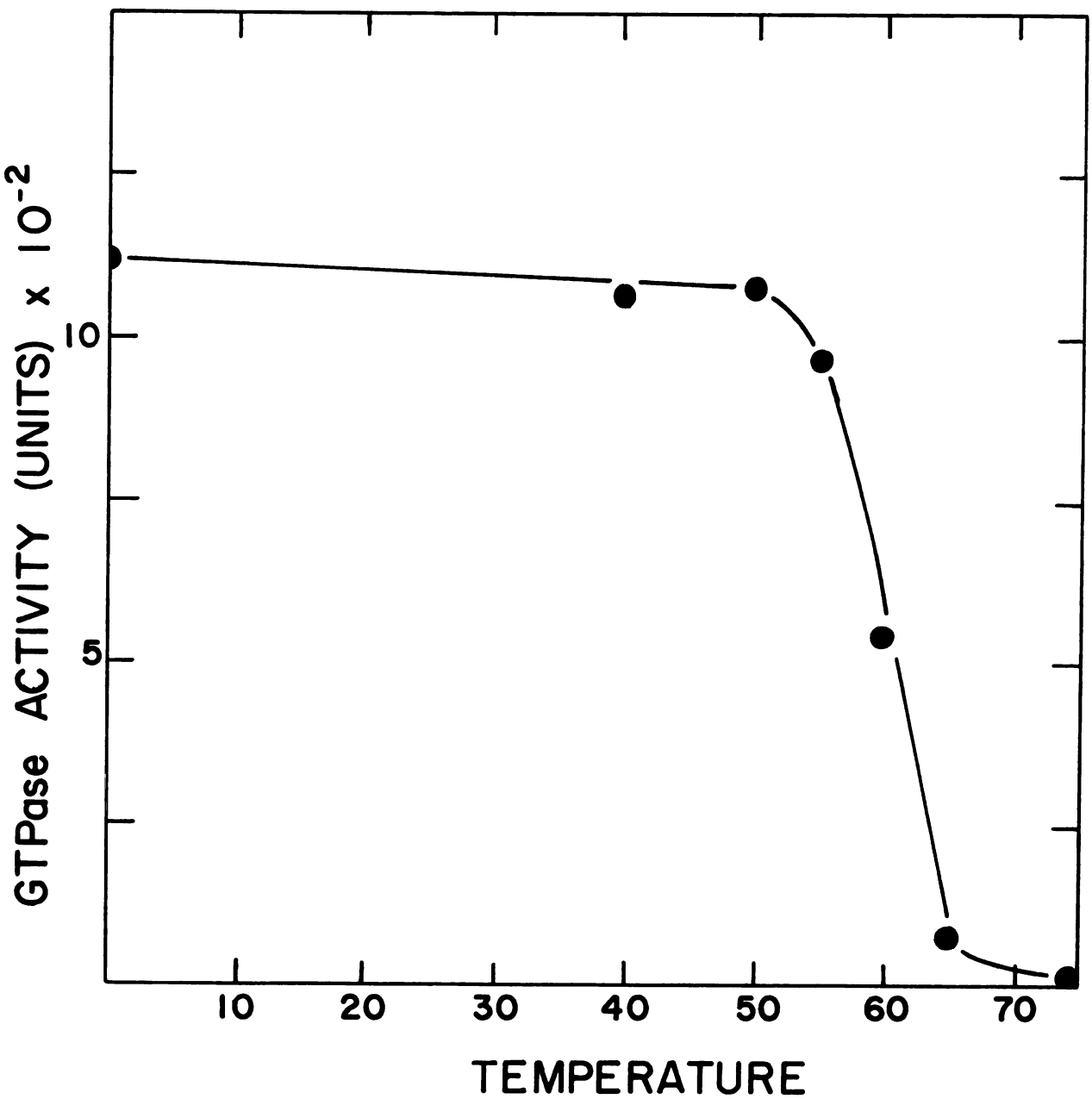

GTPase ACTIVITY (UNITS) x 10^{-2}
1
2
3
4
20
40
60
80
100
120
TIME (MINUTES)

Figure 31: Effect of temperature on the GTPase activity of the G-100-2 fraction. The enzyme was pre-treated for 5 minutes at the temperature indicated. Concentration of enzyme (128 ug) was normalized to 1 mg for specific activity.


