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NUCLEOTIDE-FACILITATED RELEASE OF INORGANIC PHOSPHATE AND HYDROLYSED ADENOSINE TRIPHOSPHATE FROM BEEF HEART MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE

by

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B.Sc., University of Guyana, 1975

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"Nucleotide-facilitated Release of Inorganic Phosphate and Hydrolysed Adenosine Triphosphate from Beef Heart Mitochondrial Adenosine Triphosphatase"

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ABSTRACT

The Sephadex centrifuge column technique introduced by Penefsky was modified and used to perform preincubation and pulse-chase type experiments with soluble beef heart mitochondrial adenosine triphosphatase, F_1. In the preincubation-type experiments, F_1 was labelled by incubation with $[^{32}P]P_i$ before being applied to Sephadex G-50 columns which contained a 1.0 cm nucleotide equilibrated middle section. Under these experimental conditions, ATP and AMPPNP bound equally well to F_1 but ADP did not bind as well. The order of effectiveness of the nucleotides in promoting the release of approximately 70% (i.e. the steep phase of the biphasic release) of the bound P_i (~0.2 mole P_i/mole F_1) from F_1 was ATP > AMPPNP >> ADP. High concentrations of nucleotides were able to effect the total release of P_i from F_1. It was concluded that binding of nucleotide, not hydrolysis, was necessary to effect P_i release.

In the pulse-chase type experiment, F_1 was labelled when it passed through a 1.0 cm nucleotide equilibrated gel at the top (pulse section) of the column. The labelled F_1 was exposed to unlabelled nucleotide present in the third section (chase section) of the column. When F_1 was given a $[\gamma\text{-}^{32}P]$ATP pulse (0.1 mole ATP/mole F_1), the release of P_i (with ADP present

at the same site) was effected equally well by chase ATP and AMPPNP. Here also the release of P_i was biphasic with about 70% of the bound label being released in the first phase. Using [^{3}H]ATP label in the pulse, it was shown that ADP release was similar to that of P_i in the presence of chase ATP. With a [^{3}H]AMPPNP pulse (0.1 mole AMPPNP/mole F_1), chase AMPPNP was not effective (less than 30%) in promoting the release of bound AMPPNP.

A comparison of the results from the two modes of investigation reveal that: (i) AMPPNP was more effective in promoting P_i release when ADP is present (pulse-chase mode) than when ADP is absent (preincubation mode); and (ii) ATP was more effective in promoting P_i release when ADP is absent (preincubation mode) than when ADP is present (pulse-chase mode).

The results are rationalized in terms of the binding-change mechanism for the F_1 catalyzed hydrolysis of ATP involving the (i) nature of the nucleotide bound, (ii) number of occupied sites of F_1 before exposure to incoming nucleotides, and (iii) the ability of the nucleotides to produce conformational changes necessary to effect the release of P_i and/or ADP.

To Martha and Matthew

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
AMPPNP	5'-Adenylyl-β,γ-imidodiphosphate
GTP	Guanosine 5'-triphosphate
ITP	Ionosine 5'-triphosphate
PK	Pyruvate Kinase
PEP	Phosphoenol pyruvate
LDH	Lactate Dehydrogenase
β-NADH	β-Nicotinamide adenine dinucleotide
NBD-Cl	7-chloro-4-nitro-2-oxa-1,3-diazole
P_i	Inorganic phosphate
SDS	Sodium dodecyl sulphate
TNP-ADP	2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-diphosphate
TNP-ATP	2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate

Introduction

In this research project the dissociation of bound molecules (ATP, ADP, AMPPNP, and P_i) from beef heart mitochondrial adenosine triphosphatase was studied in the presence of other molecules (ATP, ADP, AMPPNP, and P_i). These studies were performed with the modified Sephadex centrifuge column technique described here.

(1) Beef Heart Mitochondrial Adenosine Triphosphatase

Mitochondrial adenosine triphosphatase (ATPase - E.C. 3.6.1.3) can be separated to give two fundamental units: one, a water-soluble unit, displays ATP hydrolytic activity; and the other, a water-insoluble "membrane sector", is without ATP hydrolytic activity, but is able to alter the catalytic properties of the soluble ATPase (for appropriate reviews see Refs. 1 - 18). The catalytically active soluble ATPase is generally referred to as F_1-ATPase, or simply as F_1; whereas the membrane sector of the complex is referred to as F_0, and the intact complex (or membrane-bound form of the enzyme) as ATPase (8) - other names include F_1F_0-ATPase, H^+-ATPase, ATP synthase, and Complex V (19,20,21). These two fractions were first recognized by Racker and co-workers (22,23) as playing essential roles in the coupling of oxidation to phosphorylation (see also Refs. 19,24). Table I summarizes the reactions (or processes) in which F_1 is known to be involved (7). In the investigations

Table I

Reactions of Oxidative phosphorylation for which F_1 is required (7,8).

1. ATP synthesis

2. ATP hydrolysis

$$ATP + H_2O \rightleftarrows ADP + P_i + H^+$$

3. Exchange reactions:

(a) $^{32}P \rightleftarrows ATP$, $ATP + {}^{32}P_i \rightleftarrows ATP\ (\gamma\text{-}{}^{32}P) + P_i$

(b) $H_2{}^{18}O \rightleftarrows P_i$, $^{*}P_i + H_2{}^{18}O \rightleftarrows P_i({}^{18}O) + H_2O$

(c) $H_2{}^{18}O \rightleftarrows ATP$, $ATP + H_2{}^{18}O \rightleftarrows ATP({}^{18}O) + H_2O$

4. ATP-dependent reactions of oxidative phosphorylation:

(a) Reversal of the respiratory chains -

$$Succinate + NAD^+ \rightleftarrows Fumarate + NADH + H^+$$

(b) Ion transport.

(Taken from Ref. 7.)

reported here, only the ATP hydrolytic activity of the enzyme was utilized, and soluble beef heart mitochondrial adenosine triphosphatase, F_1, was used.

Although this enzyme has been intensively studied (there has been an increase in ATPase publications from about 500 per year in the early seventies to over 1500 per year in the early eighties (13)) by many workers from different laboratories, there still remain several deficiencies in our knowledge of F_1 (1-19). What remains most elusive is an understanding of the reaction mechanism of F_1. No doubt some of the current uncertainties concerning the reaction mechanism result from the complexity of the pathways of ATP hydrolysis by F_1 (17,25).

Some of the properties of F_1 that must be considered when studying this cold-labile enzyme, include its specific activity, molecular weight, and structure. In addition, in these particular investigations with F_1 reported here, some knowledge of the tightly bound nucleotides and inorganic phosphate (eg. their number and possible role(s)), was helpful (4,7,10).

The specific activity of F_1 seems to depend on its history, which is influenced by the (i) source of mitochondria and method of preparation, (ii) method of preparation of F_1 from the mitochondria, (iii) method of storage of F_1, and (iv) assay conditions of F_1 (7). In the investigations described here, beef heart mitochondria were prepared by the modified method of Smith

(26); and the F_1 prepared according to the method outlined by Penefsky (27,28). The purified F_1 was stored at 4°C as a suspension in ammonium sulphate (2,29). The F_1-ATPase assay procedure was the regenerating system assay (2,30) coupled to the oxidation of NADH (31) (details are given in the Materials and Methods section).

Different molecular weights (ranging from 310,000 to 400,000), determined by different techniques, have been reported for F_1 from various sources. However, for beef heart mitochondrial F_1, the accepted molecular weight values seem to be 347,000 (determined by equilibrium sedimentation ultracentrifugation (32)) and 360,000 (determined by gel filtration (33)). The structure of this multi-subunit enzyme, F_1, is complex (1,3,5,7,11,18). There are five different recognized subunits ($\alpha, \beta, \gamma, \delta$, and ε) of F_1, which have been determined and characterized by sodium dodecyl sulphate, SDS, gel electrophoresis, gel filtration, amino acid analysis, and equilibrium sedimentation studies (32,34, 35). All the approaches gave similar values for the molecular weight of each type of subunit (54,000, 50,000, 33,000, 17,500 and 5,700), except the SDS gel electrophoresis studies which gave values higher or lower than those obtained by the other methods. However, the exact stoichiometry of the subunit composition of F_1 is still unclear, despite investigations involving different methods, e.g., SDS gel

electrophoresis (27), calculations using corrected molecular weight (36), aurovertin binding measurement (37), and chemical cross-linking studies (38). The choices for the subunit composition of F_1 were $\alpha_3\beta_3\gamma\delta\varepsilon$ and $\alpha_2\beta_2\gamma_2\delta_2\varepsilon_2$ (or similar combinations); however, evidence has accumulated in favour of the $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit composition (1,3,4,7,8,10,17,21).

This enzyme on isolation contains "tightly bound" adenine nucleotides (ATP and/or ADP) - these are bound non-covalently to F_1, since they are lost on denaturation of F_1 (39). Nucleotides are considered to be "tightly bound" if they are retained by F_1 despite Dowex or charcoal treatment, ammonium sulphate precipitation, or gel filtration with EDTA in a low ionic strength buffer (40). In addition, tightly bound ATP molecules are inaccesible to ATP-utilizing enzymes such as hexokinase or luciferase (41). These nucleotides are released on the denaturation of F_1 by cold (0°C) or acid treatment (e.g. 4% $HClO_4$). A non-denaturing way of removing the "tightly bound" nucleotides from F_1 is to pass the enzyme through a column of Sephadex G-25, which has been pre-equilibrated with EDTA in the appropriate buffer of high ionic strength (42,43).

The exact number of "tightly bound" adenine nucleotides per mole of F_1 is unknown, the numbers reported vary, e.g., two (0 ATP and 2 ADP/mole F_1 (34)), three (1 ATP and 2 ADP/mole F_1 (40), and 2 ATP and 1 ADP/mole F_1 (33)), and five (3 ATP and 2

ADP/mole F_1 (32)) nucleotides (7,17,41,45,46). Garrett and Penefsky (47) have suggested that five nucleotide binding sites are found on F_1; three of these contain "tightly bound" nucleotides and the other two sites participate in rapidly reversible binding of added adenine nucleotides. Cross and Nalin (48) have found six nucleotide binding sites on F_1, three sites participate in non-exchangeable binding and three sites in exchangeable binding. , This variation in amounts/numbers of "tightly bound" nucleotide as well as total bound nucleotide per mole of F_1 from different laboratories is probably dependent on the history of the enzyme (7).

The sites of the tight binding of adenine nucleotides on F_1 are also uncertain (41,45), though most likely the α and β subunits are involved. Studies with photoaffinity labels, e.g., 8-azido-ATP and 8-azido-ADP, have shown that these subunits are involved in nucleotide binding (49-52). The role of the subunits has been studied with antibodies, chemical modification, partial proteolysis, reconstitution, binding, and photoaffinity labelling experiments. The noncatalytic nucleotide binding sites are thought to be on the α subunits (48,53-55), and the catalytic sites on the β subunits (45,56-59). For example, Kozlov and Milgrom provided strong evidence when they showed that the covalent binding of the dialdehyde derivative of ADP (oxADP, formed as a result of treatment of ADP with periodate)

to the α subunit of F_1 occurred without loss in the hydrolytic activity of F_1 (54). In addition, Dunn and Futai, in their studies with isolated subunits of E. coli coupling factor ATPase, found by equilibrium dialysis studies that the isolated α subunit (not isolated β, γ, δ, or ε) bound [2,8-^{3}H]ATP or [2,8-^{3}H]ADP (approximately 0.9 mol nucleotide/mol α) (53). Their results suggested that each α subunit contains a single tight nucleotide binding site. It must be noted that Kagawa and co-workers found in their circular dichroism studies that both isolated α and β subunits of the thermophilic bacterium PS3 bound ADP and ATP (60). However, it was found by Harris et al. in their studies with beef heart mitochondrial ATPase that only 15-30% of the bound nucleotide had exchanged with labelled medium nucleotide ([^{3}H]ATP or [^{3}H]ADP), even after 24 hours in the presence of Mg^{2+} (39,41); thus the possibility of the active involvement of the tight nucleotide binding sites in the catalytic mechanism of F_1 is restricted (4,10,24,39,41). The nucleotides bound at the noncatalytic sites of the α subunits may be serving a structural (the nucleotide ATP is required for the reconstitution of F_1 from the three major subunits α, β, and γ (53)) or regulatory role (41,48,55). Ohta et. al. thought that the binding of ADP to the β subunits of TF_1 (thermostable F_1 - soluble ATPase from thermophilic bacterium PS3) was regulated by the binding of ADP to the α subunits of TF_1, possibly through allosteric interactions (60).

The nucleotides bound at the β subunits are thought to exchange rapidly with the medium nucleotides, thereby reflecting the process of the substrate binding at the catalytic sites (48, 60). Cross and Nalin in their studies with 5'-adenylyl-β,γ-imidodiphosphate (AMPPNP, a non-hydrolysable analog of ATP in which the oxygen bridge between the β and γ-phosphorus atoms has been replaced by a NH group) were able to demonstrate that three exchangeable nucleotide binding sites (probably β-subunits) are present on beef heart mitochondrial F_1, and that these sites are different from three other noncatalytic sites (probably α-subunits) (48). Grubmeyer and Penefsky used the ribose-modified nucleotides 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) and TNP-ADP in their studies and showed the presence of two high affinity nucleotide binding sites on F_1 (61,62).

The role of the nucleotide binding sites in the mechanism of mitochondrial ATPase is an area of active research (see also Refs. 63-68). The multiplicity of binding sites raises some very interesting questions, e.g. - "How many of the nucleotide binding sites are involved in the catalytic activity of the enzyme?" (7). Perhaps the most direct method of determining whether or not the "tight" nucleotide binding sites on F_1 are involved in the ATP hydrolytic reaction is to determine the turnover rate of nucleotides bound to these sites (69,70). However, as pointed out by Boyer and co-workers, their alterna-

tive non-destructive approaches (filter binding, EDTA quenching in rapid mixing experiments, and hexokinase-accessibility procedure) have their own limitations (69,70). It is undetermined whether or not most of the bound nucleotides are catalytic intermediates. The role of the tightly bound nucleotides in the mechanism of the F_1-catalysed hydrolysis of ATP is also unclear, as pointed out by Tiege et. al. in their studies using fluorescence techniques and isotope binding analyses with ADP and ADP analogues (65). Penefsky (7) and others (54,59,63-68,71,72) believe that these sites, in some way, do participate in the functioning of the enzyme.

Inorganic phosphate, P_i, is one of the products when F_1 hydrolyses ATP. F_1 also has at least one binding site for P_i (73). Kasahara and Penefsky (74,75) have demonstrated, under certain conditions (Mn^{2+} and aurovertin [an antibiotic used as a fluorescent probe of conformational change]), the presence of two types of P_i binding - a high affinity saturable binding, and a second low affinity nonsaturable binding. Their studies on the binding of P_i by F_1 showed a pH dependence and competitive inhibition by the P_i analog, thiophosphoric acid; these findings led them to suggest that the monoanion is the charged form of P_i which is bound by F_1. The binding of P_i by F_1 was shown to be influenced by the stimulators (e.g. divalent metal ions [Mg^{2+}, Co^{2+}, Ca^{2+}] and oxyanions [chromate, bicarbonate], and inhibitors (e.g. inhibitor protein, efrapeptin) of

ATPase activity and of oxidative phosphorylation (73,75). They observed that the ATP analog AMPPNP inhibited P_i binding even in the presence of aurovertin, and suggested it was likely that the P_i was binding at a site occupied by the γ-phosphate group of ATP (74). Thus there is a strong possibility that the P_i binding site can play a major role in oxidative phosphorylation.

Lauquin et. al. in their studies used the new photoaffinity derivative of inorganic phosphate, 4-azido-2-nitrophenyl phosphate (ANPP), to determine the P_i binding site(s) on isolated F_1 and inside-out particles from beef heart mitochondria (76). They found that P_i was most probably binding to the β-subunit of F_1-ATPase, and the P_i carrier protein. This work (76) therefore lends support to the proposals made by Kasahara and Penefsky (73-75).

As seen from the several reviews, the mechanism of the ATPase-catalysed reaction is still not well understood (1-18, 77-83). The main energy-requiring steps in oxidative phosphorylation and photophosphorylation (10,24) are: (i) the release of ATP from ATPase (84-87), and (ii) the binding of ADP and P_i to ATPase (88-90); evidence for these conclusions were first obtained from isotope exchange studies. In addition, there seems to be cooperative interaction between the subunits of ATPase. For example, Adolfsen and Moudrianakis showed that the rate of dissociation of bound ADP from 13S coupling factor of Alcaligenes faecalis was increased on the addition of nucleotide

to the medium (92). They proposed that the binding of nucleotide to one site of the 13S coupling factor caused a conformational change which facilitated the dissociation of bound nucleotide at the other site. These workers, however, did not show whether or not catalytic sites were involved (10,92,93).

The alternating catalytic site model of Boyer and coworkers (24,93-96) was proposed on the basis of isotope exchange experiments. This model has now evolved into what is called the "binding change mechanism" (10,13,97-99) which incorporates the cooperative interactions between the subunits and the energy-dependent binding changes (binding of ADP and P_i, and release of ATP in net synthesis, and vice versa in net hydrolysis). In this model, during net oxidative phosphorylation, ATP is produced at one site on the ATP synthase where it is transitorily tightly bound, and is only released when ADP and P_i bind at a second site, and the membrane ATPase complex is energised. Similarly, under conditions of net hydrolysis, ATP binding at one site is accompanied by the release of the transitorily tightly bound ADP and P_i (hydrolysed ATP) at a second site. This binding change mechanism is attractive, especially since it can accommodate a wide range of experimental observations (24). Evidence in support of this mechanism has been forthcoming from different sources (10,61,62,69,70,97-104). For example, strong

evidence for cooperativity between the catalytic sites of F_1 was presented by Grubmeyer and Penefsky (61,62) in their investigation with the ribose-modified nucleotides 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate, TNP-ATP, and TNP-ADP. They found that F_1 (with two binding sites for TNP-adenine nucleotides) bound both TNP-ATP and TNP-ADP, with the TNP-[γ-^{32}P]ATP being hydrolysed by F_1. They observed that on the addition of excess non-radioactive TNP-ATP (sufficient to fill the second catalytic site) to the F_1-reaction mixture, that the rate of hydrolysis of TNP-[γ-^{32}P]ATP was accelerated 15-20 fold. Boyer and co-workers have incorporated the new data (97,99-101) in their model to produce a modified version which involves the participation of three alternating sites in the binding change mechanism of ATP synthase.

Cross et. al. have produced a modified version of the binding change mechanism of Boyer and co-workers, which recognizes catalytic cooperativity and involves three interacting catalytic subunits of F_1 (10,98). (The possiblity of the involvement of three interacting sites in the catalytic mechanism of F_1 was first recognised by Kayalar et al. (93)). One major advantage of this model is that it allows events (i.e. binding changes) to occur concurrently on the different, but interacting, catalytic subunits of ATPase.

This area of research is very rich with speculations and

proposals for the mechanisms of ATP synthesis and/or hydrolysis (see e.g. Refs. 4,7,10,13,18,24,41,45,59,68,78,105-107).

From the reviews of the various proposed mechanism of ATPase, it was seen that studies of the behaviour of enzyme-bound species can lead to the determination of the catalytic intermediate(s), and thus contribute to the resolution of the catalytic mechanism of the enzyme. Currently, there are a variety of techniques (e.g. kinetic, equilibrium dialysis, equilibrium gel-filtration, filter-binding assays, spectroscopic, Sephadex centrifuge column, stopped flow, quenched flow, and isotope exhange) used in the investigation of the enzyme-ligand interactions (108). Some of these techniques have been used in the study of F_1, but each approach has its own advantages and disadvantages and/or limitations. For example, quenched flow is destructive, equilibrium dialysis is slow, and filter binding assays are not always efficient and reproducible. Hence it was considered desirable to develop a non-destructive, simple, rapid, and convenient technique for the measurement of the enzyme-bound species during catalysis. Such a method would not only be specific for studies with F_1, but would also be suitable (with the appropriate modifications) for studies with other enzymes, whose products dissociate slowly unless substrates or other ligands which facilitate dissociation are present.

The approach described herein is limited to enzymes which (i) find the column matrix, Sephadex G-50, chemically inert; (ii) have no significant interaction with the column matrix; and (iii) must not be able to enter the gel, i.e. the molecular weight (and shape) of the enzymes must be greater than the exclusion limit of the matrix. The column matrix must be stable under the reaction conditions (temperature, pH, centrifugal force), in addition to being chemically inert to the buffer and substrate and/or products (or analogs of these).

(ii) Modified Sephadex Centrifuge Column Technique

Basically, the methodology involves column centrifugation, which was originally applied to the measurement of ligand binding by beef heart mitochondrial F_1 (109). The Sephadex centrifuge column consists of a 1.0 mL tuberculin syringe fitted with a porous polyethylene disc. The column packing is Sephadex G-50 equilibrated with the appropriate buffer, and the filled column is then centrifuged (1050 × g for 2 minutes). The enzyme sample is then applied to the column before the second centrifugation (1050 × g for 2 minutes). The centrifugate is analysed for the bound ligand, activity and protein concentration of the enzyme.

The Sephadex centrifuge column procedure in essentially its original form has been used in several studies (48,73-75,96,104, 110). Cross and Nalin (48) introduced the use of an extension tube at the top of the column, which allowed (i) a number of samples to be investigated simultaneously, and (ii) other addi-

tions to be made to the samples just prior to centrifugation. Their second modification involved the addition of bovine serum albumin (BSA) to samples with very small amounts of F_1 (10 to 30 μg/mL). This facilitated the greater recovery of F_1 from the centrifuge columns when the concentration of F_1 was lower than 0.3 mg/mL. Cardon wrote an evaluation of the Sephadex centrifuge column technique in the Appendix of his Ph.D. thesis (111), in which he pointed out the basic assumptions that he made in the construction of his model to determine the necessary rate constants. So far no major evaluation, critique, or theoretical assesment of this technique has been published.

The Sephadex centrifuge column technique was modified to demonstrate the release of enzyme-bound species, e.g. P_i and ADP, in the presence of nucleotides (ATP, ADP, and AMPPNP). It is known that F_1-ATPase contains at least one binding site for P_i (73-75,109), however, it is undetermined whether or not the bound P_i is a catalytic intermediate, or whether the P_i is bound at a catalytic site. In the absence of nucleotide in the medium, the bound P_i dissociates slowly from F_1; whereas, on the addition of ATP or ADP to the medium, the dissociation of P_i from F_1 is accelerated to the point where the release is too rapid to observe using conventional techniques (96). The approach to this problem using the Sephadex centrifuge column technique, is outlined below:

(i) A column with three sections is prepared, the middle layer contains nucleotide, e.g. ATP. (ii) The sample of F_1 is preincubated with $[^{32}P]P_i$ so that radiolabelled P_i becomes bound to F_1 as indicated in the following equation:-

$$F_1 \cdot P_i \; + \; [^{32}P]P_i \; \rightleftarrows \; F_1 \cdot {}^{32}P_i \; + \; P_i$$

(iii) The $[^{32}P]P_i$ labelled F_1 is applied to the column, which in turn is placed in the centrifuge tube, and centrifuged at 1050 × g for 2 minutes; (iv) The column centrifugate is analysed as before.

In the first (uppermost) layer of the column, the loosely held species (ATP, ADP, and P_i) are removed. In the second layer (middle), the substrate, e.g. ATP becomes bound to the F_1 with or without radiolabelled P_i, and hydrolysis of ATP occurs. Lastly, in the third (bottom) layer of the column, the loosely-bound nucleotide and P_i released from F_1 are removed. Any tightly bound molecule (nucleotide and/or P_i) can be found in the centrifugate.