GTPase ACTIVITY (UNITS) x 10^{-2}
10
5
10
20
30
40
50
60
70
TEMPERATURE

parallels that determined in the studies of GTP binding to ribosomes (see Figure 8).

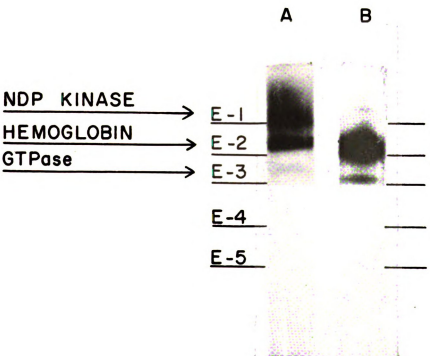
The final purification for the purposes of this study was achieved by disc electrophoresis. Development of the method involved different types of apparati. Large bloc or continous flow gels were found to inactivate the enzyme, probably because of heat generated during the electrophoretic run. The method of choice proved to be small polyacrylamide columns fitted with cooling jackets. Since the electrophoresis was carried out using a parallel circuit, each column was subjected to low current and therefore less heat was generated as opposed to 1 large column or bloc which may have a higher total resistance, thus generating more heat. The GTPase activity was found to migrate just ahead of the hemoglobin fraction and hence it carries a strong negative charge at pH 9.0, migrating near the front (toward the cathode). A running time of 8 hours, even in the cold, reduced the total recoverable GTPase activity (Table XII). The necessity of extracting the enzyme from the gel also contributed to this low recovery. However, complete removal of all detectible NDP kinase was achieved by this step. The NDP kinase carried less negative charge than hemoglobin and therefore had a considerably slower migration rate than the GTPase. The pattern of the protein distribution (Figure 32) observed following staining of the gels with buffalo black indicated that the

Table XII: Purification of GTPase by Disc Electrophoresis

Sample	Total Protein	GTPase Activity		ATPase Activity	NDP Kinase Activity
	mg	Specific	Total Units	Units	Units
G-100-2	28.6	1,900	54,500	0	2,020
E-1	8.8	160	1,500	0	505
E-2	12.0	106	1,200	0	480
E-3	12.4	1,200	15,000	0	0
E-4	2.3	0		0	0
E-5	1.5	0		0	0
E-3 (0-70% Ammonium Sulfate ppt.)	7.4	1,070	7,920		0

G-100-2 used in these studies is not the same G-100-2 fraction used in Table XI, thus there is a difference in specific activity between the two values.

Figure 32: Disc electrophoresis of G-100-2. Column A was cut along the line indicated. Column B was fraction E-3 of a following concentration by precipitation with ammonium sulfate at 70% of saturation subjected to electrophoresis.


A
B
NDP KINASE
HEMOGLOBIN
GTPase
E-1
E-2
E-3
E-4
E-5

GTPase containing fraction (E-3) still exhibits 2 major and possibly 2 minor bands of protein.

The GTPase specific activity of the E-3 fraction was actually lower than the GTPase which was subjected to the electrophoresis. The reduction is probably due to the sensitivity of the enzyme to the method utilized for this final purification. That is, the enzyme may experience inactivation during electrophoresis because of heating, etc. However, the fraction is active and contains no measurable NDP kinase activity.