If labelled P_i is found in the F_1-centrifugate, it means that the P_i remains on the F_1 as a tightly bound molecule; and therefore most likely does not participate as a catalytic intermediate, nor occupies a catalytic site. On the other hand, if labelled P_i is not found in the F_1-centrifugate, it implies

that on the binding of nucleotide, the P_i is released from F_1, and thus most probably participates as a catalytic intermediate or occupies a catalytic site. If the dissociation of the bound P_i can be shown to occur within one enzyme turnover of ATP hydrolysis, then the dissociation of the bound P_i can be judged capable of being part of the catalytic mechanism of ATP hydrolysis.

In addition to the preincubation experiments outlined above, the Sephadex centrifuge column technique was used for performing pulse-chase experiments. The approach was very similar to that described above except that the first section contained the pulse molecules, and the third section the chase molecules.

Thus two approaches, preincubation and pulse-chase modes, were used to investigate the effect of the nucleotides ATP, ADP, and AMPPNP on the release of P_i, ADP, ATP, and AMPPNP from soluble beef heart mitochondrial adenosine triphosphatase.

Experimental Procedures

Materials

All reagents used in these investigations were reagent grade, ACS, or enzyme grade quality - whichever was applicable. The common laboratory reagents were obtained from the usual sources, except if otherwise mentioned. The following chemicals and enzymes were obtained from Sigma Chemical Co.: adenosine 5'-triphosphate (disodium salt, Grade IX); adenosine 5'-diphosphate (disodium salt, Grade IX); adenylyl imidodiphosphate (tetralithium salt); ammonium sulphate; 1,4-bis-2-(5-phenyloxazoyl)-benzene (POPOP); bovine serum albumin; 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl); DEAE-Sephadex G-50-120; 2,5-diphenyloxazole (PPO); β-nicotinamide adenine dinucleotide, reduced form (Grade III, from yeast); phosphoenol pyruvate; Sephadex G-50-80 (fine); sucrose; tris (hydroxymethyl) amino-methane; Triton X-114; hexokinase (Type C-300, from yeast); L-lactate dehydrogenase (Type XI, from rabbit muscle); and pyruvate kinase (Type III, from rabbit muscle). DEAE-Sephadex G-50-120 (fine) and Sephadex G-50-80 (fine) were also obtained from Pharmacia. The 1.0 mL tuberculin syringes were from Mandel Scientific Co. The polyethyleneimine thin layer chromatography plates were from E. Merck Co.

The radiochemicals: [2,8-^{3}H] adenosine 5'-triphosphate (ammonium salt); and adenosine 5'-[γ-^{32}P] triphosphate (triethyl

ammonium salt) were from Amersham Corp. [2,8-^{3}H] adenosine 5'-diphosphate (trisodium salt) was from New England Nuclear Co. [2,8-^{3}H] adenylyl 5'-imidodiphosphate was obtained from ICN, Chemical and Radioisotope Division. Phosphorus-32 (as orthophosphate in dilute HCl solution, pH 2.3) was purchased from both Amersham Corp. and New England Nuclear Co.

Methods

Preparation of Soluble Beef Heart Mitochondrial Adenosine Triphosphatase, F_1-ATPase

Fresh beef hearts were obtained from a local slaughterhouse in Surrey, British Columbia, Canada. The beef heart mitochondria were isolated by the method outlined by Smith (26). Both light and heavy fractions of beef heart mitochondria were used in the preparation of F_1-ATPase as described by Knowles and Penefsky (27,28).

From the ammonium sulphate suspension of F_1, the enzyme was prepared for studies in a procedure similar to that described by Penefsky (73). An aliquot of the ammonium sulphate suspension of F_1 was placed in a capped 2-mL polyethylene microcentrifuge tube, which in turn was placed in a larger polyethylene centrifuge tube. The large centrifuge tube (in its adaptor tube) was centrifuged in a SS-34 rotor for 10 minutes at 12,000 rpm at 4°C. The supernatant was decanted, and the inner walls of the 2-mL polyethylene microcentrifuge tube carefully dried with filter paper. The protein precipitate was dissolved by adding a volume of 50 mM Tris-acetate, pH 7.5, buffer at 30°C. The F_1 solution was desalted by centrifugation of 100-125 µL aliquots through Sephadex G-50-80 equilibrated with 50 mM Tris-acetate, pH 7.5, buffer at 30°C, using the Sephadex centrifuge

column technique of Penefsky (73). The desalted F_1 centrifugate was then ready for use with the appropriate reaction mixture(s).

Assay for ATPase Activity

The rates of ATP hydrolysis by F_1-ATPase were monitored using the coupled assay (31). The reagents and their respective concentrations and volumes used are shown in Table II. The first six reagents were added in the quantities indicated to a 1-mL cuvette. The reaction mixture in the cuvette was allowed to stand in the cuvette holder of a spectrophotometer (Varian Technotron Model 635) at 340 nm for about 10 minutes at 30°C. The spectrophotometer reading was adjusted to read zero absorbance at 340 nm. The required amount of β-NADH was added to the cuvette, and a stable base line at 340 nm established. A measured volume (X μL) of the F_1 solution was then added to the cuvette, and the decrease in absorbance monitored spectrophotometrically via a chart recorder. The rate of hydrolysis of ATP was calculated using an extinction coefficient of 6.22×10^3 litre mole.$^{-1}$.cm^{-1} for NADH (0.1 μ mole of NADH in 1.0 mL of solution gives A_{340} of 0.622). In these F_1 preparations, the ATPase activity was usually between 80-100 μmole.min.$^{-1}$.mg^{-1} protein.

Determination of Protein Concentration

All protein concentration determinations were according to the Lowry et. al. procedure (112). The protein bovine serum

Table II

The Coupled Enzyme Assay System

	Reagent	Volume μL
1.	0.1 M $MgCl_2$	50
2.	0.1 M ATP	37
3.	0.1 M PEP	37
4.	(5 mg/mL) LDH	10
5.	(5 mg/mL) PK	10
6.	50 mM CH_3COOK	
	50 mM Tris-Cl, pH 8.0	806 - χ
7.	8.75 mM NADH	50
8.	F_1-ATPase solution (mg/mL)	χ

NADH, $E_{340} = 6.22 \times 10^3$ litre mole.$^{-1}$.cm^{-1} or ($M^{-1}.cm^{-1}$)

i.e. 0.1 μmole.min^{-1} of NADH in 1.0 mL of solution gives ΔA_{340} of 0.622/min.

albumin (BSA) was used as the standard in all assays. The protein concentration of the BSA standards was determined by measuring the absorbances at 280 nm; these absorbance values (A_{280}) together with an extinction coefficient (E_{280}) of 0.677 $mL.mg^{-1}.cm^{-1}$ were then used in the Beer-Lambert law calculations. [Concentration of Protein = Absorbance at 280 nm/(Extinction Coefficient at 280 nm × path length)].

Molecular Weight

A molecular weight of 347,000 for F_1 was used in all calculations (73).

Purity of the F_1 Preparations

The purity of the isolated native F_1 preparations was tested with the use of polyacrylamide gel disc electrophoresis techniques as described by Davis (113), modified as mentioned by Knowles and Penesky (27). One major enzyme band was seen which had a relative mobility of 0.3 with respect to the dye front. With 25 μg of protein applied per gel, a minor band with a relative mobility of 0.7 was seen. These observations were similar to those of Knowles and Penefsky (27). Repurification of the enzyme on a DEAE-Sephadex A-50 column (28) did not result in the disappearance of the trace band.

Purity of the Radionucleotides

The purity of the radionucleotides ([γ-^{32}P]ATP, [2,8-^{3}H]-ATP, and [2,8-^{3}H]ADP) was tested on polyethyleneimine (PEI)

plates using the procedure of Randerath and Randerath (114). A sample of the labelled nucleotide was added to a mixture of the nucleotides (AMP, ADP, and ATP), which was spotted on the PEI plate beside separated spots of AMP, ADP, and ATP (1 μL of 2.5 mM each, pH 7.0). The plate was placed in a tank with 1.0 M LiCl and developed for 20-25 minutes. The dried plate was placed under UV light and the nucleotide spots encircled. The spots were cut out and placed in scintillation cocktail for counting. The percentage purity in each case was found to be about 90% (% Purity = [cpm of label recovered/cpm of label applied] × 100).

Monitoring ATP and/or ADP Concentrations

The concentrations of ATP and ADP (10^{-6} M) were monitored using the Hexokinase Assay (31).

Measurement of Radioactivity

The radioactivity of the samples was determined by adding an aliquot of the sample to 10 mL of water (Cerenkov counting), or to 10 mL of Triton/Toluene scintillant. The scintillant was prepared by adding 15 g PPO and 1 g of POPOP to 1.875 L xylene; to that mixture was then added 1.25 L Triton X-114 and 1.875 L xylene to bring the final volume to 5 L. Each radioactive preparation (Cerenkov counting or liquid scintillation counting sample) was counted for 5 minutes using a LKB Wallac 17 liquid scintillation counter.

Preparation of Sephadex Centrifuge Columns

The 1-mL Sephadex centrifuge columns used for (i) desalting F_1, and (ii) preparation of the gels for the longer and/or reassembled columns, were prepared as outlined by Penefsky (28,73). The columns used for desalting F_1 were packed with Sephadex G-50-80 equilibrated with 50 mM Tris-acetate, pH 7.5. The columns used in the investigations with F_1 were packed with Sephadex G-50-80 equilibrated with a buffer containing 90 mM Tris-acetate (pH 7.5), 1.6 mM $MgSO_4$, and 47 μM P_i. Nucleotide-equilibrated gels had the particular nucleotide added to the buffer to give the indicated concentration. All centrifugations using the Sephadex centrifuge column technique were performed with a table top clinical I.E.C. centrifuge (with a swinging bucket rotor, model 221) at 1050 x g (Setting No. 5) for 2 minutes.

Making a Longer Column Barrel

To make a longer column barrel, two 1-mL tuberculin syringes were cut (one at the 1.0 mL mark and the other at the 0.55 mL mark), and joined by means of a piece of tygon tubing. The length of the column barrel was 10 cm, with the length of the whole assembly (from head to outlet tip) being about 11 cm.

Modified Sephadex Centrifuge Column Technique

(a) Preincubation Mode

Basically the technique was the same as described in Appendix I (Fig. 1A), with the optimum conditions mentioned below being used.

Three 1-mL Sephadex centrifuge columns were prepared using the conventional Sephadex centrifuge column technique (28,73). Two of the columns were packed with Sephadex G-50-80 equilibrated with a buffer containing: 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 47 μM P_i. The third column was packed with Sephadex G-50-80 equilibrated with a buffer consisting of: 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, 47 μM P_i, and varying concentrations of nucleotide. In both cases, 5 g of Sephadex G-50-80 were added to 100 mL buffer (or reduced amounts in the same proportions) and allowed to stand overnight at 4°C.

Care was taken in pouring the gels (equilibrated to room temperature), since trapped air bubbles usually resulted in broken gels. In addition, the columns were not allowed to run dry until the column barrels were filled with gel.

The columns were centrifuged at 1050 x g (Setting No. 5, I.E.C., Rotor 221) for 2 minutes. The gels were removed from the column barrels by gently tilting the columns so that the gels slipped out. The first two gels were cut to give lengths of 2.5 and 4 cm, respectively. The third gel (i.e. with nucleo-

tide) was cut to give a 1-cm portion. The measured lengths of gels were reassembled in the lengthened column barrel (prepared as described above). The 4-cm gel was placed in the column barrel (on top of a porous polyethylene frit); the 1-cm nucleotide-containing gel was placed on top of the 4-cm gel; and lastly the 2.5-cm gel was placed on the nucleotide-containing gel. The bottom portion of a cut 1-mL pipette tip (with about 200 μL capacity) was inserted in the column. The tip of the pipette tip was not allowed to touch the top gel nor the inside of the column barrel. The entire assembly was inserted in a 15-mL conical centrifuge tube, which in turn was placed in the swinging bucket rotor (Model 221) of the table top clinical centrifuge (I.E.C.). At 150-μL aliquot of the reaction mixture which was preincubated for at least 30 minutes at 22-25°C was placed in the cut pipette tip. The reaction mixture consisted of: 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, 47 μM P_i with $[^{32}P]P_i$, and F_1. The control experiment was performed without F_1 in the reaction mixture. Whenever F_1 was used, the concentration was approximately 2.8 μM, or 0.96 mg protein/mL. However, in each experiment the specific activity of the $[^{32}P]P_i$ and the concentration of F_1 used are indicated. The loaded reassembled column was centrifuged at 1050 x g for 3 minutes. A sample (20 or 25 μL) of the centrifugate was used to determine the amount of $[^{32}P]P_i$ which passed through the

column. Other samples of the centrifugate were used to determine the protein concentration and/or activity of the F_1 in the centrifugate. Each experiment was performed in triplicate for each nucleotide concentration.

The amount of P_i in a fixed volume of centrifugate was calculated using the amount of radioactivity found in that volume of the centrifugate and the specific activity of the $[^{32}P]P_i$ label in the reaction mixture applied to the column. The amount of F_1 in an equivalent volume of centrifugate was calculated using the protein concentration of F_1 in the centrifugate found by the Lowry assays. The value of the amount of P_i divided by the value of the amount of F_1 gives the ratio of mole P_i/mole F_1. On the x-axis are shown the nucleotide concentrations used in the equilibration buffers of the middle gels. Each value plotted is the average obtained from the triplicate experiments, and the lowest and highest experimental results give values which fell within the enclosed circle, square, or triangle.

(b) Pulse-Chase Mode

The experimental conditions and procedure were basically the same as described for the preincubation mode of the modified Sephadex centrifuge column technique. The lengths of the four sections (from top to bottom) were 1,3,1 and 2.5 cm, respective-

ly. These were arranged as shown in Appendix II, Fig. 7A. In some cases, the third and fourth sections were combined to give one longer chase section (3.5 cm). The radiolabelled nucleotide (e.g. [γ-^{32}P]ATP) was added to the nucleotide-containing buffer used to prepare the pulse gel (N.B. No [^{32}P]P_i was added to the reaction mixture applied to the column). The pulse gel equilibration buffer contained: 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, 47 μM P_i, and 1 μM nucleotide; whereas the chase gel equilibration buffers had the various concentrations of nucleotide indicated. The experiments were performed in triplicate for each different chase nucleotide concentration.

Results

Release of P_i from F_1: Preincubation Method

The effects of ATP, ADP, and AMPPNP on the release of P_i bound to F_1 were studied using the preincubation mode of the modified Sephadex centrifuge column technique described under Methods and Materials and in Appendix I. In these studies, the concentrations of each adenine nucleotide in the equilibration buffers of the middle sections of the columns were as indicated and, unless otherwise stated, the length of the nucleotide containing middle gel was always 1.0 cm. Figure 1 shows the effects of the three adenine nucleotides ATP, ADP, and AMPPNP on the release of P_i from F_1. It is evident from Figure 1 that the sensitivity of the P_i release reaction to the concentration of nucleotide in the middle section of the column is different for each of the three nucleotides, and that P_i release shows a biphasic response to ATP and AMPPNP in the middle section. In these biphasic responses, approximately 70% of the total P_i bound is released in a relatively highly sensitive phase (steep slope), and the remaining P_i is released in a less sensitive phase (shallow slope).

Figure 2 shows that at relatively low concentrations (i.e. less than 1.0 μM nucleotide), the effects of ADP and AMPPNP on the release of bound P_i from F_1 are essentially the same. This

Figure 1

The effects of ATP, ADP, and AMPPNP on the release of bound P_i from F_1. The modified Sephadex centrifuge column technique was used as described in Methods. For the ADP plot (□): the concentration of F_1 in the reaction mixture was 0.64 mg protein·mL^{-1} or 1.85 μM, the specific activity of the [^{32}P]P_i was 1.9×10^5 cpm/nmole, and the 100% P_i bound corresponded to a ratio of 0.22 mole P_i/mole of F_1. For the AMPPNP plot (△): the concentration of F_1 was 0.75 mg protein·mL^{-1} or 2.2 μM, the specific activity of the [^{32}P]P_i was 1.5×10^6 cpm/nmole, and the 100% P_i bound corresponded to a ratio of 0.26 mole P_i/mole F_1. For the ATP (○): the concentration of F_1 was 0.8 mg protein·mL^{-1} or 2.32 μM, the specific activity of the [^{32}P]P_i was 1.9×10^6 cpm/nmole, and the 100% P_i bound corresponded to a ratio of 0.28 mole P_i/mole F_1. (□ ADP, △ AMPPNP, and ○ ATP).

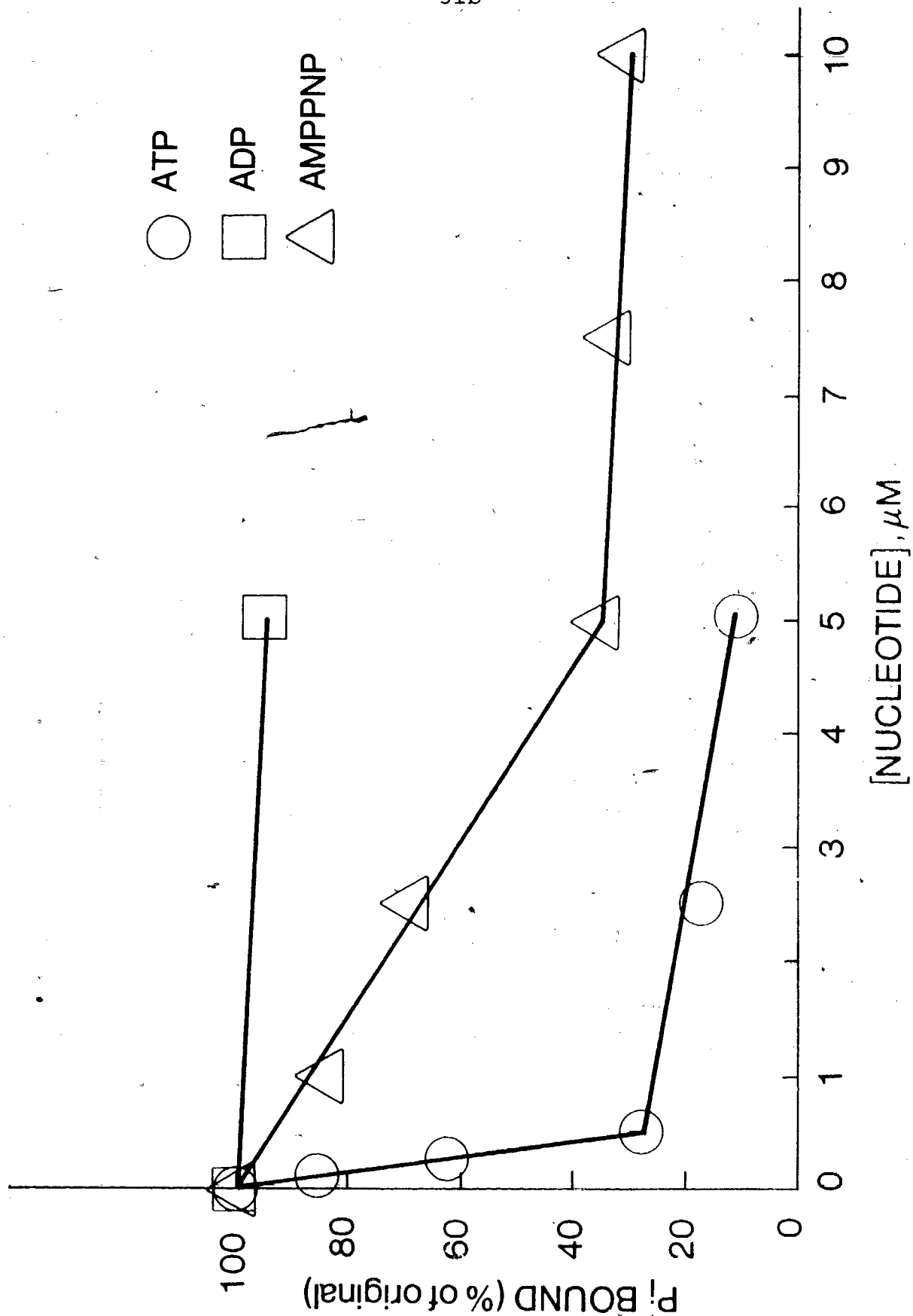
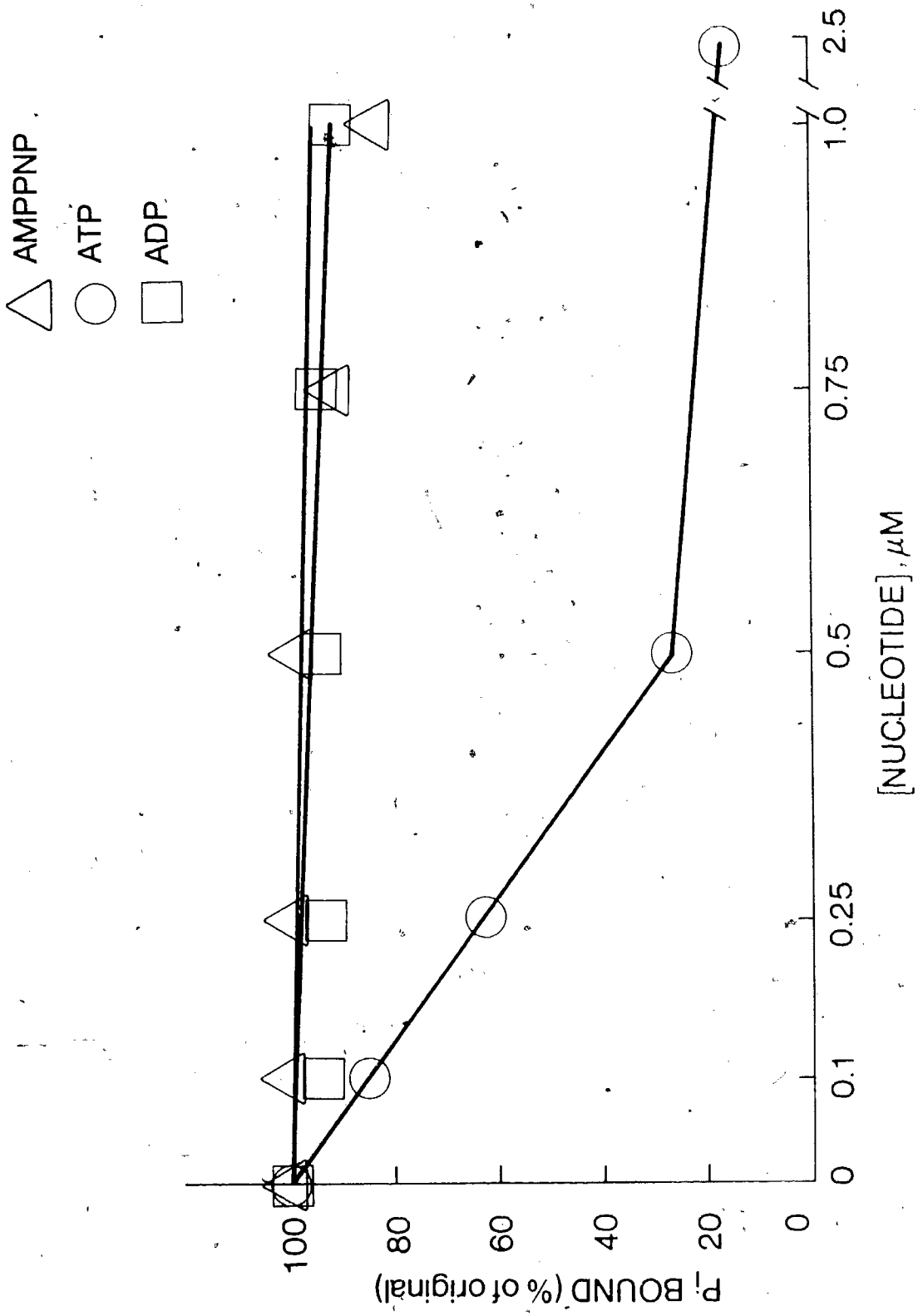

ATP
ADP
AMPPNP
Pi BOUND (% of original)
100
80
60
40
20
0
0
1
3
4
5
6
7
8
9
10
[NUCLEOTIDE], μM

Figure 2

The effects of ADP, AMPPNP, and ATP on the release of P_i from F_1. The modified Sephadex centrifuge column technique was used as described in Methods. For the ADP plot (□): the concentration of F_1 was 1.1 mg protein·mL^{-1} or 3.2 μM, the specific activity of the $[^{32}P]P_i$ was 1.8×10^6 cpm/nmole, and the 100% P_i bound corresponded to a ratio of 0.27 mole P_i/mole F_1. For the AMPPNP plot (△): the concentration of F_1 was 0.76 mg protein·mL^{-1} or 2.23 μM, the specific activity of the $[^{32}P]P_i$ was 1.6×10^5 cpm/nmole, and the 100% P_i bound corresponded to a ratio of 0.29 mole P_i/mole F_1. For the ATP plot (○): the concentration of F_1 was 0.8 mg protein·mL^{-1} or 2.3 μM, the specific activity of the $[^{32}P]P_i$ was 1.9×10^6 cpm/nmole, and the 100% P_i bound corresponded to a ratio of 0.28 mole P_i/mole F_1. (□ ADP, △ AMPPNP, and ○ ATP)


AMPPNP
ATP
ADP
Pi BOUND (% of original)
100
80
60
40
20
0
0
0.1
0.25
0.5
0.75
1.0
2.5
[NUCLEOTIDE], μM

pointed out the necessity of exploring a range of experimental conditions, before attempting to draw general conclusions about the relative effectivness of the different adenine nucleotides in facilitating release of P_i from F_1. Figures 3-5 show the effects of high concentrations of ATP and ADP on P_i release from F_1. From these results, it is evident that high concentrations of ATP and ADP appear to have similar effects, and that in both cases they are able to effect the release of essentially all the bound P_i from F_1.