Isolation of a GTPase from Ribosomes

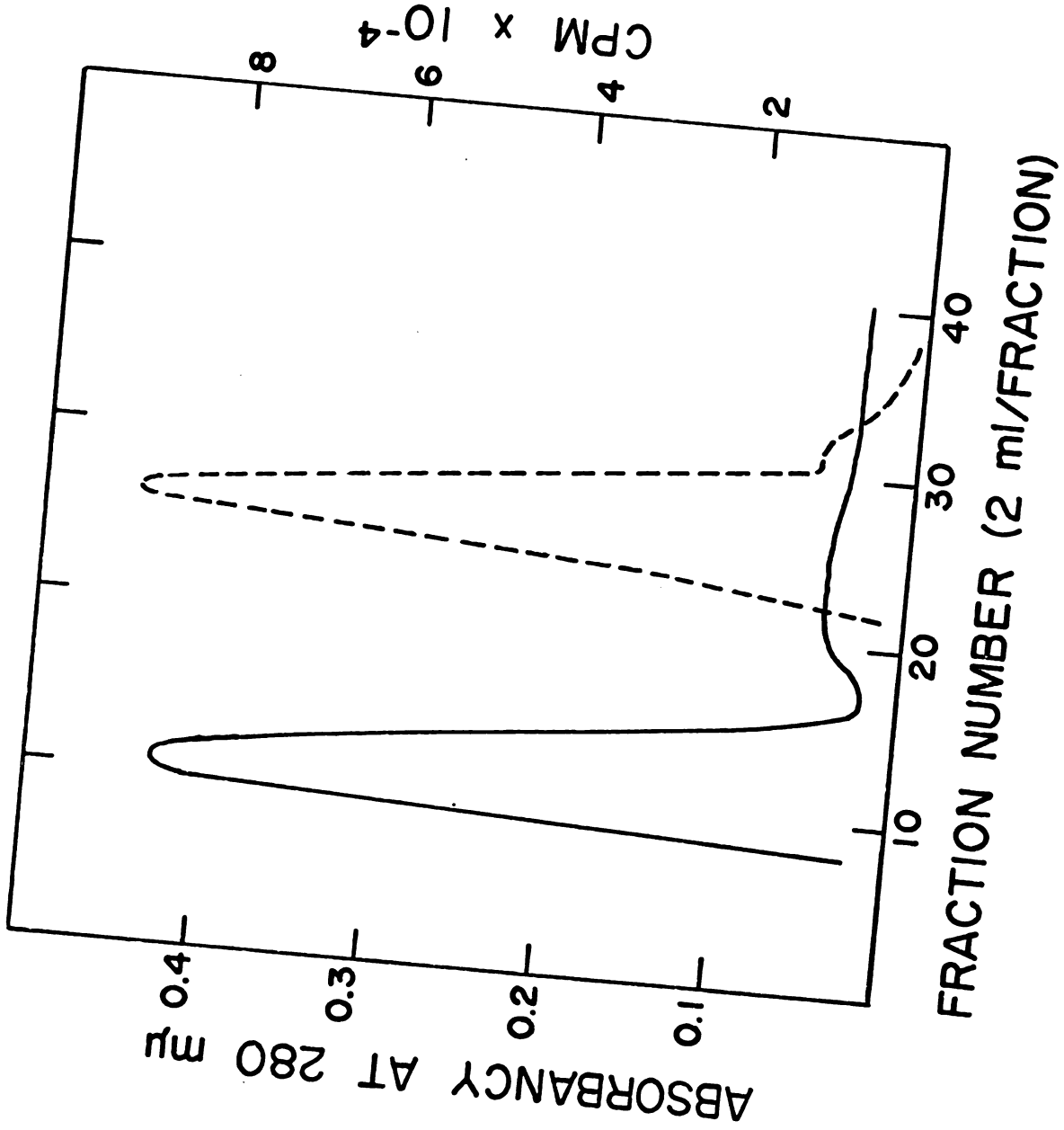
An attempt to isolate a GTPase from ribosomes was successful insofar as the isolation procedure which had been established for the purification of a GTPase from the high speed supernatant fraction did contain GTPase activity when applied to ribosomes. Ribosomes were treated with RNase as indicated in Methods. The soluble hydrolysate from an RNase treatment (see Methods) was precipitated with ammonium sulfate at 50-70% of saturation and chromatographed over Sephadex G-100. The optical density profile of the elution pattern from the Sephadex G-100 chromatography indicated 2 peaks of material having 280 mu absorbance were present. One peak was eluted at the column front while the second peak was eluted in the same volume as a G-100-2 fraction from the high speed supernatant frac-

tion. When this ribosomal G-100-2 fraction was rechromatographed on the same column the material with 280 mu absorbance eluted in the portion of the eluate fractions as before. The second peak from the G-100 Sephadex chromatography was concentrated by precipitation at 70% ammonium sulfate saturation and subjected to disc electrophoresis. GTPase activity from this second peak was detected at a migration distance identical to an E-3 fraction from the high speed supernatant fraction. No other GTPase activity was detected on the gel bed. However, the total GTPase activity extracted from the gel bed was only 4% of the GTPase activity present in the concentration of ribosomes utilized as starting material. In addition, the specific activity calculated for the GTPase isolated from ribosomes was only 20% of the specific activity determined for the E-3 fraction isolated from the supernatant fraction.

Phosphate Intermediate from GTPase

(^{14}C) GTP binding was attempted using the G-100-2 enzyme fraction. After incubation the reaction mixture was chromatographed over Sephadex G-25. The profile (Figure 33) indicates that no radioactivity was bound to protein. Thus the binding of (^{14}C) GTP must occur in a different environment on the ribosome or may not be bound to this particular enzyme at all.

Figure 33: Attempted binding of (^{14}C) GTP to G-100-2. The column dimension were 1 x 25 cm. The void volume was 19 ml. 40 mumoles of (^{14}C) GTP, of specific activity of 25 uc/umole was incubated with 0.5 mg G-100-2 fraction for 5 minutes, cooled and subjected to fractionation on the column containing G-25 Sephadex. Samples were counted in Bray's solution for radioactivity (dotted line). Absorbance at 280 mu was also determined (solid line).


CPM x 10-4
8
6
4
2
FRACTION NUMBER (2 ml/FRACTION)
10
20
30
40
ABSORBANCY AT 280 mμ
0.4
0.3
0.2
0.1

If a true phosphoprotein intermediate is present, the possibility may be considered that the following mechanism or a variation thereof may be operative;

$$(^{32}P)\ GTP \longrightarrow GDP + {}^{32}P\text{-}ENZ \longrightarrow ENZ + {}^{32}Pi$$

If an equilibrium exists in the form of the proposed reaction the equilibrium would be expected to lie far to the right (66). The addition of carrier free ^{32}Pi might phosphorylate the GDP forming small amounts of (^{32}P) GTP. In order to test this hypothesis, 3 uc of carrier free ^{32}Pi were added to 400 mumoles of GTP and 50 ug of the E-3 fraction from the disc electrophoresis procedure using the normal assay conditions (see Methods). Following incubation the reaction mixture was chromatographed on PEI treated paper (see Methods). No radioactivity appeared in the GTP area of migration on the chromatogram. The final attempt to illustrate a phosphate intermediate involved the same type of analysis as was carried out previously with the ribosomes. (^{32}P) GTP was added to several assays containing successively increasing concentrations of the E-3 fraction. No differences in radioactivity in the samples and pre-heated controls were experienced following analysis by the phenol extraction procedure (Table XIII). It is apparent therefore that no intermediate is present, at least in so far as these methods of detection are concerned. These results are not entirely surprising since phosphoryl inter-

Table XIII: Attempt to Demonstrate a Phosphoprotein Intermediate in the E-3 Fraction

Micrograms of E-3 fraction/assay	Nontreated cpm	Preheated cpm
50	1,500	1,200
100	1,200	1,200
200	1,110	1,170
Ribosomes (0.5 mg/assay)	9,742	3,564
Bovine serum albumin (0.5 mg)	1,700	

Fifty mumoles of (^{32}P) GTP (specific activity 160 uc/umole) added to each assay. Untreated controls (ribosome and bovine serum albumin) are included for comparison.

mediates for kinase enzymes have not as yet been demonstrated where the products formed are of low energy. Acetylphosphate has been implicated in an ATPase dependent ion transport (64) but has been questioned (67).

DISCUSSION

There are many reactions which take place in the red cells, but few which utilize GTP. Certainly the NDP kinase can transfer the phosphate of GTP to ATP which can then be utilized by the ribose pathway. However, no phosphate was detected from glucose-6-phosphate incubated with the crude enzyme fractions. The enzymes necessary for RNA synthesis may be present but are probably in extremely low concentration since no RNA synthesis could be shown to occur in reticulocytes (68). The study of the metabolism of GTP in reticulocytes must include the possibility that GTP can be utilized by enzymes which have no direct role in protein synthesis. Thus the attempt was made to study GTP metabolism in those reactions which would be most informative, that is, binding, hydrolysis and detection of any intermediate whether the reaction occurs on the ribosome or in the supernatant fraction.