Figure 3

The effect of ATP on the release of P_i from F_1. The modified Sephadex centrifuge column technique was used as described in Methods. The concentration of F_1 in the reaction mixture was 0.96 mg protein$\cdot mL^{-1}$ or 2.78 μM. The specific activity of the $[^{32}P]P_i$ was 1.3×10^6 cpm/nmole. The 100% P_i bound corresponded to a ratio of 0.2 mole P_i/mole F_1.

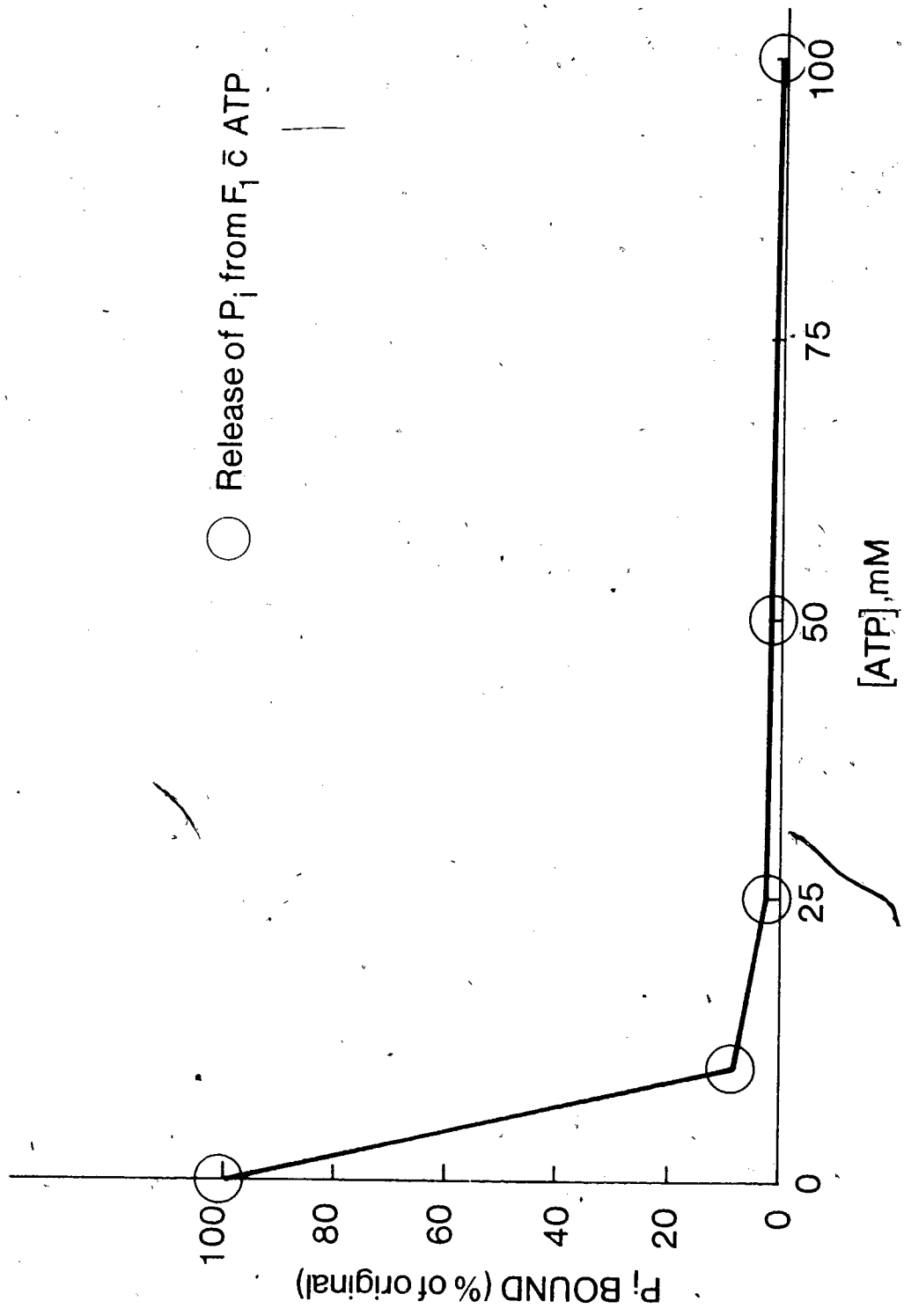

Release of P_i from F_1 c̄ ATP
P_i BOUND (% of original)
100
80
60
40
20
0
0
25
50
75
100
[ATP],mM

Figure 4

The effect of ATP on the release of P_i from F_1. The modified Sephadex centrifuge column technique was used as described in Methods. The concentration of F_1 in the reaction mixture was 0.96 mg protein$\cdot mL^{-1}$ or 2.78 μM. The specific activity of the $[^{32}P]P_i$ was 1.5×10^5 cpm/nmole. The 100% P_i bound corresponded to a ratio of 0.18 mole P_i/mole F_1.

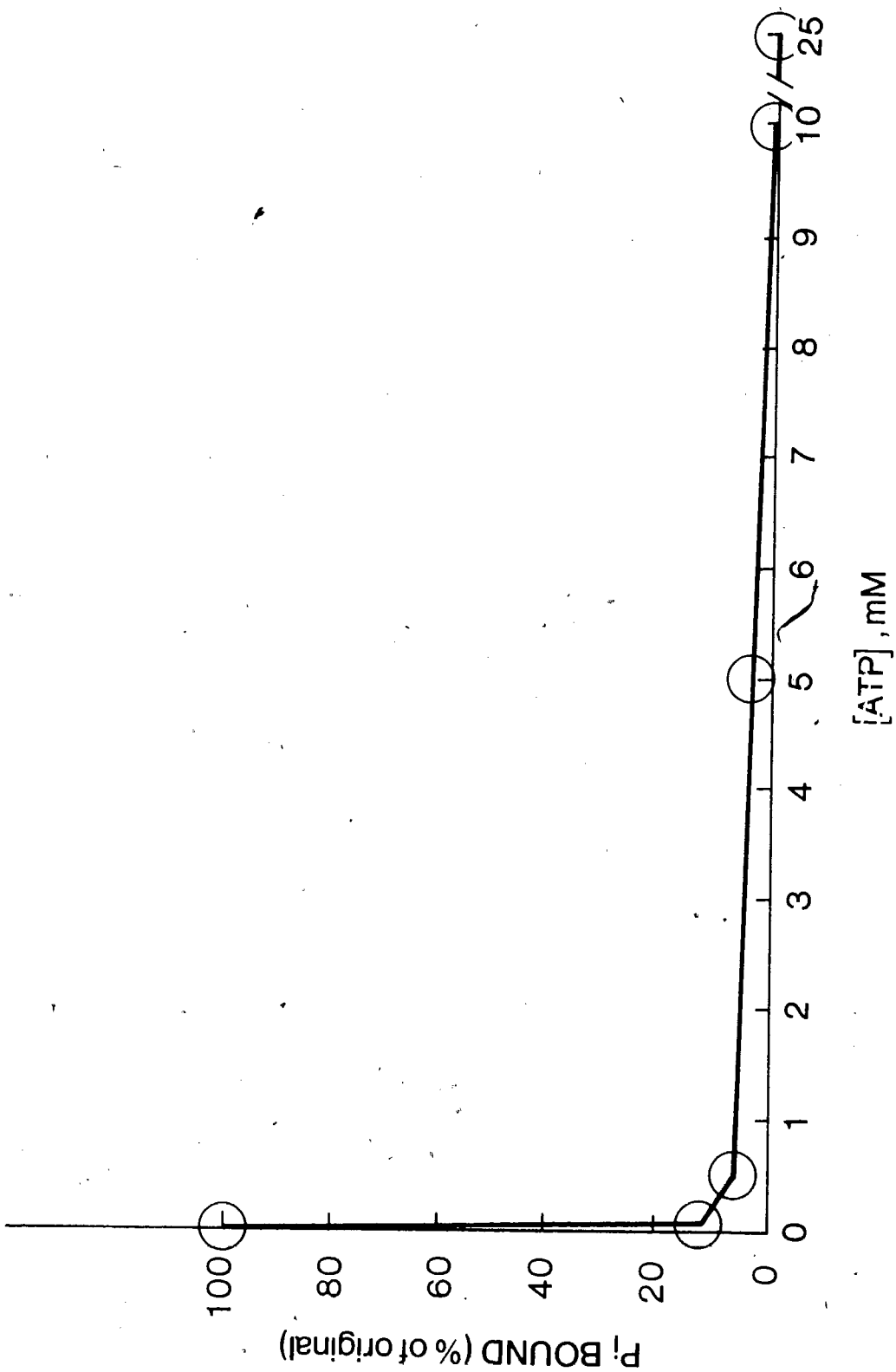
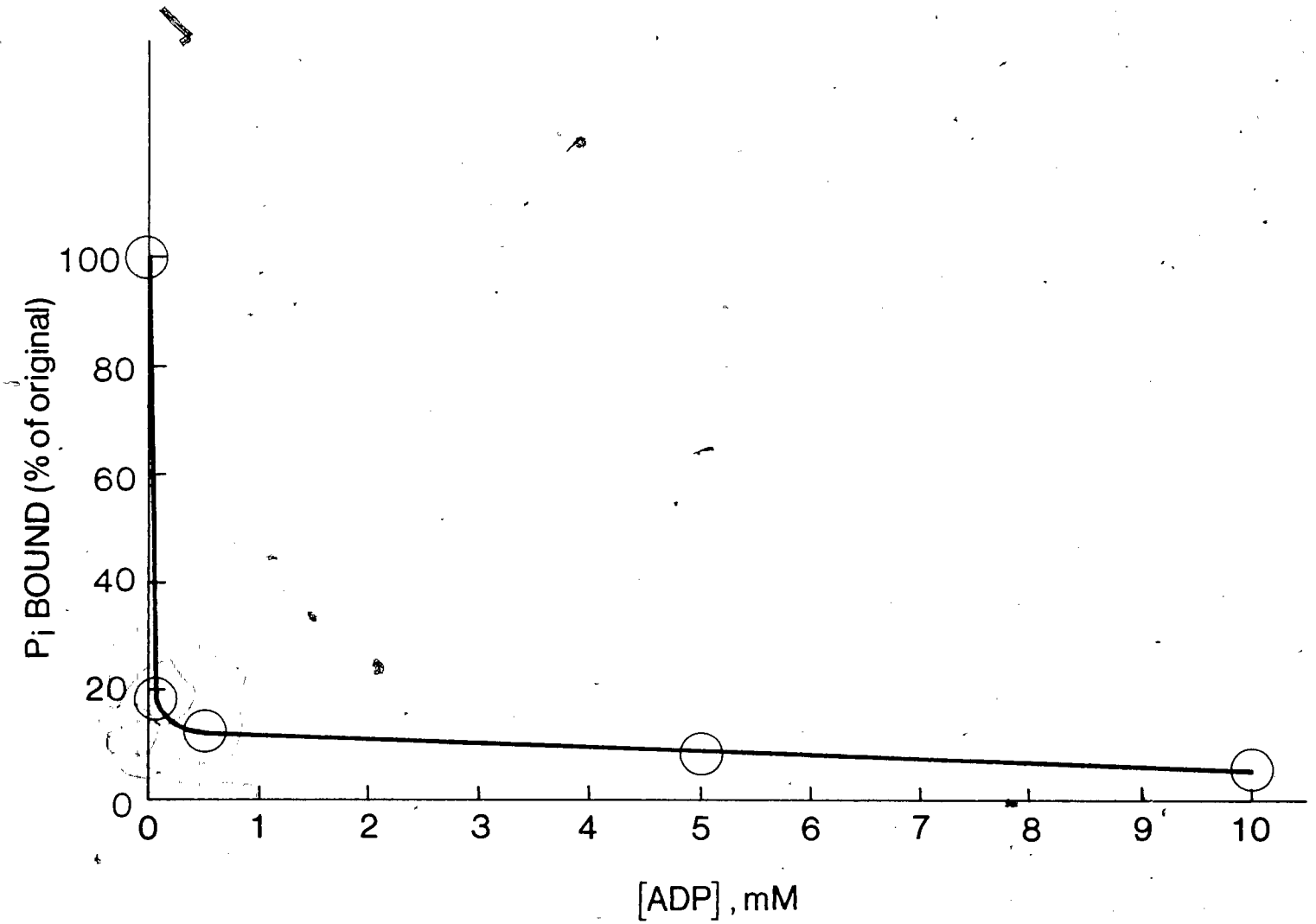

Pi BOUND (% of original)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
25
[ATP], mM

Figure 5

The effect of ADP on the release of P_i from F_1. The modified Sephadex centrifuge column technique was used as described in Methods. The concentration of F_1 in the reaction mixture was 0.64 mg protein.mL^{-1} or 1.85 μM. The specific activity of the $[^{32}P]P_i$ was 1.9×10^5 cpm/nmole. The 100% P_i bound corresponded to a ratio of 0.22 mole P_i/mole F_1.


100
80
60
40
20
0
P_i BOUND (% of original)
0
1
2
3
4
5
6
7
8
9
10
[ADP], mM

Release of P_i from F_1: Pulse-Chase Method

The pulse-chase mode of the modified Sephadex centrifuge column technique, as described under Methods and in Appendix II, was used to study the effects of ADP, ATP, and AMPPNP on the release of bound label from F_1. The label in this approach, was given to F_1 in its passage through the 1 cm "pulse section" at the top of the column. This pulse section was prepared from a gel which had the desired labelled nucleotide in the equilibration buffer of the Sephadex. Below the pulse section, the column contained three sections (3, 1, and 2.5 cm) serving similar functions as those used in the columns for the studies employing the preincubation mode of the Sephadex centrifuge column technique. The third section from the top is referred to as the "chase section" of the column.

The studies of P_i release using the pulse-chase mode are valuable for comparison with those using the preincubation mode. In the preincubation studies: when P_i (from the reaction mixture) is bound to F_1, the P_i presumably occupies an empty site which contains no other ligands. In the pulse-chase studies [γ-^{32}P]ATP is present in the pulse gel, therefore any bound [^{32}P]P_i (found in the centrifugate) originated from the [γ-^{32}P]ATP which bound and hydrolysed, and it also shares its binding site with ADP (the other product of hydrolysis).

Published studies have shown that when ATP binds to F_1 under single site occupancy conditions (such as those used in the studies reported here), it hydrolyzes rapidly and ADP and P_i are released slowly (103). Thus by comparing the results from the preincubation and pulse-chase methods, it is possible to assess the effect of ADP being bound at the same site with P_i, on the sensitivity of the P_i release reaction to nucleotides in the chase section. Figures 6 and 7 show the effects of ATP and AMPPNP (in the chase section), respectively, on the release of label bound as [γ-^{32}P]ATP from the pulse section. For ease of comparison, the results from the corresponding studies with the preincubation mode are included in the figures. The sensitivity of P_i release to ATP is decreased in the pulse-chase experiment relative to the release observed in the preincubation experiment (Fig. 6), whereas the opposite effect is observed with AMPPNP in the pulse-chase and preincubation experiments (Fig. 7). The sensitivity of P_i release to ATP and AMPPNP is essentially the same in the pulse-chase experiments (cf. Figs. 6 and 7); whereas the sensitivity of P_i release to ATP is much greater than to AMPPNP in the preincubation experiments. The biphasic response of P_i release on exposure to ATP and AMPPNP is observed with both experimental methods.

Figure 6

Comparison of the effects of ATP, in preincubation and pulse-chase modes, on the release of label from F_1. For the preincubation experimental plot, ○ , the data were taken from Figure 2. The pulse-chase technique was as described in Methods, with four sections (1, 3, 1 and 2.5 cm) being used. Note that in both modes, a 1 cm nucleotide-containing middle section (preincubation mode) or chase section (pulse-chase mode) was used. The concentration of F_1 in the reaction mixture 0.96 mg protein$\cdot mL^{-1}$ or 2.78 μM. The pulse-gel equilibration buffer contained 1.0 μM ATP with $[\gamma\text{-}^{32}P]ATP$, the specific activity of which was 3.07×10^5 cpm/nmole. The 100% ATP bound corresponded to a ratio of 0.1 mole ATP/mole F_1 (○ ATP on the release of label bound as $[^{32}P]P_i$ (Preincubation mode); and ❑ ATP on release of label bound $[\gamma\text{-}^{32}P]ATP$ (Pulse-chase mode)).

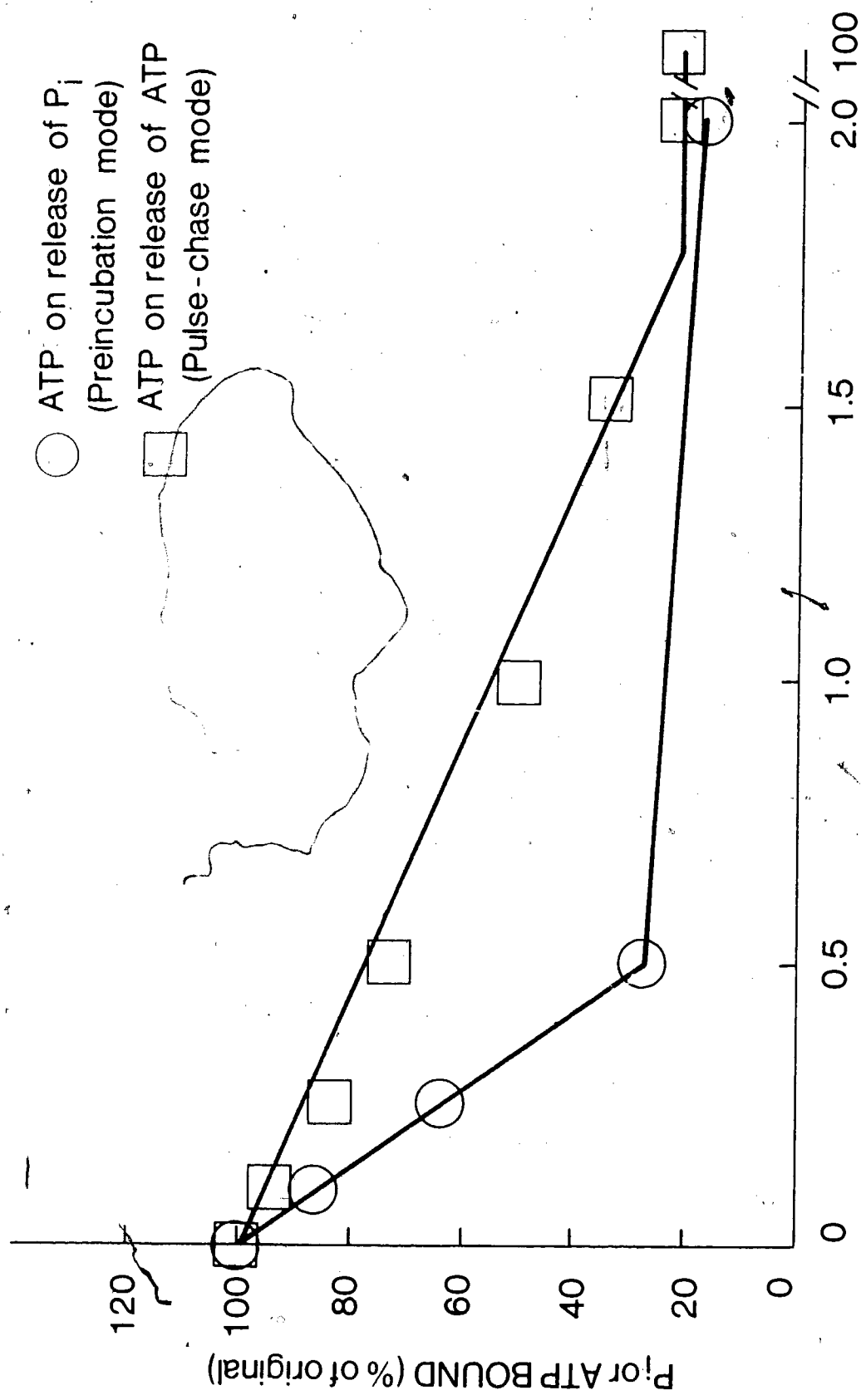
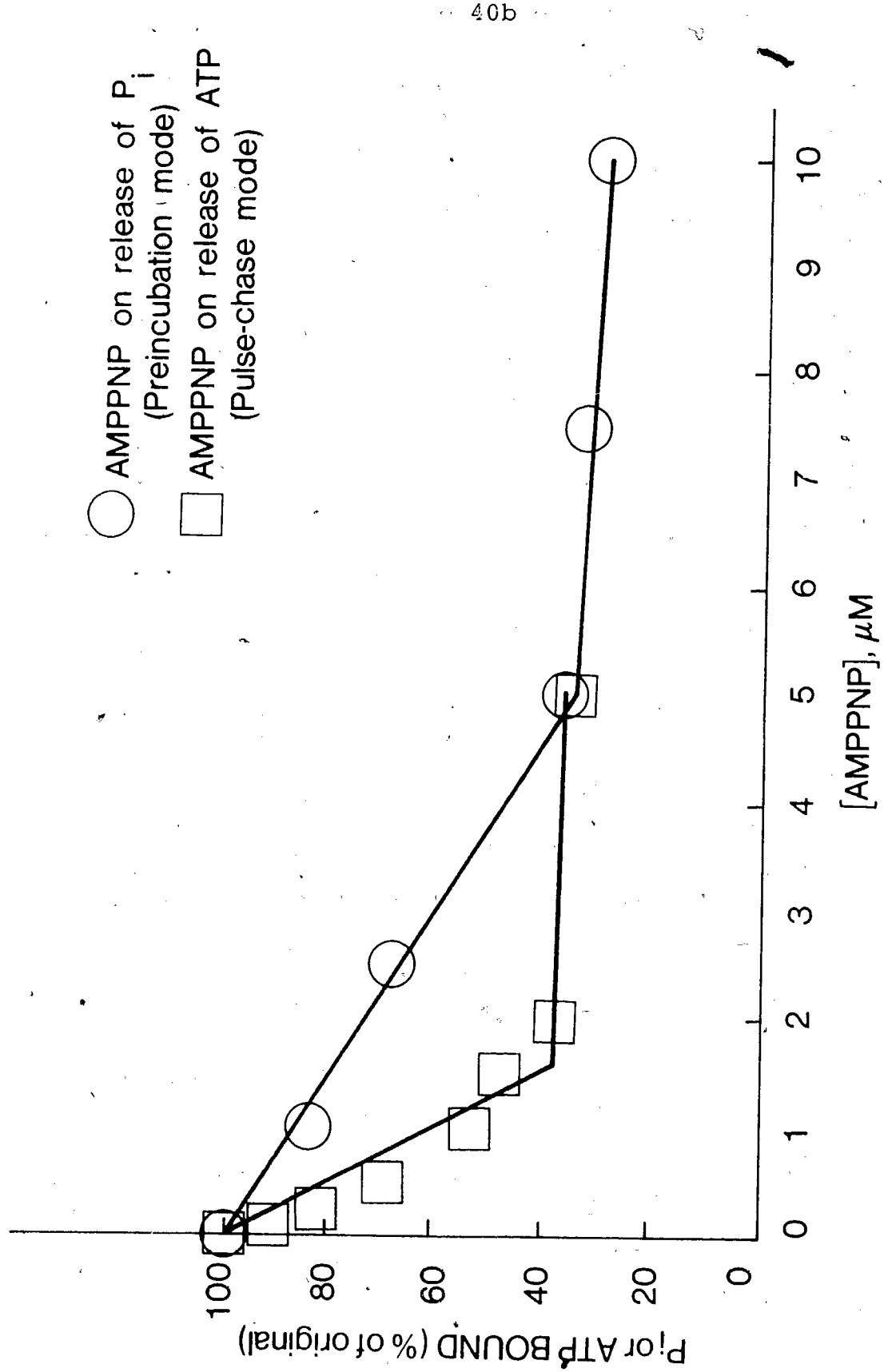

ATP on release of P_i
(Preincubation mode)
ATP on release of ATP
(Pulse-chase mode)
P_i or ATP BOUND (% of original)
120
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0 100
[ATP], μM

Figure 7

Comparison of the effects of AMPPNP, in preincubation and pulse-chase modes, on the release of label from F_1. For the preincubation experimental plot, ○ , the data were taken from Figure 2. The pulse-chase technique was as described in Methods, with four sections (1, 3, 1 and 2.5 cm) being used. Note that in both modes, a 1.0 cm nucleotide-containing middle section (preincubation mode) or chase section (pulse-chase mode) was used. The concentration of F_1 in the reaction mixture was 1.07 mg protein$\cdot mL^{-1}$ or 3.09 μM. The pulse-gel equilibration buffer contained 1.0 μM ATP with [γ-^{32}P]ATP (2.68×10^5 cpm/nmole). The 100% ATP bound corresponded to a ratio of 0.1 mole ATP/mole F_1. (○ AMPPNP on release of label bound as [^{32}P]P_i (Preincubation mode), and □ AMPPNP on release of label bound as [γ-^{32}P]ATP (Pulse-chase mode)).


AMPPNP on release of P_i
(Preincubation mode)
AMPPNP on release of ATP
(Pulse-chase mode)
P_i or ATP BOUND (% of original)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
[AMPPNP], μM

In the pulse-chase experiments just described (Figs. 6 and 7), 1 cm chase sections were used in order to have the nucleotide-containing sections identical to those used (1 cm middle section) in the preincubation experiments. Thus the experimental conditions and procedures were made as close as possible in both methods. Figures 8 and 9 show the results of pulse-chase experiments in which entire lower 3.5 cm (1 cm chase gel plus 2.5 cm bottom gel) of the column contained chase nucleotide. A comparison of Figure 8 with Figures 6 and 7 shows the results with [γ-^{32}P]ATP pulse and an ATP or AMPPNP chase were essentially identical in both cases, i.e. with the column containing either a 3.5 cm chase section (i.e. the entire bottom) or a 1 cm chase section followed by a 2.5 cm spacer section. Figure 8 shows the relative sensitivity of the release of label, bound as [γ-^{32}P]ATP, to ADP, ATP, and AMPPNP in the 3.5 cm chase section. The nucleotides ATP and AMPPNP were equally effective in facilitating the release of label, whereas ADP was considerably less effective in promoting the release of label. Figure 9 shows that whether label was bound as [^{3}H]-ATP or as [γ-^{32}P]ATP from the pulse section, approximately the same sensitivity of label release to chase ATP was seen in both cases.

Figure 8

The effects of ADP, ATP, and AMPPNP on the release of label bound as [γ-^{32}P]ATP from F_1. The pulse-chase technique was as described in Methods, with three sections (1, 3, and 3.5 cm) being used. Note that the entire bottom section (3.5 cm) contained chase nucleotide. For the ADP plot (□): the concentration of F_1 in the reaction mixture was 1.09 mg protein$\cdot$mL^{-1} or 3.16 μM; the specific activity of the [γ-^{32}P]ATP in the 1.0 μM ATP-containing equilibration buffer of the pulse gel was 2.04 $\times$ 10^5 cpm/nmole; and the 100% ATP bound corresponded to a ratio of 0.1 mole ATP/mole F_1. For the ATP plot (○): the concentration of F_1 was 1.16 mg protein$\cdot$mL^{-1} or 3.36 μM; the specific activity of the [γ-^{32}P]ATP was 2.15 $\times$ 10^5 cpm/nmole; and the 100% ATP bound corresponded to a ratio of 0.12 mole ATP/mole F_1. For the AMPPNP plot (△): the concentration of F_1 was 0.95 mg protein$\cdot$mL^{-1} or 2.75 μM; the specific activity of the [γ-^{32}P] was 2.35 $\times$ 10^5 cpm/nmole; and the 100% ATP bound corresponded to a ratio of 0.1 mole ATP/mole F_1. (□ ADP, ○ ATP, and △ AMPPNP)

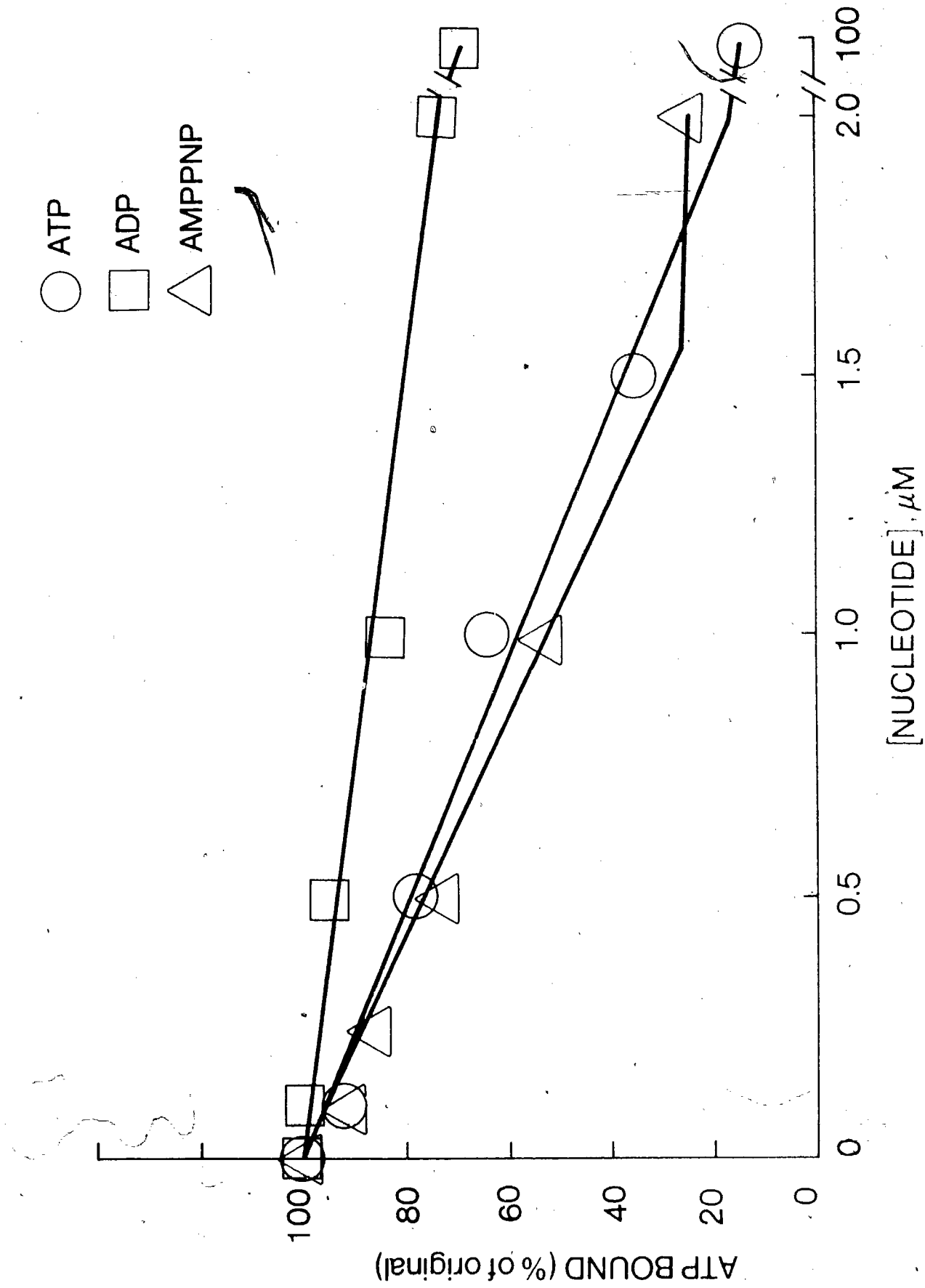
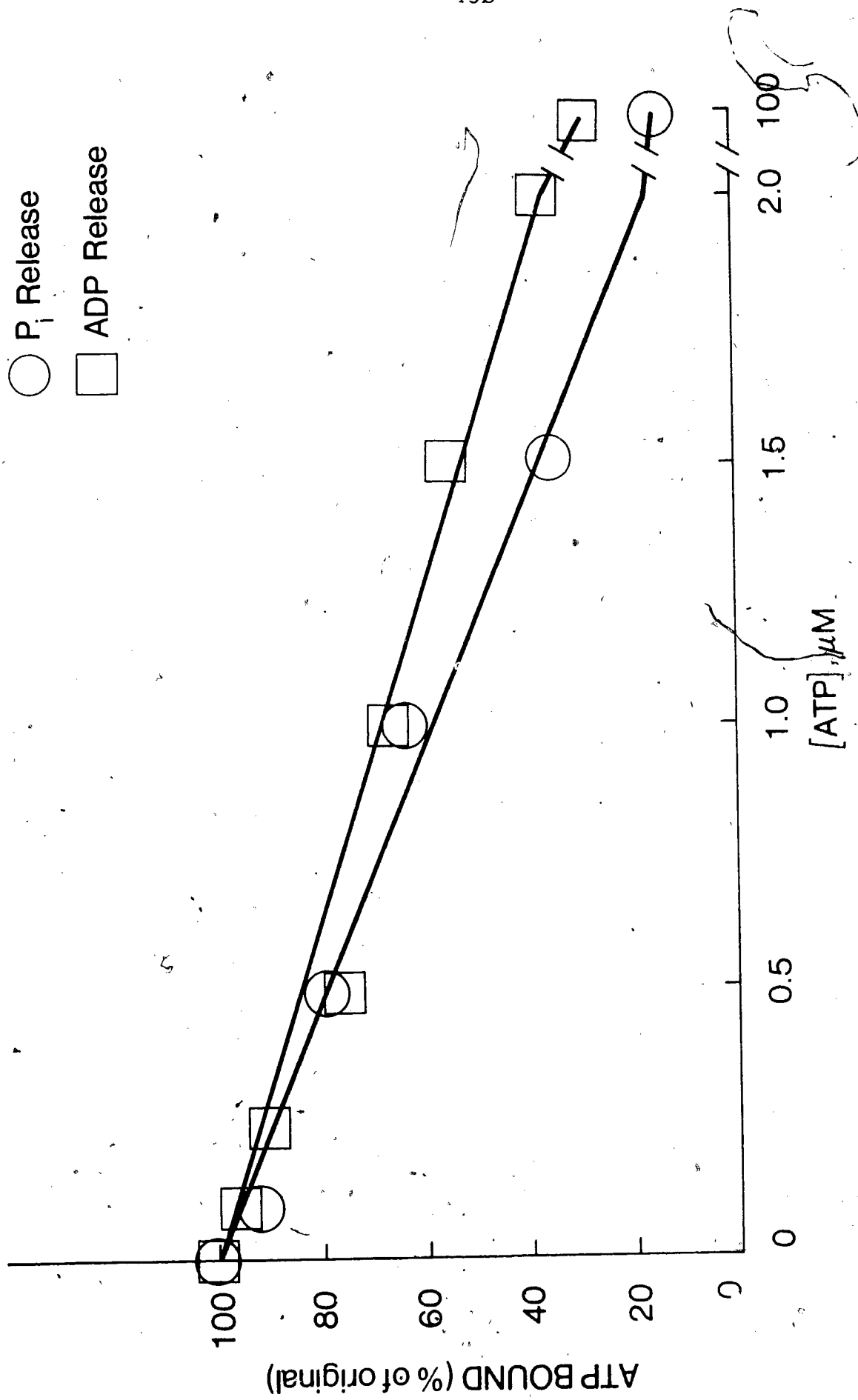

ATP
ADP
AMPPNP
ATP BOUND (% of original)
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
100
[NUCLEOTIDE], μM

Figure 9

The effects of ATP on the release of label bound as [γ-^{32}P]ATP and (2,8-^{3}H]ATP from F_1. The pulse-chase technique was as described in Methods, with three sections (1, 3, and 3.5 cm) being used, and the entire bottom 3.5 cm section containing chase nucleotide. For the release of label bound as [γ-^{32}P]ATP plot (○), the data were taken from Figure 8. For the release of label bound as [^{3}H]ATP plot (□): the concentration of F_1 in the reaction mixture was 0.94 mg protein·mL^{-1} or 2.72 μM; the specific activity of the [^{3}H]ATP in the 1.0 μM ATP-containing equilibration buffer of the pulse gel was 2.28 × 10^5 cpm/nmole; and the 100% ATP bound corresponded to a ratio of 0.15 mole ATP/mole F_1. (○ ATP on the release of label bound as [γ-^{32}P]-ATP, and □ ATP on the release of label bound as [^{3}H]ATP).

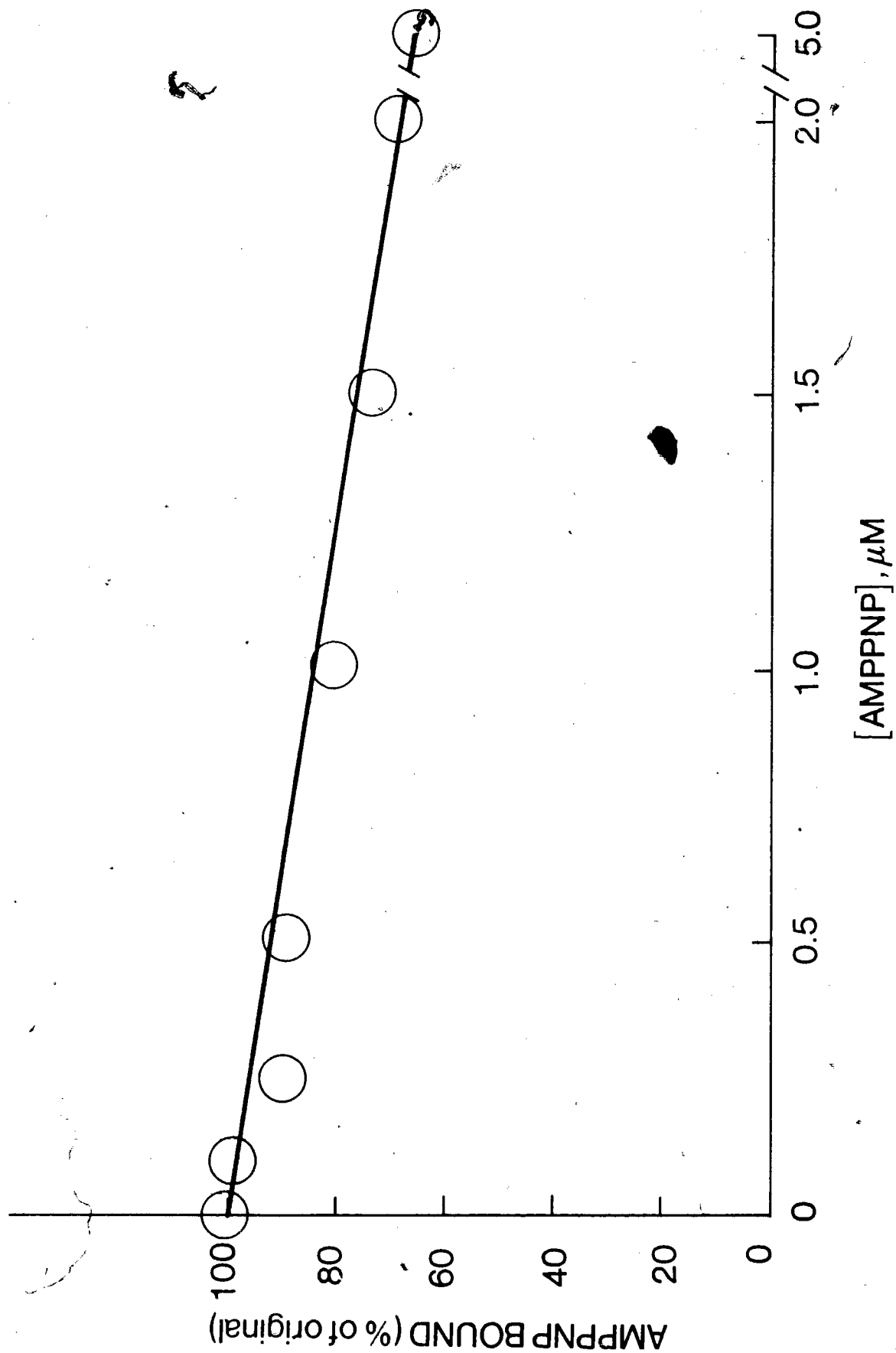

P$_i$ Release
ADP Release
ATP BOUND (% of original)
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
100
[ATP], μM

Release of AMPPNP from F_1: Pulse-Chase Method

Figure 10 shows the results of a pulse-chase experiment in which labelled AMPPNP ([^{3}H]AMPPNP) was used in the pulse section and unlabelled AMPPNP in the 1.0 cm chase section. A comparison of Figure 10 with Figure 7 shows that chase AMPPNP is more effective in facilitating the dissociation of label bound as [γ-^{32}P]ATP than the dissociation of label bound as [^{3}H]AMPPNP. These findings support (but do not prove) the hypothesis that label bound as [γ-^{32}P]ATP dissociates from F_1 as [^{32}P]P_i rather than as the unhydrolysed [γ-^{32}P]ATP, on the binding of chase AMPPNP. It was shown that under single site occupancy conditions, an equilibrium is attained in which the ratio of bound ATP to bound hydrolysis products, ADP and P_i (i.e. the equilibrium constant, K) is 2. The calculated forward and reverse rate constants for this step of the F_1-catalysed reaction were 10 s^{-1} and 20 s^{-1}, respectively (103). In the absence of medium nucleotides, the dissociation of ADP and P_i from F_1 is slow with the rate constants of 4×10^{-4} s^{-1} and 3×10^{-3} s^{-1}, respectively (103). If the behaviour of AMPPNP is similar to that of ATP, then the results displayed in Figure 10 indicate that in an AMPPNP chase, the dissociation of bound ATP is far less favourable than the dissociation of bound ADP and P_i.

Figure 10

The effect of AMPPNP on the release of label bound as [^{3}H]AMPPNP from F_1. The pulse-chase technique was as described in Methods, with four sections (1, 3, 1 and 2.5 cm) being used, i.e. the 1.0 cm chase gel was followed by 2.5 cm spacer gel. The concentration of F_1 in the reaction mixture was 0.83 mg protein·mL^{-1} or 2.49 μM. The pulse-gel equilibration buffer contained 1.0 μM AMPPNP with [^{3}H]AMPPNP (1.63×10^5 cpm/nmole). The 100% AMPPNP bound corresponded to a ratio of 0.1 mole AMPPNP/mole F_1.


AMPPNP BOUND (% of original)
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
5.0
[AMPPNP], μM

Binding of Nucleotides to F_1 in the Sephadex Centrifuge Column

Figure 11 shows the results of an experiment in which F_1 was passed through a column of the type used in the preincubation mode. In this case though, the F_1 was not preincubated with labelled P_i, i.e. [^{32}P]P_i, but the nucleotide-containing 1.0 cm middle section had labelled ATP ([γ-^{32}P]ATP). Assuming that the volume of the centrifugate was the same as the volume applied to the column, Table III shows the results of calculations of the percentage of the total amount of the [γ-^{32}P]ATP contained in the column which was bound. The method of calculation is described in the table legend. Table III shows that under the conditions of the experiment, where ATP was present in substoichiometric amount with respect to F_1, approximately all of the ATP bound to F_1. This observation is consistent with the very rapid binding of ATP to F_1 (second order rate constant of 6×10^6 $M^{-1} \cdot s^{-1}$) and the very slow release of ATP from F_1 (rate constant of 7×10^{-6} s^{-1}), when ATP is present at substoichiometric amounts (103). It is also consistent with the results shown in Figure 1 for the effect of ATP (in the middle section) on the release of [^{32}P]P_i bound to F_1 in a preincubation experiment. Similar calculations (see Table III) were made using data (for the ATP-induced P_i release only) from Figure 1, and the results are shown in Table IV. These results reveal that in the steep phase of the ATP-induced P_i plot, the molar

Figure 11

The binding of ATP by F_1 from the 1.0 cm middle section of the column (preincubation mode). The modified Sephadex centrifuge column technique was used as described in Methods. Note that no $[^{32}P]P_i$ was added to the reaction mixture. The ATP-containing equilibration buffers of the middle gels had $[\gamma\text{-}^{32}P]ATP$ (3.52×10^6 cpm/nmole). The concentration of F_1 in the reaction mixture was 1.82 mg protein$\cdot mL^{-1}$ or 5.26 μM.