The binding of GTP observed in reticulocytes parallels the binding of GTP to a fraction derived from E. coli ribosomes which is known to contain an initiation factor for protein synthesis in the E. coli cell-free system (36). The properties of the 2 reactions are somewhat similar. The following properties are indicative of both systems; the

reaction occurs very rapidly at 4°, the reaction is inactivated by heating for short periods of time, the addition ATP at 10 to 50 times the concentration of GTP does not totally inhibit the reaction, GDP is able to bind as well as GTP and GMP does not bind to any great extent. The binding of GTP to the initiation factor fraction in E. coli mentioned here was strongly inhibited by GDP, which also inhibits polypeptide synthesis in E. coli (69). No data was given in this preliminary report as to whether or not the GTP is hydrolyzed under these conditions. As mentioned earlier, the binding of N-formyl-methionine to ribosomes in E. coli is stimulated by either GTP or 5'-guanylyl methylenediphosphonate (GMP-PCP) (Thack, R. E. or Clark, B. F. C., unpublished data) which presumably cannot be hydrolyzed to GDP and inorganic phosphate. An allosteric reaction may be present in both the E. coli system and the rabbit reticulocyte system reported here, but the binding of GTP to ribosomes in reticulocytes has not been related to protein synthesis.

The GTPase partially purified by Conway and Lipmann in E. coli (69) and further purified by Nishizuka (21) has been implicated in peptide bond synthesis. The system utilized for these studies was a poly U directed phenylalanine polymerization. No binding with either (^{14}C) GTP or (^{32}P) GTP nor any radioactive phosphoprotein intermediate has been demonstrated in this system. The re-

lationship of GTPase to protein synthesis in the E. coli system appears to be the hydrolysis of GTP with the formation of GDP and inorganic phosphate. The same relationship seems to be true for the reticulocyte cell-free system of Schweet (17) and the rat liver cell-free system of Moldave (27). Neither phosphoprotein intermediates nor GTP binding have been reported for these systems. The hydrolysis reported here which is catalyzed by a factor attached to ribosomes as well as by high speed supernatant fractions has not been shown to be due to the same enzyme as the binding factor although a number of correlations in properties have been pointed out.

There is the possibility that GTP acts allosterically to activate an initiator factor as well as being hydrolyzed as an energy supply coupled to other events in protein synthesis. Thus the intermediate phosphoprotein need not be the GTPase itself but a receptor site on the ribosome as proposed by Schweet (17). The data presented here for a ribosomal phosphoprotein intermediate does not exclude this possibility, but was not characterized to the degree that is necessary for any study of function.

The enzyme which we have partially purified may not be the same GTPase as is active in protein synthesis. This point could only be demonstrated if the GTPase were limiting in the reaction. In view of the tenacious binding of GTPase

to ribosomes, the experimental conditions necessary to test this point have been difficult to establish. The demonstration of a limiting reaction of this type necessitates the isolation of highly purified ribosomes, synthetic messenger RNA, aminoacyl-sRNA, etc. In addition to these problems, the fact remains that more than one GTPase may be present in reticulocytes. The front peak from Sephadex G-100 (G-100-1) contains GTPase activity. This activity could be due to a nonspecific ATPase, NDP kinase plus a specific ATPase or it may be an aggregate of the GTPase found in the second peak (G-100-2).

A number of functional properties are shared by the fractions containing GTPase activity isolated from sources other than reticulocytes. Sulfhydryl and magnesium ion requirements are present in the factors from E. coli (21, 31), the transferase factors from liver (27) and yeast (33). The G factor from E. coli has a pH optimium of 9.0 (21). No sulfhydryl requirement has been demonstrated for the T-1 factor of reticulocytes (25) but rather for the site on the ribosome where it presumably functions.

We have in fact answered fewer questions than we have created. This is the intention of a preliminary study of this type. Now that the enzyme properties and cofactors have been ascertained to a large extent, other methods which

had failed before may now be successful. Preliminary studies on DEAE cellulose currently in progress indicate better activity recovery than electrophoresis and may lead to a preparative method for isolating GTPase of high purity (Chern, C. J., Unpublished Data).

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