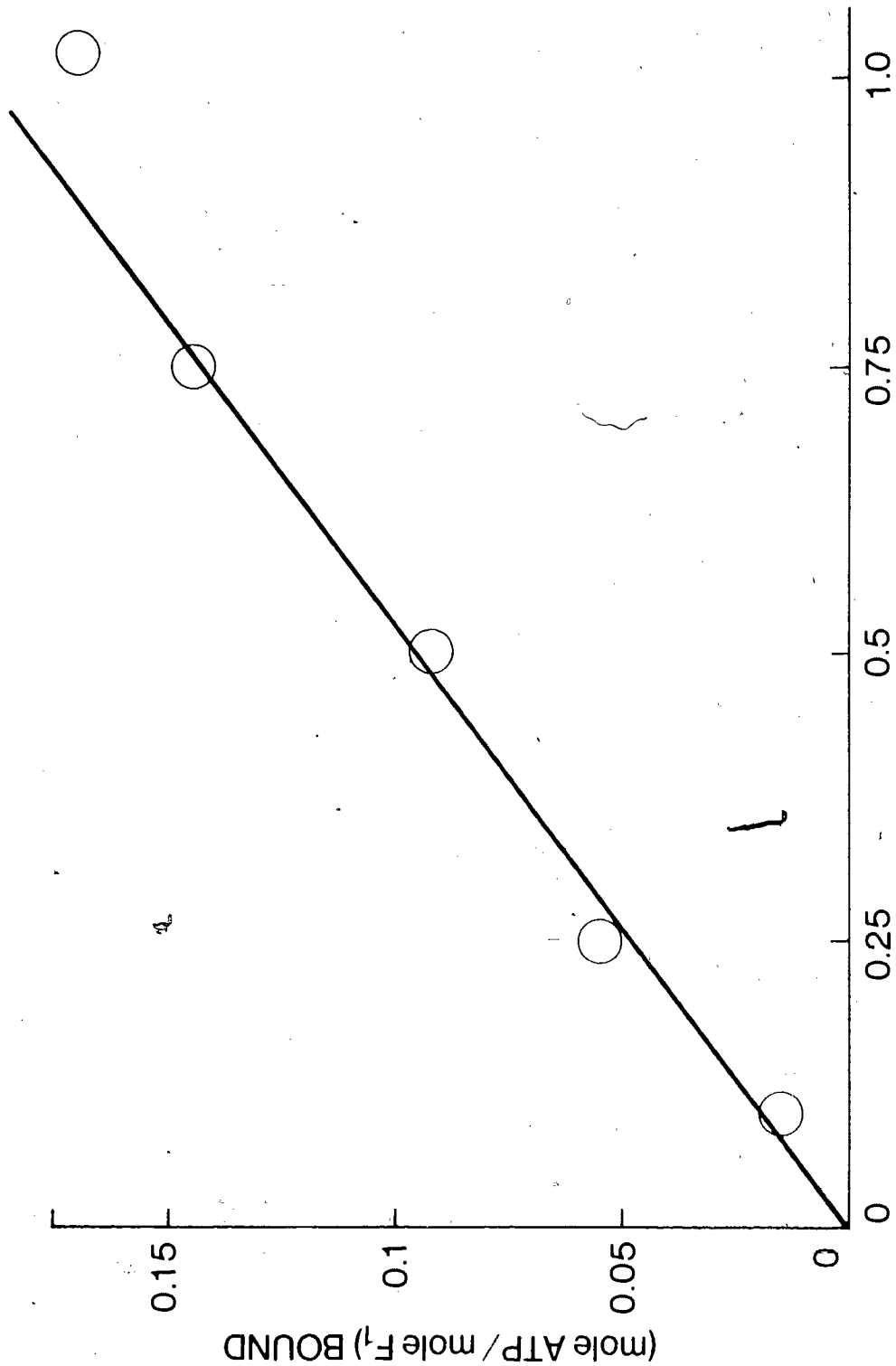

(mole ATP/ mole F_1) BOUND
0.15
0.1
0.05
0
0
0.25
0.5
0.75
1.0
[ATP], μM

Table III

The Binding of ATP in the Column to F_1

ATP Concentration in Column (μM)	Total ATP in Column (pmole)	ATP Bound by F_1 (pmole)	% ATP Bound
0	0	0	0
0.1	12.1	10.23	84.5
0.25	30.5	30	100
0.5	63.1	57.95	91.8
0.75	89.9	91.66	100
1.0	119.8	107.11	89.4

The experimental conditions, procedures, and data were the same as in Figure 11. The amount of ATP (before application of F_1) in the 1.0 cm middle gel was determined as outlined below: (i) 25 μL aliquots of $\chi\mu$M ATP-containing buffers with [^{32}P]ATP were counted, and the specific activity calculated; (ii) Samples of 1.0 cm lengths of the $\chi\mu$M ATP with [^{32}P]ATP equilibrated gels were counted. Lastly, the amount of ATP in the gels were calculated by dividing the amount of label (cpm) given by each 1.0 cm gel by the specific activity of the [^{32}P]ATP in the corresponding ATP-containing buffer. Note that the volume of the centrifugate was never as much as the 150 μL applied to the column, hence the amount of label in the 25 μL centrifugate counted was multiplied by 6 to give a total value.

Table IV

The Binding of ATP in the Column to F_1 and the Release of P_i from F_1

ATP Concentration in Column	Total ATP in Column (pmole)	Total P_i bound by F_1 (pmole)	Total P_i released by F_1 (pmole)	mole P_i released / mole ATP available
0	0	97	0	--
0.1	12.1	83	14	1.16
0.25	30.5	61	36	1.18
0.5	63.1	26	71	1.13
2.5	305	17	80	0.26
5.0	610	11	86	0.14

The experimental conditions, procedures, and data were the same as in Figure 1. The total ATP in the column was determined as described in the legend of Table III.

ratio of the P_i released to the total ATP present in the column is approximately one. Initially it was difficult to rationalize the results shown in Table IV, because it seemed reasonable to expect that ATP (in the middle section of the column) would bind to F_1 containing bound P_i and to F_1 without bound P_i, and therefore not every ATP bound would cause P_i release. In the experiment shown in Figure 1 and Table IV, only 0.28 of the total F_1 had P_i bound. The results are consistent with the hypothesis, that only part of the F_1 is in a state such that it can bind ATP rapidly during its brief passage through the column. This part corresponds to the F_1 which has P_i bound such that it can be released in the steep phase of the line drawn through the circles in Figure 1. The remainder of the F_1 is either inactive, or requires different conditions, such as exposure to higher ATP concentrations, in order to become fully activated. Figure 12 shows that F_1, whether or not it was preincubated with P_i, bound [^{3}H]ATP to approximately the same extent in its passage through the column. Apparently with P_i bound to F_1 (i.e forming $F_1 \cdot P_i$ complex), the affinity of the F_1 (or $F_1 \cdot P_i$ complex) for ATP does not increase under the conditions of the experiment. (Note that Cross *et al.* (104) found that the rate constant of the second ATP binding to F_1 is the same as the first, i.e. $6 \times 10^6 M^{-1}.s^{-1}$).

Figure 12

The effect of P_i on the binding of ATP by F_1. The modified Sephadex centrifuge column technique was used as described in Methods. Note that the concentration of P_i was 47 μM, whenever P_i was present in the equilibration buffers of the gels or in the reaction mixture. The ATP-containing equilibration buffers of the middle gels had [^{3}H]ATP (5.8×10^5 cpm/nmole). The concentration of F_1 in the reaction mixture was 1.64 mg protein.mL^{-1} or 4.74 μM. (○ No P_i in the equilibration buffers and reaction mixture, and □ P_i in the equilibration buffers and reaction mixture).

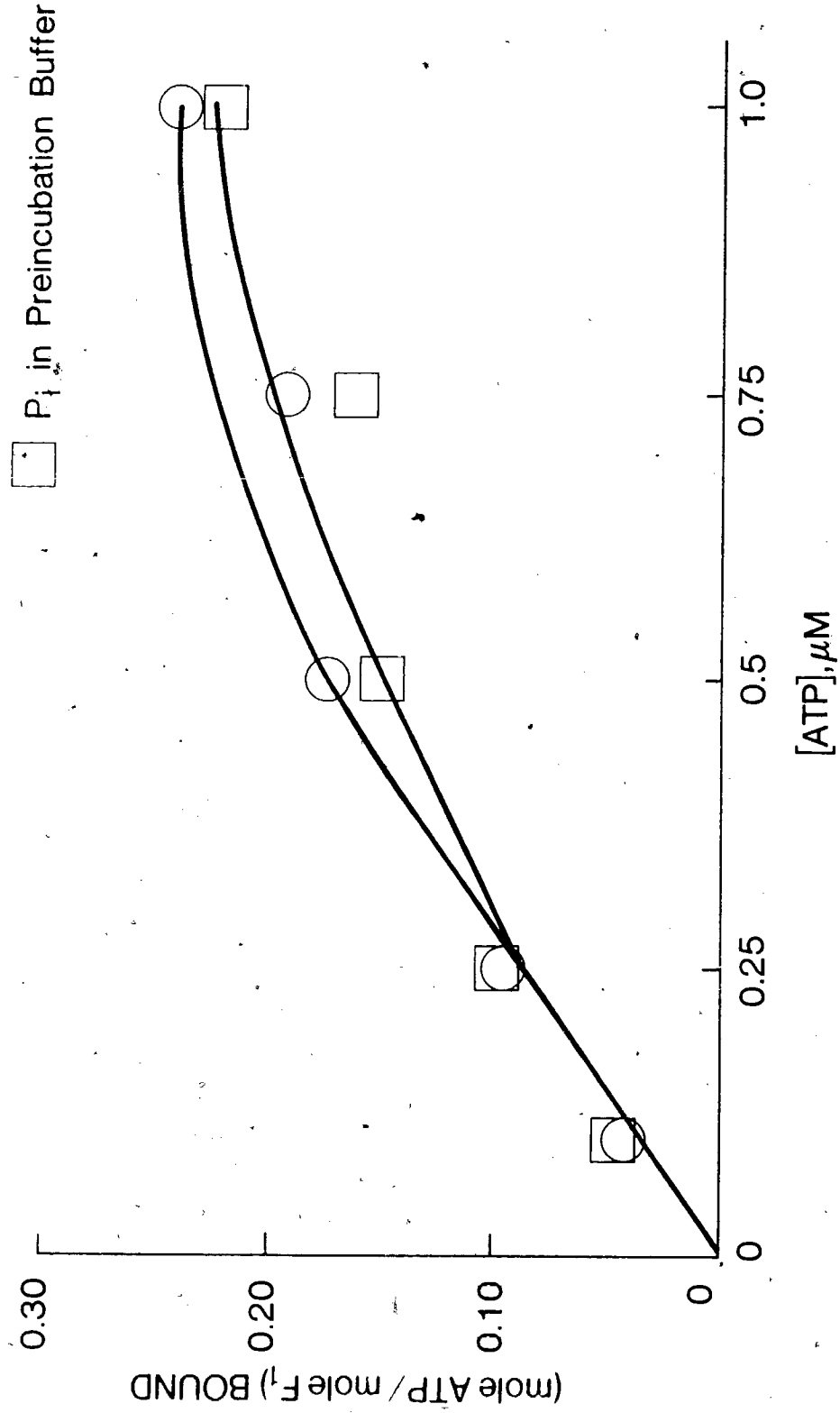

No P_i in
Preincubation Buffer
P_i in Preincubation Buffer
(mole ATP/ mole F_1) BOUND
0.30
0.20
0.10
0
0
0.25
0.5
0.75
1.0
[ATP], μM

Figure 13

The release of label bound as [γ-^{32}P]ATP and the binding of label as [^{3}H]ATP. The pulse-chase technique was as described in Methods, with four sections (1, 3, 1 and 2.5 cm) being used. The pulse-gel equilibration buffer contained 1.0 μM ATP with [^{3}H]ATP (3.06×10^{5} cpm/nmole). The chase-gel equilibration buffers contained the indicated concentrations of μM ATP with [^{3}H]ATP (9.94×10^{5} cpm/nmole). The concentration of F_1 in the reaction mixture was 1.93 mg protein.mL^{-1}. Correction was made for label from the chase gel taken through the column by solvent drag. The 100% ATP bound (from pulse gel) corresponded to a ratio of 0.1 mole ATP/mole F_1, and this value was used as 100% to calculate the ratio of mole ATP/mole F_1 for the chase ATP. (◯ % label ([γ-^{32}P]ATP) retained from pulse, and □ % label ([^{3}H]ATP) bound in chase).

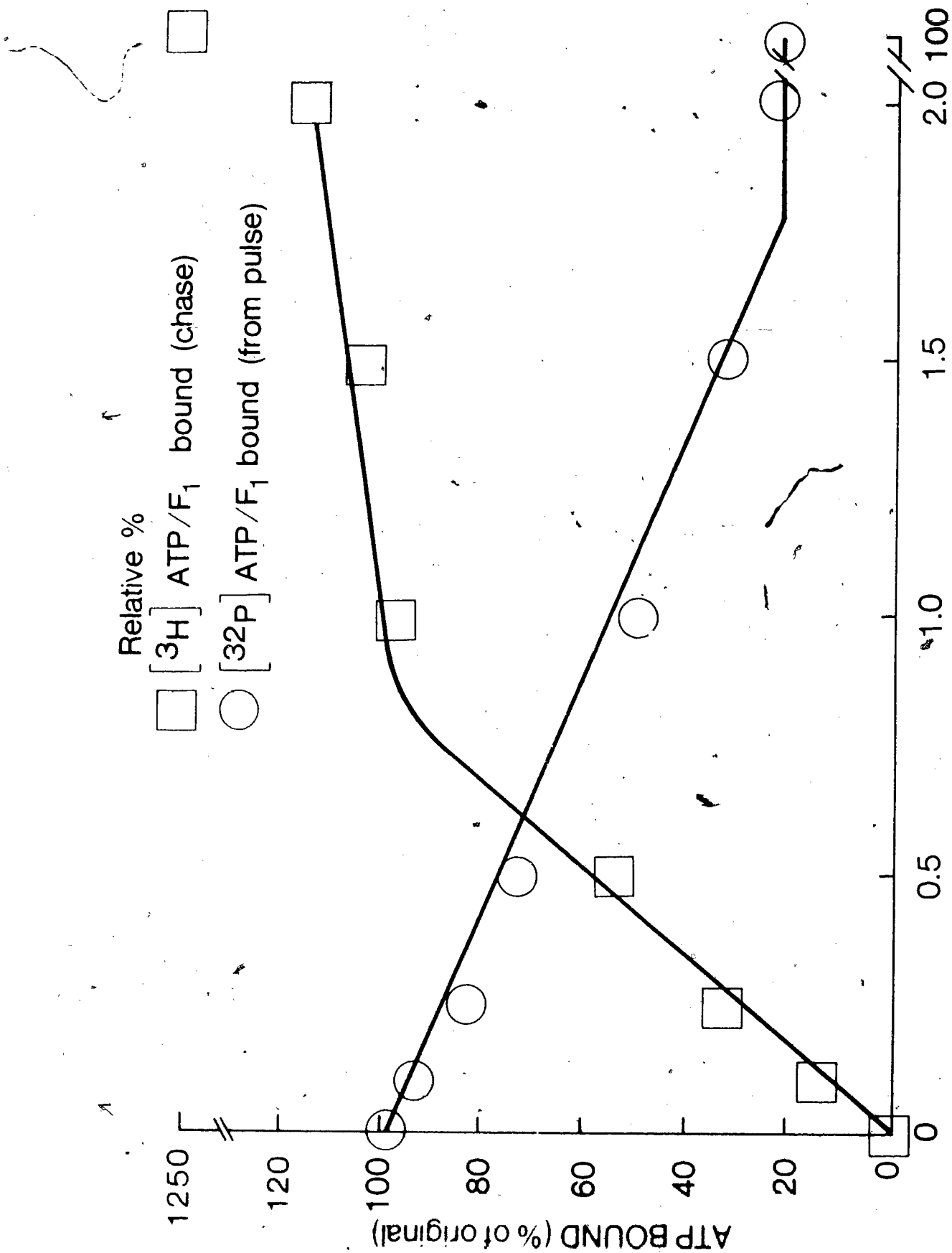
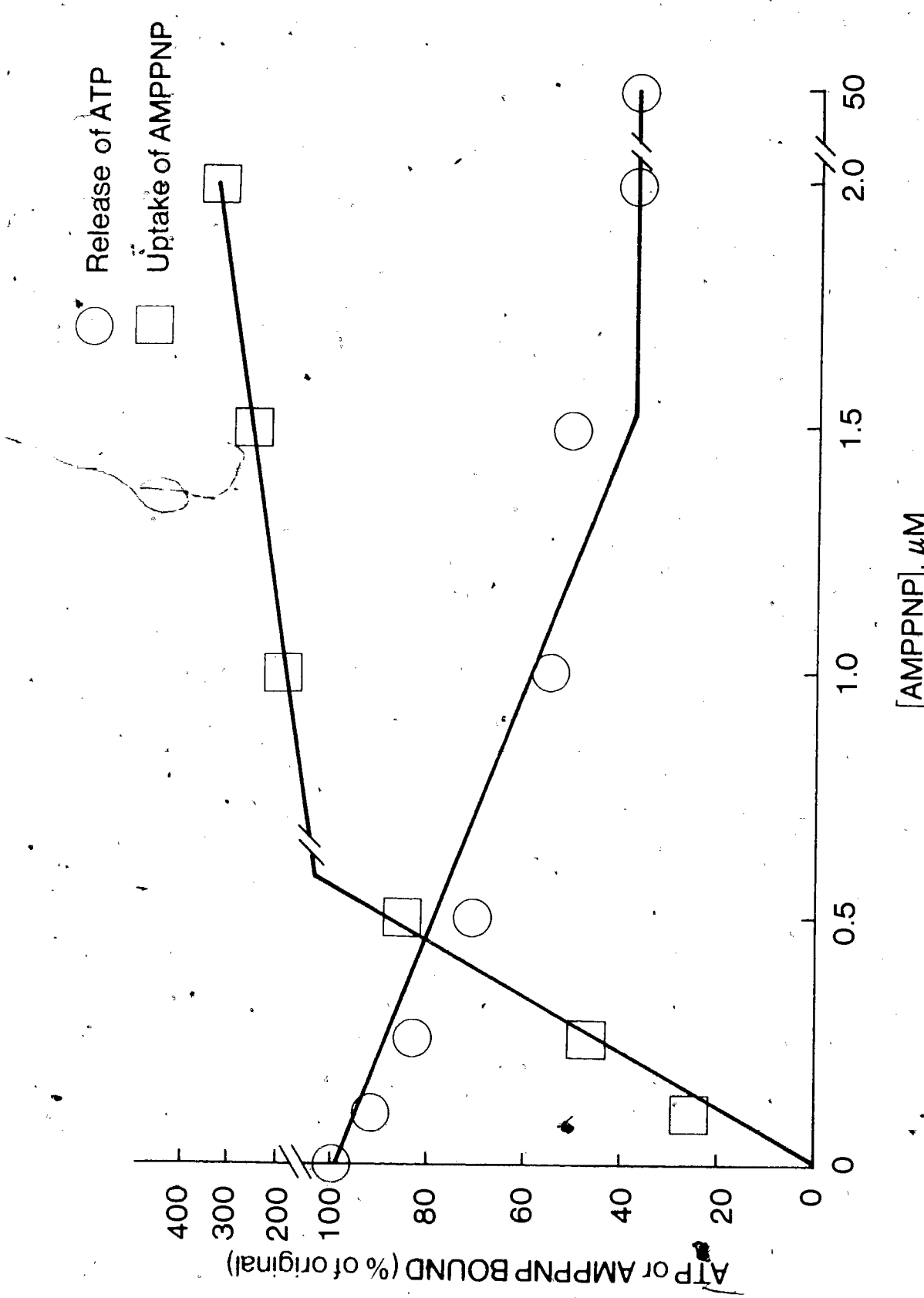

Relative %
[3H] ATP/F1 bound (chase)
[32P] ATP/F1 bound (from pulse)
ATP BOUND (% of original)
1250
120
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0 100
[ATP], μM

Figure 14

The release of label bound as [γ-^{32}P]ATP and the binding of label as [^{3}H]AMPPNP. The pulse-chase technique was as described in Methods, with four sections (1, 3, 1, and 2.5 cm) being used. The pulse-gel equilibration buffer contained 1.0 μM ATP with [γ-^{32}P]ATP (2.6×10^5 cpm/nmole). The chase-gel equilibration buffers contained the indicated concentrations of μM AMPPNP with [^{3}H]AMPPNP (5.97×10^5 cpm/nmole). The concentration of F_1 in the reaction mixture was 1.78 mg protein.mL^{-1}. Correction was made for label from the chase-gel taken through the column by solvent drag. The 100% ATP bound (from pulse gel) corresponded to a ratio of 0.1 mole ATP/mole F_1, and this was used as 100% to calculate the ratio of mole AMPPNP/mole F_1. (○ % label retained from pulse, and □ % label ([^{3}H]AMPPNP) bound in chase).


Release of ATP
Uptake of AMPPNP
ATP or AMPPNP BOUND (% of original)
400
300
200
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
50
[AMPPNP], μM

Figures 13 and 14 show the results of pulse-chase experiments with [γ-^{32}P]ATP in the pulse sections, and [^{3}H]ATP and [^{3}H]AMPPNP, respectively, in the chase sections of the columns. In this way, the release of label ([γ-^{32}P]ATP) which was bound in the pulse was monitored simultaneously with the binding of label ([^{3}H]ATP or [^{3}H]AMPPNP) in the chase. Figure 15 shows a comparison of the binding curves (ATP and AMPPNP bound in the chase) from Figures 13 and 14, respectively. In these experiments (as in all of the pulse-chase experiments described thus far), the pulse section consisted of a 1 cm gel prepared from a column made using Sephadex equilibrated in a buffer with 1.0 μM nucleotide (see Methods). Under these conditions, the molar ratio of [γ-^{32}P]ATP bound to F_1 in the pulse was usually about 0.1. Thus it is reasonable to assume that some of the chase nucleotide bound to F_1 which had not bound label in the pulse section. Figure 13 shows that with increasing ATP concentration in the chase section, initial binding of the ^{3}H label in the chase section increases more than does release of $^{32}P_i$ label which had bound in the pulse section. This result can be rationalized if at low chase ATP concentrations, the [^{3}H]ATP in the chase binds to both F_1 without label bound from pulse and to F_1 with label bound from pulse. The binding of ATP by F_1 in the chase levels off as the chase ATP concentration increases (Figs. 13, 15, and Table V); whereas the binding of AMPPNP by F_1 in the chase was essentially linear with the AMPPNP concentra-

Figure 15

The binding of ATP and AMPPNP from the chase gels in pulse-chase experiments. For the ATP binding plot (○), the information was taken from Figure 13, and for the AMPPNP binding plot (□), the information was taken from Figure 14. (○ ATP ([^{3}H]ATP) binding, and □ AMPPNP ([^{3}H]AMPPNP) binding).

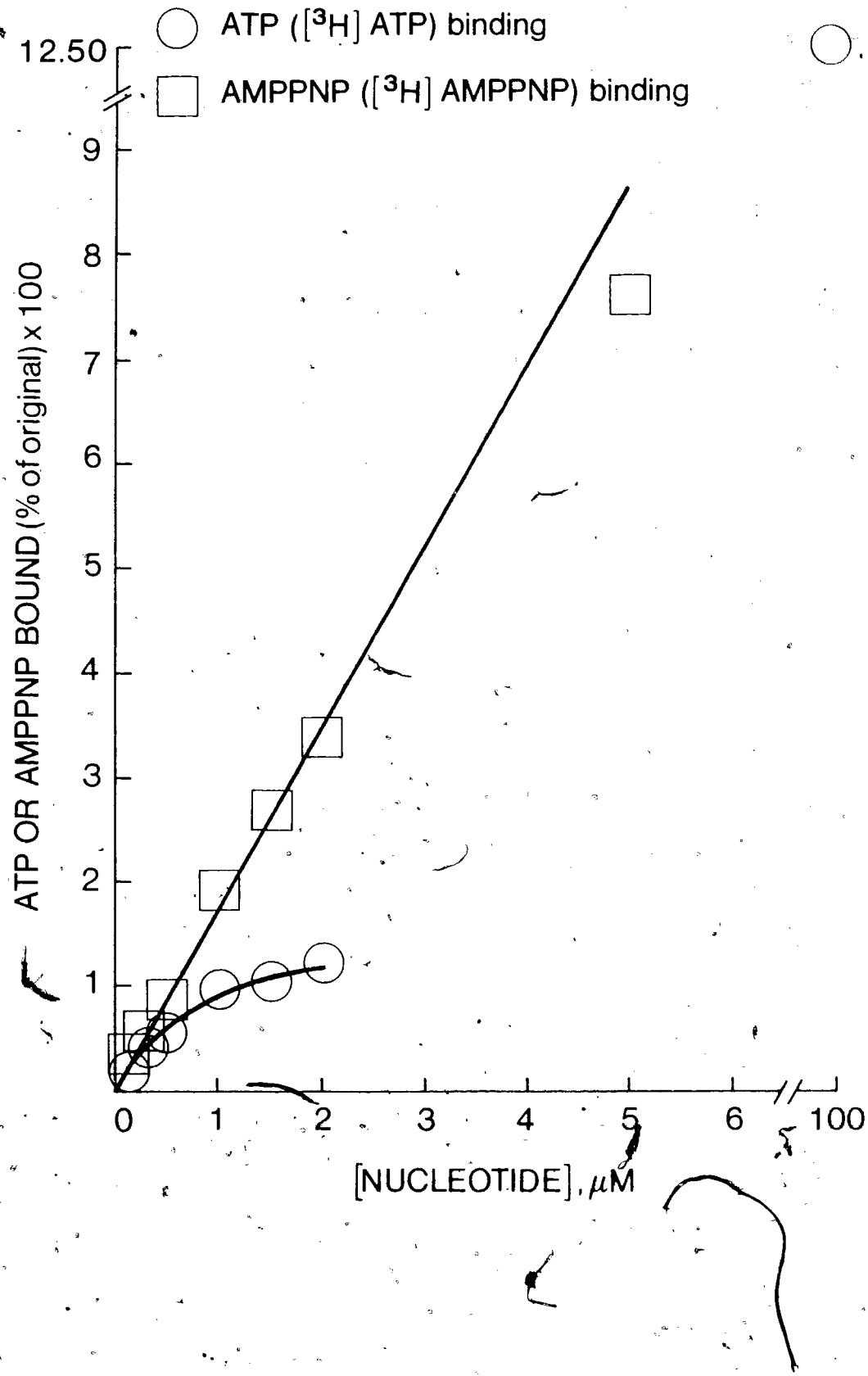

ATP ([3H] ATP) binding
AMPPNP ([3H] AMPPNP) binding
12.50
9
8
7
6
5
4
3
2
1
ATP OR AMPPNP BOUND (% of original) x 100
0
1
2
3
4
5
6
100
[NUCLEOTIDE], μM

Table V

The Binding of Chase ATP in the Column to F_1

ATP Concentration in Column (μM)	Total ATP in Column (pmole)	ATP bound by F_1 (pmole)	% ATP Bound	mole $[^3H]$ATP / mole F_1
0	0	0	0	0
0.1	12.5	12.5	100	0.01
0.25	31.2	30	96	0.03
0.5	62.5	50	80	0.05
1.0	125	90	72	0.10
1.5	187.5	93	50	0.10
2.0	250	114	46	0.12
100	12500	1038	8	1.25

The data for the calculations were from Figures 13 and 15. The amount of ATP in the 1 cm chase gel was determined as described for the 1 cm middle gel in the legend of Table III.

Table VI

The Binding of Chase AMPPNP in the Column to F_1

AMPPNP Concentration in Column (μM)	Total AMPPNP in Column (pmole)	AMPPNP bound by F_1 (pmole)	% AMPPNP Bound	mole [^{3}H]AMPPNP / mole F_1
0	0	0	0	0
0.1	12.5	12.5	100	0.02
0.25	31.2	28	90	0.04
0.5	62.5	51	82	0.07
1.0	125	115	92	0.15
1.5	187.5	162	86	0.21
2.0	250	201	80	0.26
5.0	625	456	73	0.59

The data for the calculations were from Figures 14 and 15. The amount of AMPPNP in the 1 cm chase gel was determined as described for the 1 cm middle gel in the legend of Table III.

tion throughout the range studied (Figs. 14, 15, and Table VI). This is probably because several molecules of AMPPNP can bind to each F_1 (48, 73, 102); while on the other hand, if [^{3}H]ATP binds to a F_1 which already bound one [^{3}H]ATP, the previously bound ATP would very likely have hydrolysed and the label would be released on the binding of the second [^{3}H]ATP at another site on the F_1.

From Figure 15, it can be observed that the binding of AMPPNP to F_1 is as favourable as the binding of ATP in the columns. This observation eliminates the possible explanation that F_1 has a lower affinity for AMPPNP, thereby causing the reduced sensitivity of response of the AMPPNP-induced P_i release in the preincubation experiments shown in Figure 1.

The Biphasic Release of P_i from F_1

In an effort to discover the cause of the biphasic release of P_i from F_1, the experiments described below were performed. Figure 16 shows that increasing the length of the ATP-containing middle section from 1.0 to 2.0 cm in a preincubation experiment, did not appreciably alter the proportions of P_i released in the two phases. Figure 17 shows the amounts of P_i released from F_1 when different lengths of 0.25 μM ATP-containing middle sections were used. Figure 18 shows that using a 5.0 cm ATP-containing gel (i.e. the entire lower section) in the preincubation mode also did not alter the biphasic nature of the P_i release reaction.

Figure 19 shows that inactivation of F_1 by treatment with NBD-chloride (120, 121) reduced the amount of ATP bound in a pulse-chase experiment by $\geq$ 95%. This result supports the hypothesis that the ATP binds at catalytic sites.

Figure 20 shows that preincubation of F_1 with ATP did not alter the amount of ATP bound to F_1; and neither was the amount of P_i released from F_1 altered in the pulse-chase experiment using a 3.5 cm 0.25 μM ATP-containing chase section. Figures 21 and 22 show that preincubation of F_1 with either ADP or EDTA did not affect the amount of label $[^{32}P]P_i$ bound in preincubation experiments, and that these treatments also did not affect the biphasic P_i release from F_1. Figure 23 shows that in a pulse-chase type

Figure 16

The effect of ATP on the release of bound P_i from F_1 using 1.0 cm and 2.0 cm ATP-containing middle sections in the columns. The modified Sephadex centrifuge column technique was used as described in Methods, except that the 2.0 cm ATP-containing middle section columns had 3.0 cm bottom sections. The concentration of F_1 in the reaction mixture was 0.94 mg protein.mL^{-1} or 2.72 μM. The specific activity of the $[^{32}P]P_i$ in the reaction mixture was 1.85×10^5 cpm/nmole. The 100% P_i bound corresponded to a ratio of 0.25 mole P_i/mole F_1. (○ 1.0 cm ATP-containing middle section, and □ 2.0 cm ATP-containing middle section).

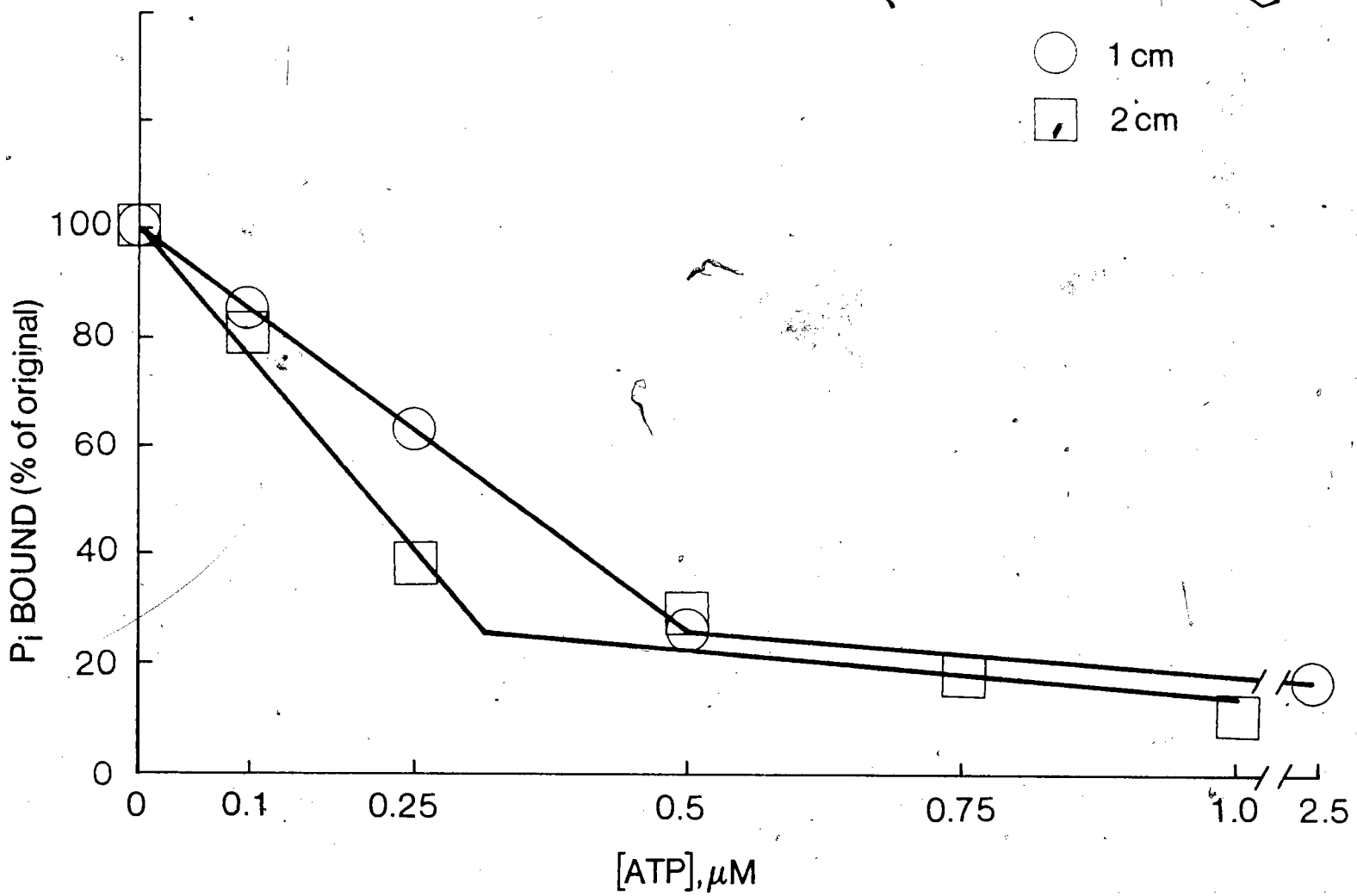

1 cm
2 cm
100
80
60
40
20
0
P_i BOUND (% of original)
0
0.1
0.25
0.5
0.75
1.0
2.5
[ATP], μM

Figure 17

The effect of ATP on the release of bound P_i from F_1 using various lengths of ATP-containing middle sections in the columns. The modified Sephadex centrifuge column technique was used as described in Methods, except that the bottom section was decreased by the same length by which the 1.0 cm ATP-containing middle section was increased. In addition, the concentration of ATP used in the equilibration buffer of the middle gels was kept constant at 0.25 μM. The concentration of F_1 used in the reaction mixture was 0.92 mg protein.mL^{-1} or 2.66 μM. The specific activity of the $[^{32}P]P_i$ used was 1.9×10^5 cpm/nmole. The 100% P_i bound corresponded to a ratio of 0.21 mole P_i/mole F_1.

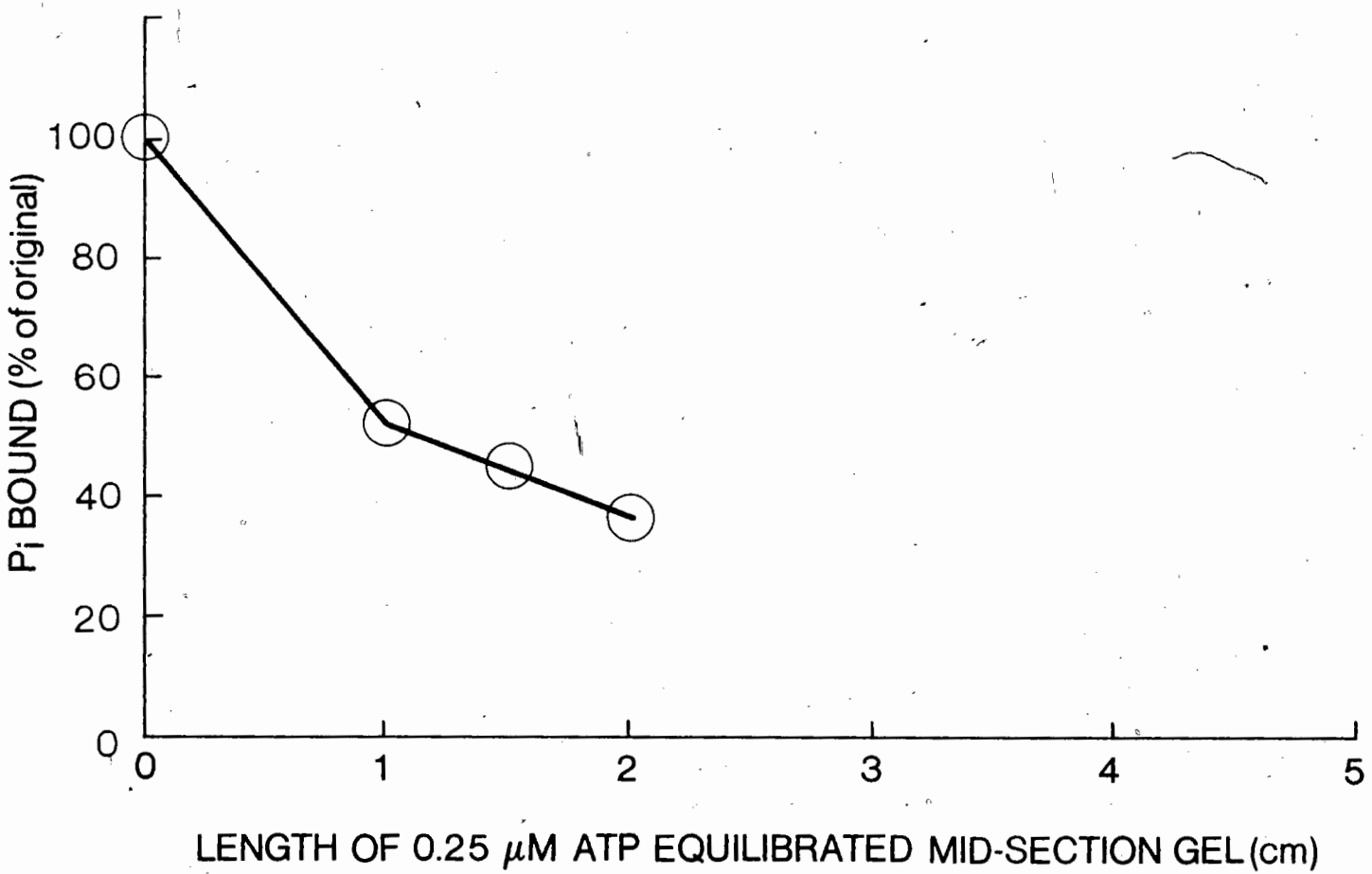

100
80
60
40
20
0
P_i BOUND (% of original)
0
1
2
3
4
5
LENGTH OF 0.25 μM ATP EQUILIBRATED MID-SECTION GEL (cm)

Figure 18

The effect of ATP on the release of bound P_i from F_1 using a 5.0 cm ATP-containing section in the column. The modified Sephadex centrifuge column technique was used as described in Methods, except the 4.0 cm bottom section also contained ATP. The concentration of F_1 in the reaction mixture was 1.29 mg protein. mL^{-1} or 3.73 μM. The specific activity of the $[^{32}P]P_i$ was 1.2×10^5 cpm/nmole. The 100% P_i bound corresponded to a ratio of 0.17 mole P_i/mole F_1.

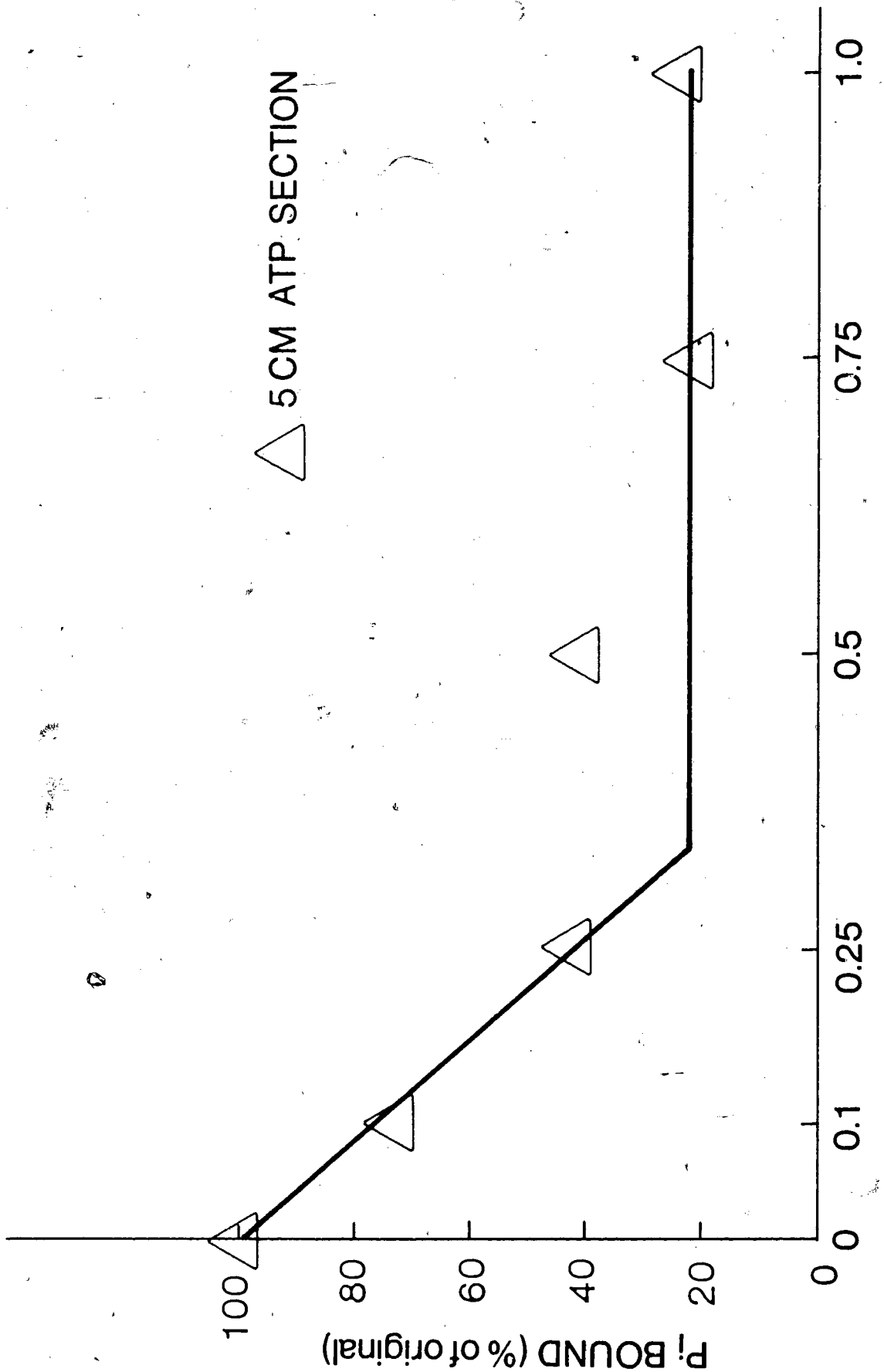

5CM ATP SECTION
Pi BOUND (% of original)
100
80
60
40
20
0
0
0.1
0.25
0.5
0.75
1.0
[ATP], μM

Figure 19

The effect of preincubation of F_1 with NBD-chloride before its application to a pulse-chase column. The F_1 was treated as described by Kohlbrenner and Boyer (121). The F_1 in the reaction mixture was inactivated by the addition of a freshly prepared ethanolic solution of NBD-chloride to give a final concentration of 100 μM. The reaction mixture was incubated for 2 hours in the dark at 25°C, before it was applied to the column. The pulse-chase technique was as described in Methods with three sections (1, 3, and 3.5 cm) being used, i.e. the 1 cm chase gel was combined with the 2.5 cm bottom gel to give 3.5 cm chase gel. The concentration of F_1 in the reaction mixtures was 0.94 mg protein. mL^{-1} or 2.72 μM. The pulse-gel equilibration buffer contained 1.0 μM ATP with [γ-^{32}P]ATP (1.6×10^5 cpm/nmole). The two F_1 reaction mixtures were treated the same except for the addition or exclusion of NBD-chloride. For the reaction mixture without NBD-chloride, the 100% ATP bound corresponded to a ratio of 0.1 mole ATP/mole F_1. (○ F_1 not treated with NBD-chloride, □ F_1 treated with NBD-chloride).

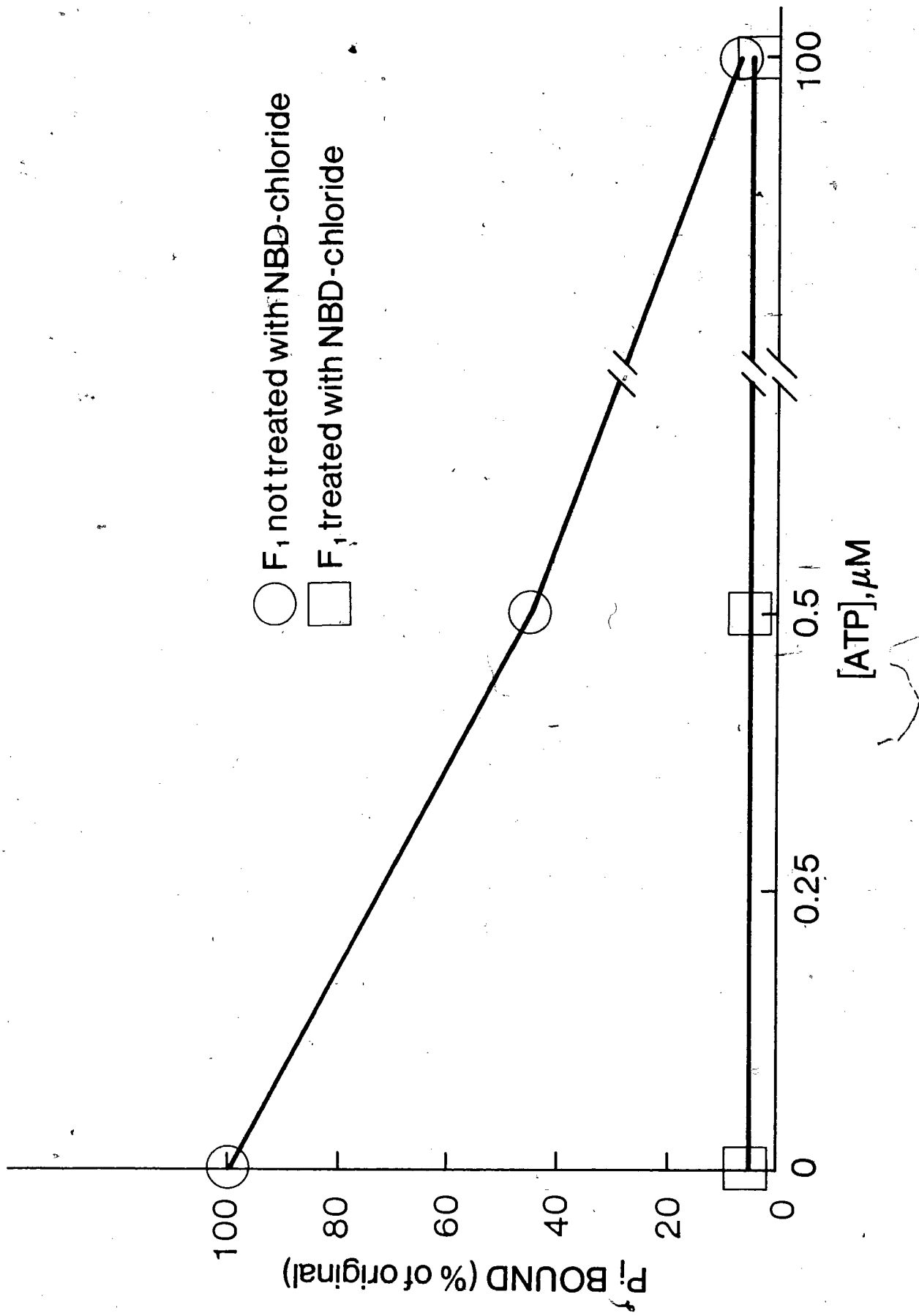

F1 not treated with NBD-chloride
F1 treated with NBD-chloride
Pi BOUND (% of original)
100
80
60
40
20
0
0
0.25
0.5
100
[ATP], μM

Figure 20

The effect of preincubation of F_1 with ATP before its application to a pulse-chase column. The F_1 was added to the usual reaction mixture which now also contained 4.0 mM ATP, and the mixture was allowed to stand for at least 20 minutes before application to the column. The pulse-chase technique was as described in Methods, with three sections (1, 3, and 3.5 cm) being used, i.e., the entire bottom section (3.5 cm) contained chase nucleotide. The concentration of F_1 in the reaction mixture was 0.9 mg protein.mL^{-1} or 2.61 μM. The pulse-gel equilibration buffer contained 1.0 μM ATP with [γ-^{32}P]ATP (2.14×10^5 cpm/nmole). The two F_1 reaction mixtures were treated the same except for the addition or exclusion of ATP. For the reaction mixture without ATP, the 100% ATP bound corresponded to a ratio of 0.1 mole ATP/mole F_1. (○ F_1 not preincubated with ATP, □ F_1 preincubated with ATP).

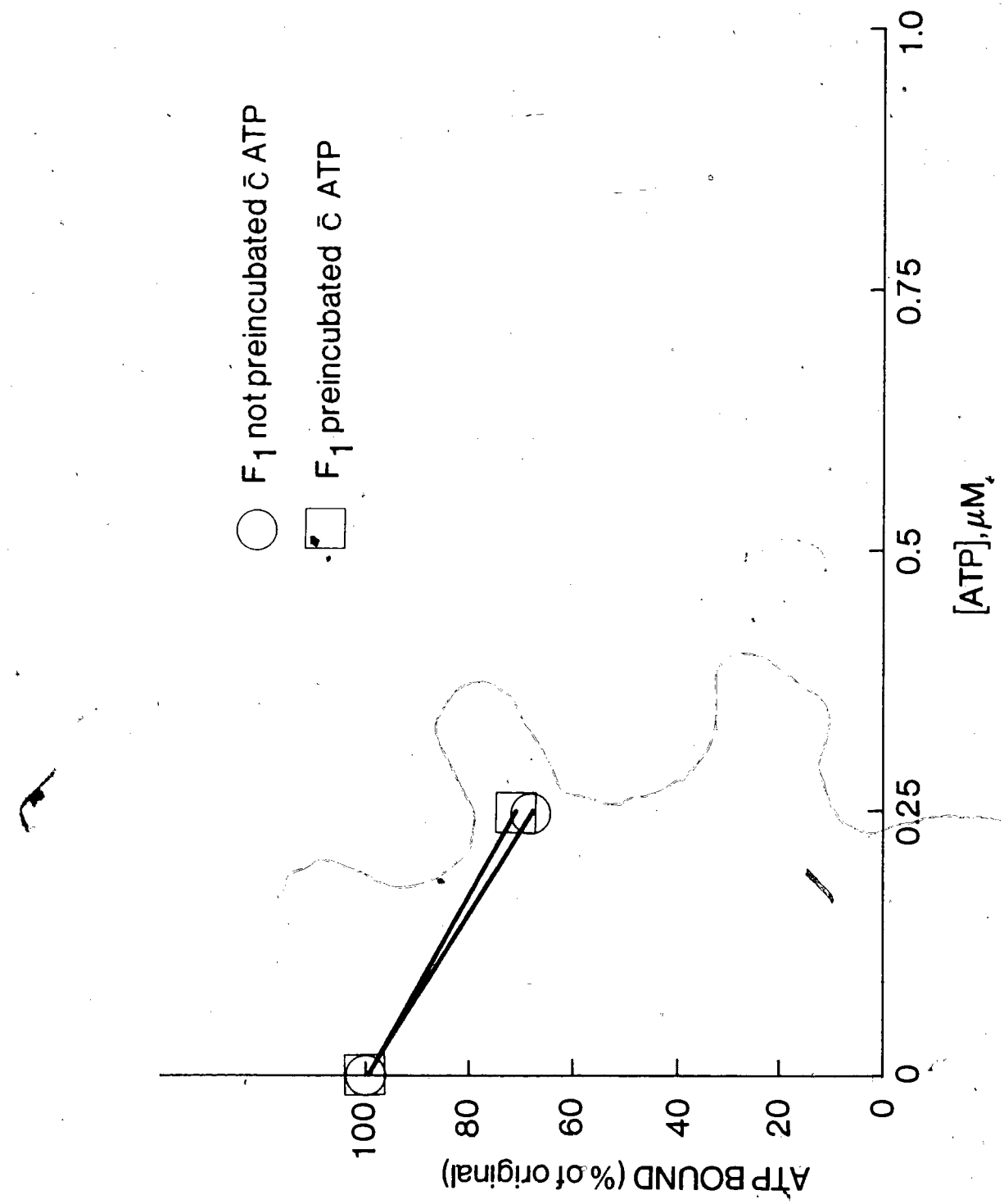

F1 not preincubated c̄ ATP
F1 preincubated c̄ ATP
ATP BOUND (% of original)
100
80
60
40
20
0
0
0.25
0.5
0.75
1.0
[ATP], μM

Figure 21

The effect of preincubation of F_1 with ADP before its application to a column. The F_1 was added to a reaction mixture containing 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 100 μM ADP. The mixture was allowed to stand for 5 minutes before its application to the usual Sephadex centrifuge column (28, 73). The centrifugate was added to the usual reaction mixture (90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 47 μM P_i with $[^{32}P]P_i$), and this allowed to stand for 8 minutes before application to the column. The modified Sephadex centrifuge column technique (preincubation mode) was used as described in Methods. The concentration of F_1 in the usual reaction mixture was 0.75 mg protein.mL^{-1} or 2.17 μM. The specific activity of the $[^{32}P]P_i$ used in the usual reaction mixture was 1.18×10^5 cpm/nmole. The F_1 reaction mixtures were treated the same except for the addition or exclusion of ADP. For the reaction mixture without ADP-treated F_1, the 100% P_i bound corresponded to a ratio of 0.25 mole P_i/mole F_1 (○ F_1 not preincubated with ADP, and □ F_1 preincubated with ADP).

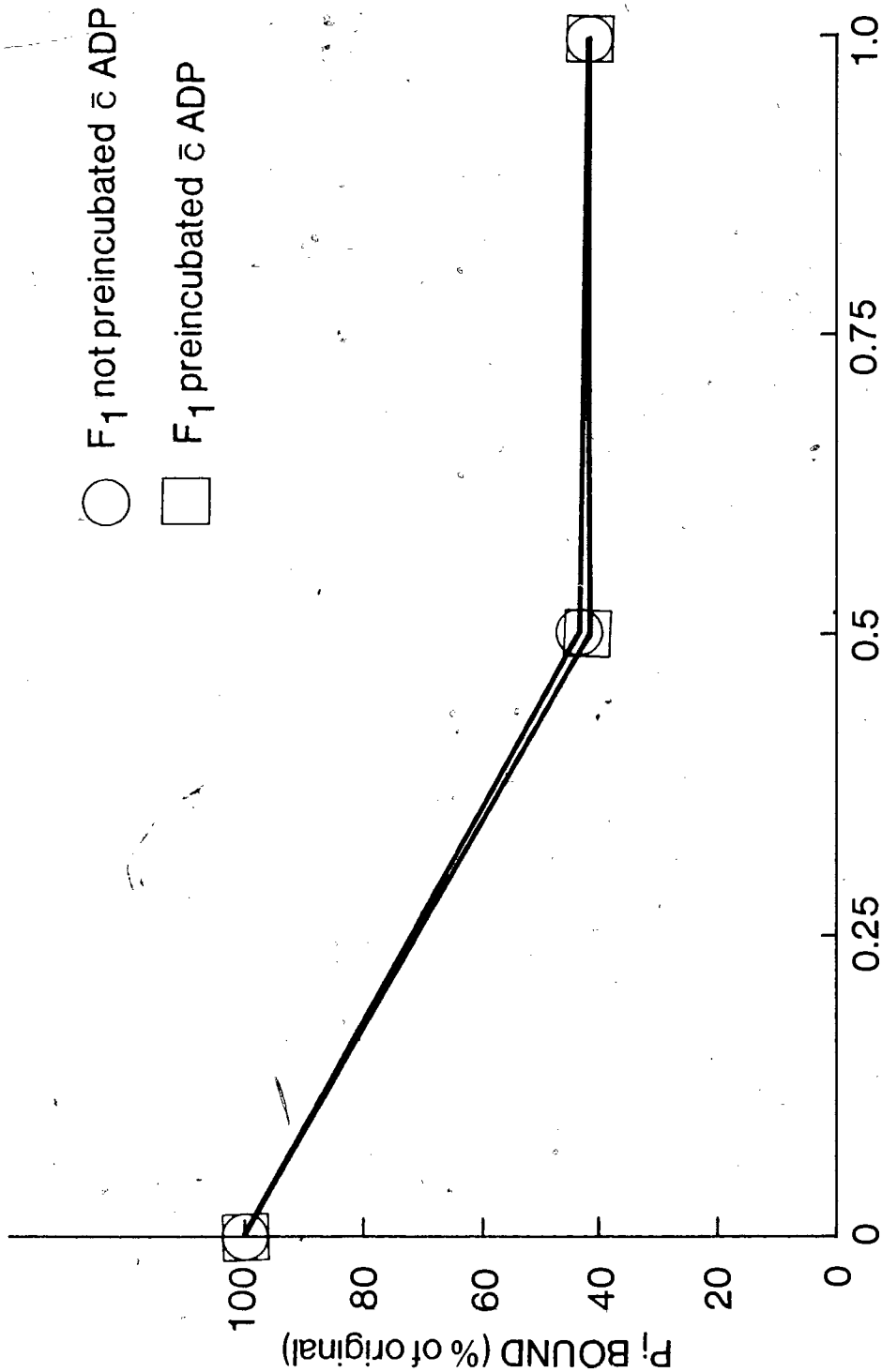

F1 not preincubated c̄ ADP
F1 preincubated c̄ ADP
Pi BOUND (% of original)
100
80
60
40
20
0
0
0.25
0.5
0.75
1.0
[ATP], μM

Figure 22

The effect of preincubation of F_1 with EDTA before its application to the column. The F_1 (in 50 mM Tris-acetate, pH 7.5) was added to a solution of EDTA (also in 50 mM Tris-acetate, pH 7.5) to give a final concentration of 17 mM EDTA. The mixture of F_1 and EDTA was allowed to stand for 1 minutes before its application to the usual Sephadex centrifuge column (28, 73). The centrifugate was added to the reaction mixture to give final concentrations of 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 47 μM Pi with $[^{32}P]P_i$, and this allowed to stand for 8 minutes before application to the column. The modified Sephadex centrifuge column technique (preincubation mode) was used as described in Methods. The concentration of F_1 in the reaction mixture was 0.68 mg protein mL^{-1} or 1.97 μM. The specific activity of the $[^{32}P]P_i$ in the reaction mixture was 1.22×10^5 cpm/nmole. The F_1 reaction mixtures were treated the same except for the addition or exclusion of EDTA. For the reaction mixture without EDTA-treated F_1, the 100% P_i bound corresponded to a ratio of 0.24 mole P_i/mole F_1. (○ F_1 not preincubated with EDTA, and □ F_1 preincubated with EDTA).

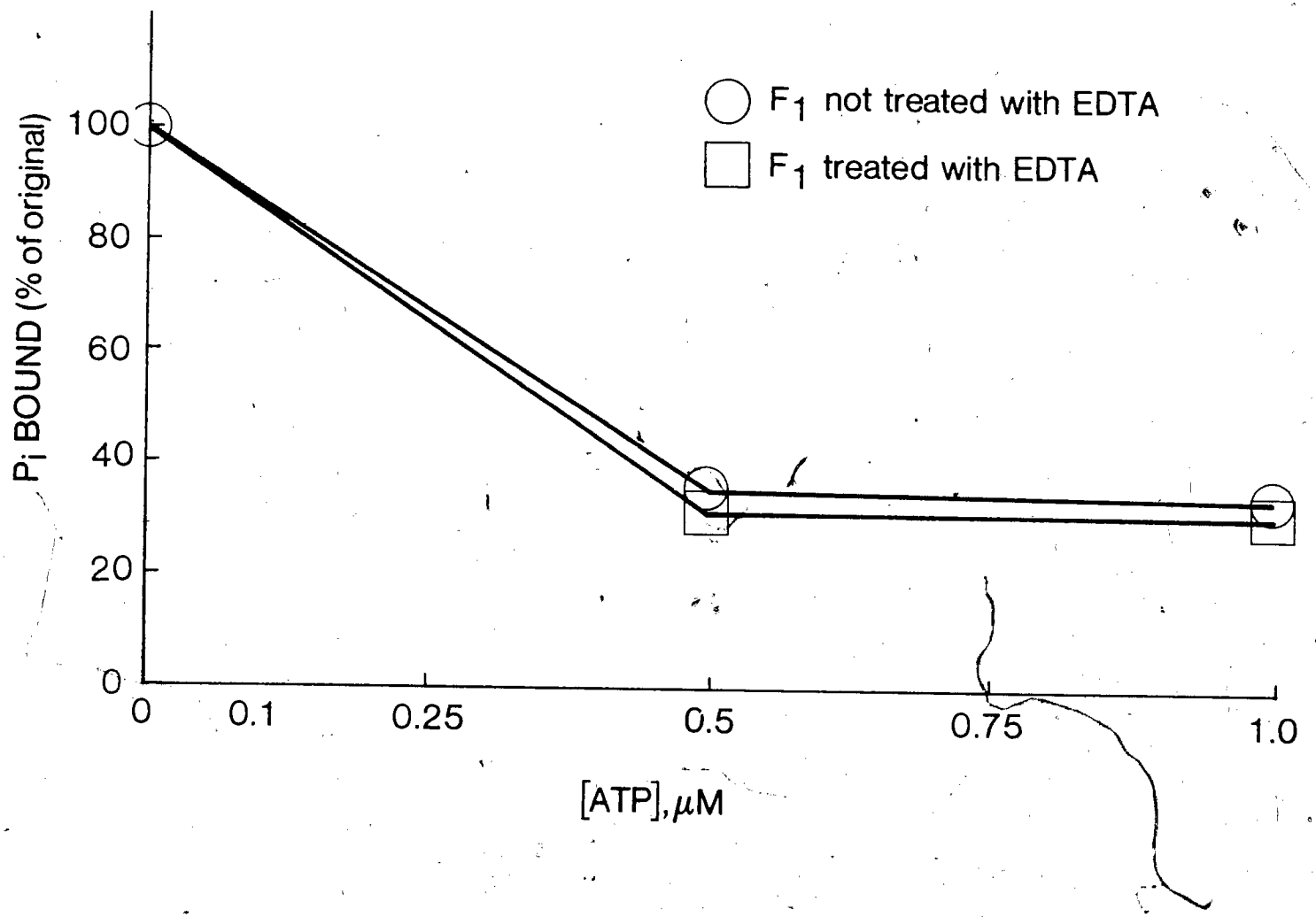
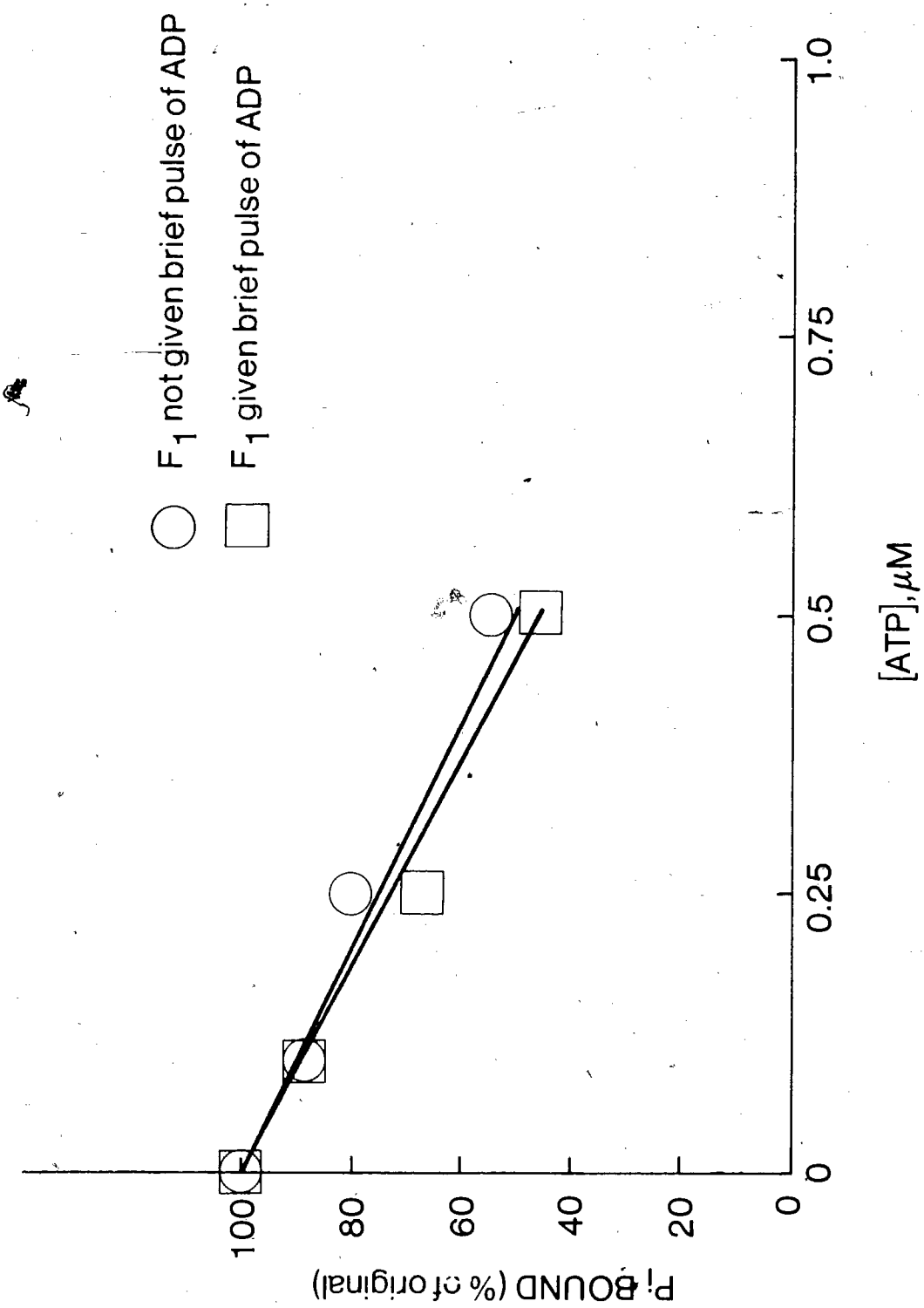

F_1 not treated with EDTA
F_1 treated with EDTA
P_i BOUND (% of original)
100
80
60
40
20
0
0
0.1
0.25
0.5
0.75
1.0
[ATP], μM

Figure 23

The effect of ATP on the release of P_i from F_1, when the $F_1 \cdot P_i$ complex was given a pulse of ADP. The pulse-chase technique was used as described in Methods, with four sections (1, 3, 1 and 2.5 cm) being used. Note that in this case the reaction mixture contained labelled P_i as $[^{32}P]P_i$ (1.24×10^5 cpm/nmole). The concentration of F_1 in the reaction mixture was 1.0 mg protein.mL^{-1} or 2.89 μM. The pulse-gel equilibration buffer contained 50 μM ADP with $[^3H]$ADP (3.5×10^4 cpm/nmole). For the $F_1 \cdot P_i$ complex not given pulse of ADP (i.e. a 1.0 cm spacer gel used instead), 100% P_i bound corresponded to a ratio of 0.21 mole P_i/mole F_1; and for $F_1 \cdot P_i$ complex given a pulse of ADP, 100% P_i bound corresponded to a ratio of 0.18 mole P_i/mole F_1 (○ F_1 not given pulse of ADP, and □ F_1 given pulse of ADP). [Note that the total amount of ADP available in the 1 cm of ADP-pulse-gel was 800 pmole, and the total ADP bound by F_1 in the centrifugate was 10.4 pmole.] The ratio of mole ADP/mole F_1 was 0.02. The methods of determination and calculation were as described in the legend of Table III.]


F1 not given brief pulse of ADP
F1 given brief pulse of ADP
Pi BOUND (% of original)
100
80
60
40
20
0
0
0.25
0.5
0.75
1.0
[ATP], μM

experiment, with a 1.0 cm 5.0 μM ADP ([^{3}H]ADP)-containing top section, that both the amount of P_i bound to F_1 and the amount of P_i released were not influenced by the ADP-pulse treatment. In addition, the experiment showed that a ratio of 0.02 mole ADP/mole F_1 was obtained under the experimental conditions (or about 0.01 of the available ADP from the 1.0 cm top section was bound). This last series of experiments (Figs. 20-23) was performed in order to test the possibility that P_i binding or ATP-facilitated P_i release might be affected by the presence of Mg•ADP bound to F_1. Bound ADP, which could be removed from F_1 by treatment with EDTA or incubation with pyruvate kinase and PEP, has been proposed as the cause of lags in ATP hydrolysis catalysed by F_1 (115-119).

The purity of the F_1 preparations used in these experiments was similar to that of Knowles and Penefsky (27) as described under methods. The NBD-chloride inactivation experiment shown in Figure 19 indicates that even if some protein impurities were present, they did not bind significant amounts ($\leq$ 5%) of ATP, and therefore cannot account for the observed biphasic release of label.

At present, there is no satisfactory explanation available for the biphasic P_i release observed. It is interesting that at sufficiently high ATP or ADP concentrations, all of the bound P_i was released (Figs. 3 and 4). Possibly all of the P_i was bound at catalytic sites, but the F_1 exists in different states and some

of these cannot be activated by exposure to low ATP concentrations on the time scale of the Sephadex centrifuge column experiment. It was found by other workers that F_1, labelled by exposure to substoichiometric amounts of ATP, did not release all of its label upon subsequent exposure to high concentrations of medium ATP (103).

Other published studies are also consistent with the biphasic P_i release observed in the investigations reported here. In the original study of P_i binding to and its release from F_1, about 10% of the bound P_i did not dissociate even at 48 minutes after the addition of unlabelled P_i to the reaction mixture (73). A semilog plot of the published data (Fig. 3 of Ref. 73), using the 48 minute point as the infinity value, is shown in Figure 24. This figure shows that the P_i dissociation which occurred did not follow clear first order kinetics. This result is consistent with there being more than one kind of bound P_i. Similar results were obtained in another published study (96), and in the preliminary work which led to the development of the methods used in the studies reported here. The results from the present studies are shown in Figure 25. A semilog plot of the P_i release data from this experiment also does not show clean first order kinetics. The biphasic nature of the ADP-facilitated P_i release is evident from the experiment shown in Figure 25, in which ADP was added to unlabelled P_i which was then in turn

Figure 24

A semilog plot showing the release of P_i from F_1 with time. The data were taken from Figure 3 of Ref. 73, with the 48 minute point being used as the infinity value. $\Delta[P_i]$bound on y-axis represents {mole P_i/mole bound at t min - mole P_i/ mole bound at 48 min}.

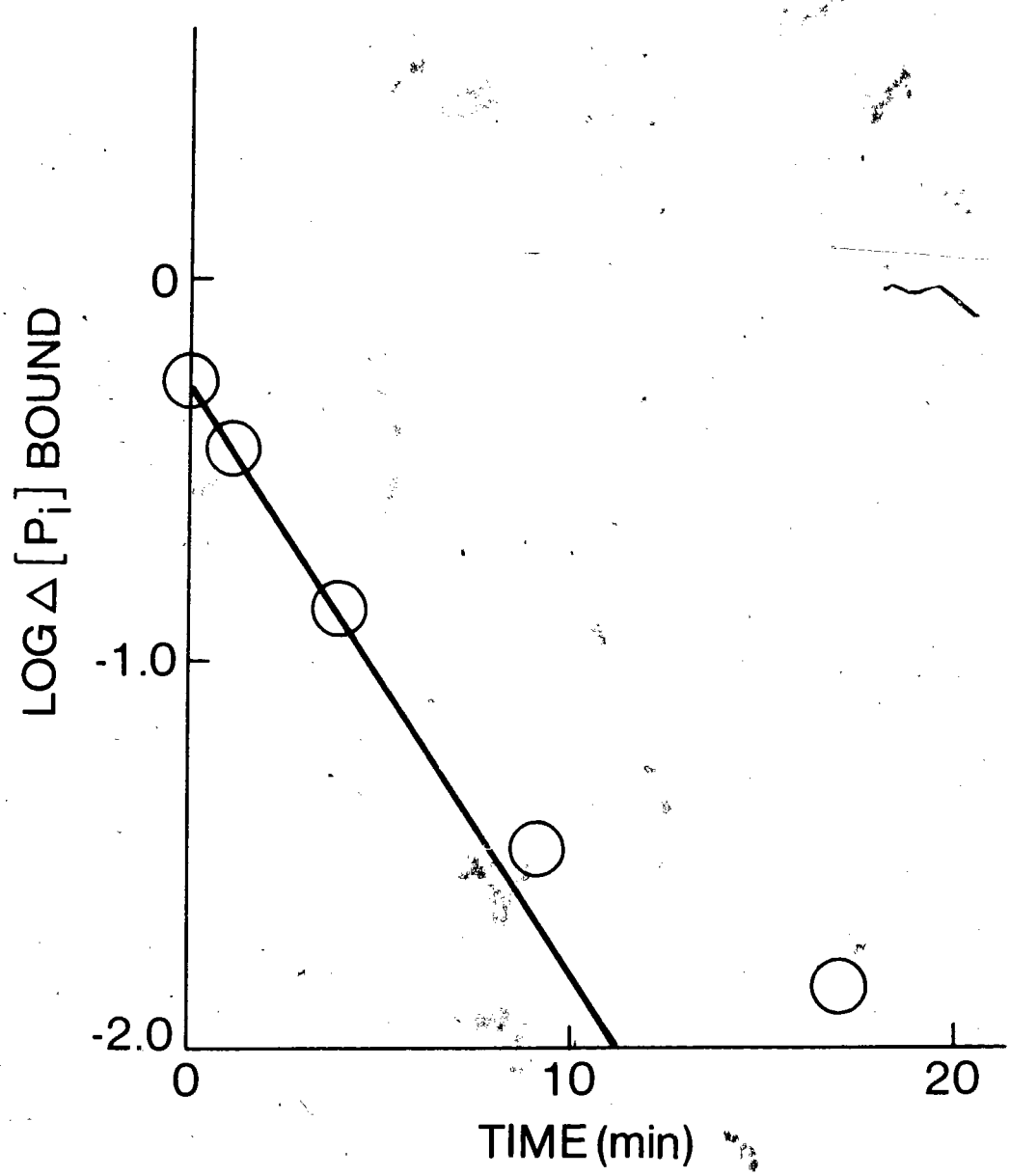
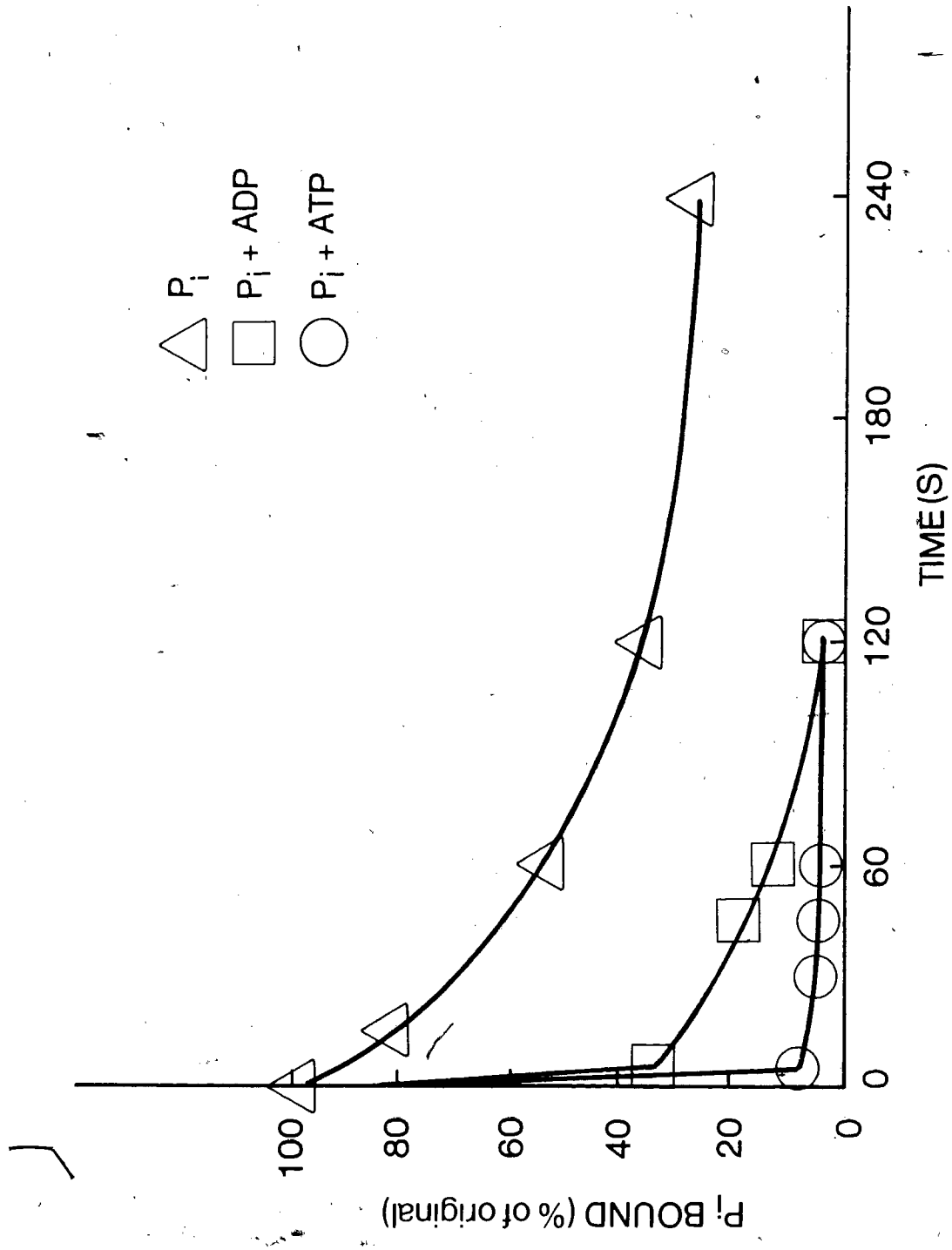

LOG Δ [Pi] BOUND
0
-1.0
-2.0
0
10
20
TIME (min)

Figure 25

The effect of nucleotides on the release of P_i bound to F_1. (Repeat of Hutton and Boyer's experiment shown in Figure 2 of Ref. 96.) A reaction mixture containing 90 mM Tris-Acetate, pH 7.5, 1.6 mM $MgSO_4$, 47 μM P_i with [^{32}P]P_i (1.4×10^3 cpm/pmole), and 3.7 μM F_1-ATPase was incubated for 20 minutes at 23°C. 80 μL aliquots of the reaction mixture were applied in the Sephadex centrifuge column technique as described by Penefsky (28, 73). The Sephadex G-50-80 gel was equilibrated in a buffer containing 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 47 μM P_i. Other 80 μL aliquots were mixed with 20 μL of P_i, P_i and ATP, or P_i and ADP to give final concentrations of 12 mM P_i and 20 mM ATP or ADP, respectively. 80 μL aliquots of these mixtures were added to the Sephadex columns at the times indicated, and centrifugation performed immediately. The 100% P_i bound corresponded to a ratio of 0.16 mole P_i/mole F_1. (△ 12 mM P_i, □ 12 mM P_i + 20 μM ADP, and ○ 12 mM P_i + 20 μM ATP).


P_i
P_i + ADP
P_i + ATP
P_i BOUND (% of original)
100
80
60
40
20
0
0
60
120
180
240
TIME (S)

added to the F_1 reaction mixture to initiate $[^{32}P]P_i$ release from F_1. This experiment (Fig. 25) was performed using exactly the same conditions and procedure used in a similar study (96), and the results obtained in the two studies were essentially identical.

Although it has thus far proved impossible to either eliminate or adequately rationalize the biphasic P_i release, it is consistent with published results (73, 96, 103). In the analysis of the results in the discussion section, attention will be focused on the sensitive or steep phase of nucleotide-facilitated P_i release from F_1. A case will be made for the hypothesis that the P_i released from F_1 in this phase is released from a catalytic site, and that this reaction is a step in the catalytic mechanism of F_1-catalysed ATP hydrolysis. The results will be rationalized in terms of the catalytic mechanism.

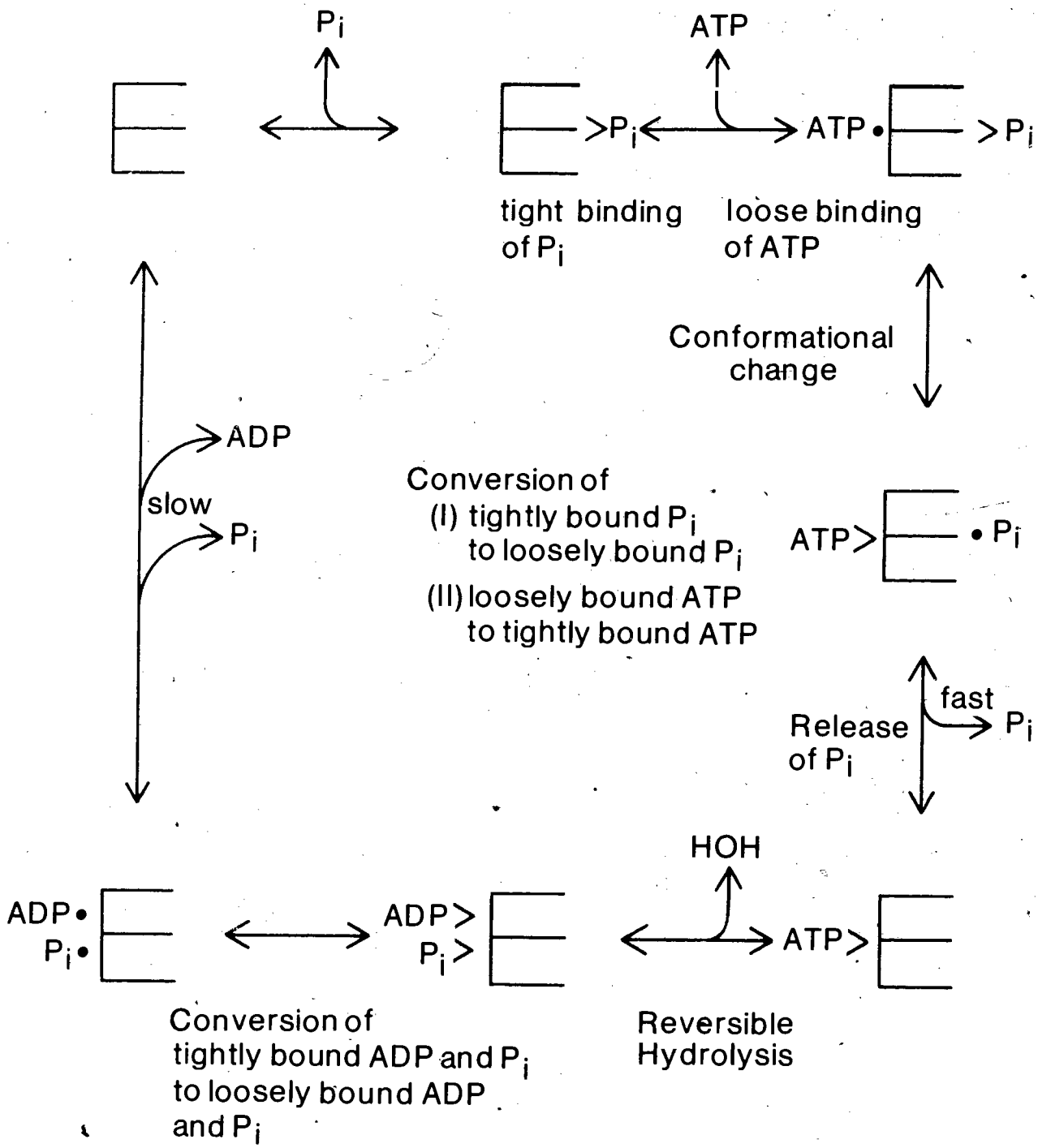
DISCUSSION

The Release of P_i from F_1: Preincubation Method

Figure 2 shows that at relatively low concentrations of the nucleotides, ATP was able to effect the release of P_i from F_1, whereas ADP and AMPPNP had no observable effect. The effect of ATP can be explained by the "binding change mechanism" (10,13, 97-99,122) illustrated as shown in Figure 26. F_1 binds P_i during the preincubation period to give the $F_1 \cdot P_i$ complex (123). This tightly bound P_i is released very slowly ($t_{1/2}$ 2 minutes) from the $F_1 \cdot P_i$ complex (73,96). However, when the $F_1 \cdot P_i$ complex passes through the ATP-containing section of the column, ATP becomes bound at one of the available ATP-binding sites. The binding of ATP at an alternate site is able to effect the conformational change necessary to promote the faster release of P_i from F_1. Grubmeyer and Penefsky (62) have demonstrated that at least two binding sites are involved in site-site cooperativity in the mechanism of action of F_1. The results obtained with ADP in the middle section of the column are explained by the failure of F_1 to bind ADP under these experimental conditions. This explanation is substantiated by the observation in Figure 23 in that the ratio of mole ADP/mole F_1 was 0.02, when a 1.0 cm pulse-section (with 5μM ADP) was used. Thus with lower ADP concentrations in the middle sections

Figure 26

P_i release from F_1 on binding of ATP (for simplicity only two binding sites are shown). The designations are as follows: (1) E represents F_1, (ii) > or < represents a transitorily tight bound substrate or product molecule, and (iii) a **center dot** represents a more loosely bound substrate or product molecule. (See Refs. 70,93,94.)

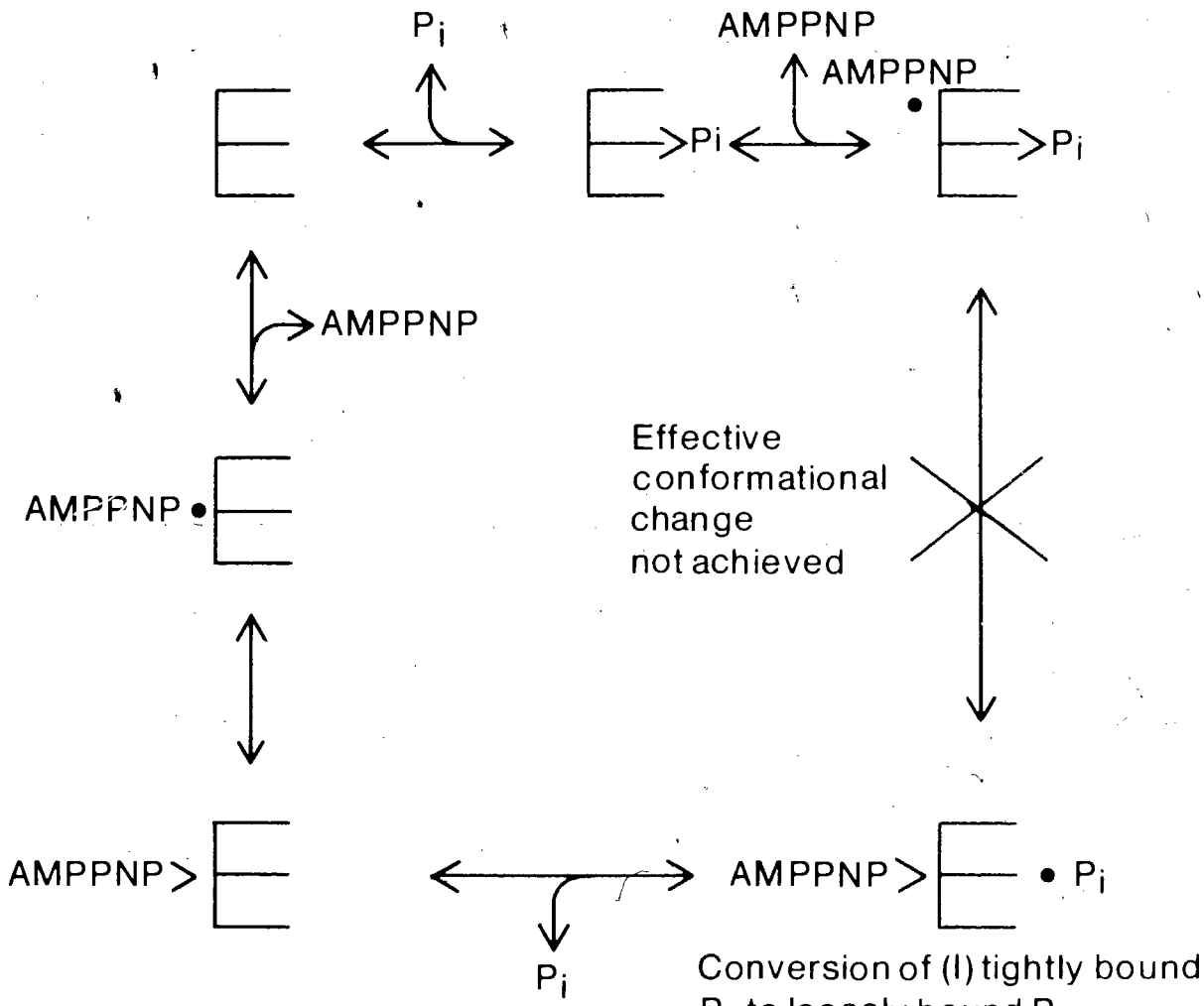

Pi
ATP
>Pi
ATP•
>Pi
tight binding of Pi
loose binding of ATP
Conformational change
ADP
slow
Pi
Conversion of
(I) tightly bound Pi to loosely bound Pi
(II) loosely bound ATP to tightly bound ATP
ATP>
• Pi
fast
Release of Pi
Pi
HOH
ADP•
Pi•
ADP>
Pi>
ATP>
Conversion of tightly bound ADP and Pi to loosely bound ADP and Pi
Reversible Hydrolysis

the amount of ADP bound was not significant. Grubmeyer et. al. had found that the rate of ADP binding to F_1, (10^3 $M^{-1}.s^{-1}$), is three orders of magnitude lower than that of ATP (104). Since low concentrations of AMPPNP bind as well as ATP to F_1 under these experimental conditions (Tables III and IV, Figure 15 and Ref. 124), it can be concluded that the bound AMPPNP (low concentrations) was, unlike ATP, unable to effect the conformational change which results in the release of P_i (Fig. 27).

High concentrations of all three nucleotides were able to effect the total release of P_i from F_1 (Figs. 3-5). At these high ATP concentrations (Figs. 3,4), since ATP is present in greater than stoichiometric amounts, the probability of each $F_1 \cdot P_i$ complex binding an ATP molecule is greatly enhanced, thus resulting in the greater release of P_i from the $F_1 \cdot P_i$ complex (Fig. 26). It is also possible that the high nucleotide concentrations were able to make more F_1 active under these experimental conditions, thereby accounting for the greater release of P_i from the $F_1 \cdot P_i$ complex. In addition, it is possible in the presence of excess ATP for each $F_1 \cdot P_i$ complex to bind a second ATP molecule, thus accelerating the release of P_i from F_1 at least two-fold (104).

Figure 27

P_i release from F_1 on exposure to low AMPPNP concentrations (Figure 2). (See Figure 26 for explanation of symbols.)

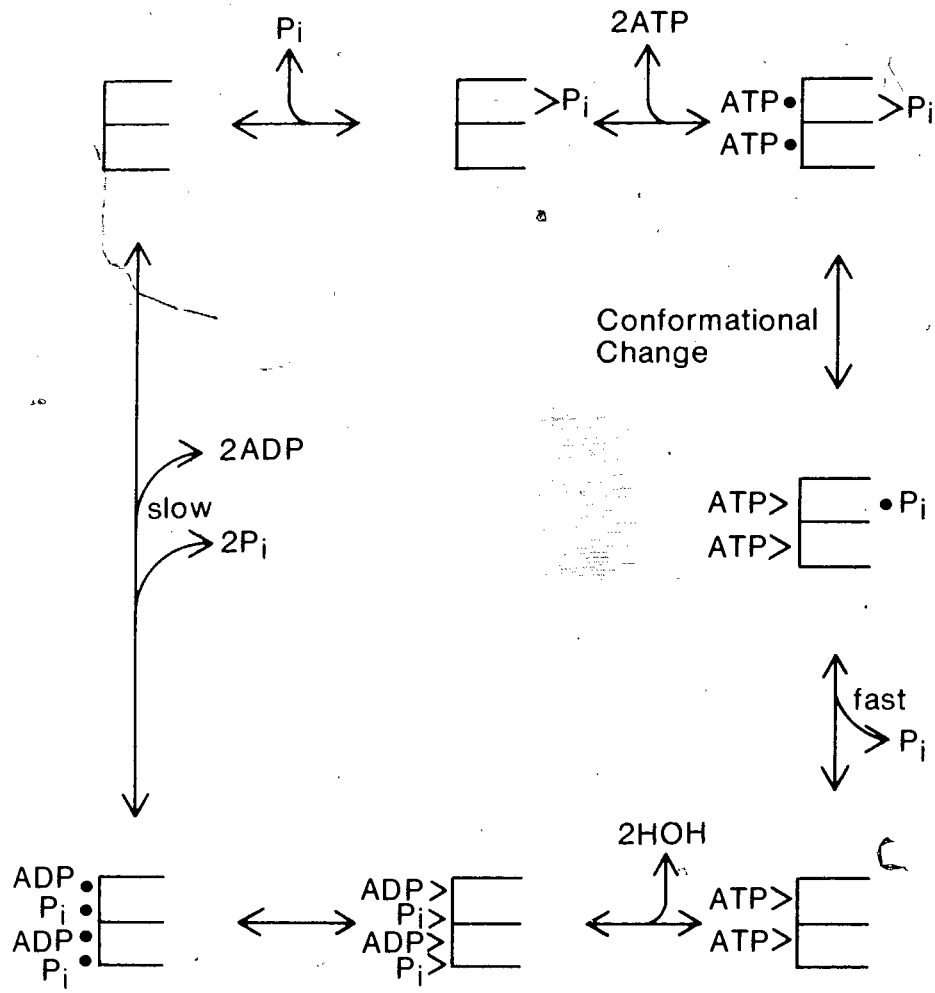


Conversion of (I) tightly bound P_i to loosely bound P_i not achieved, (II) loosely bound AMPPNP to tightly bound AMPPNP not achieved

Grubmeyer and Penefsky found that a third binding site was occupied after prolonged (30 to 60 minute) incubation with high concentrations (5 to 100 μM) of TNP-adenine nucleotides (Mg^{2+} present in buffer). The presence of a third site for the nucleotides AMPPNP and ADP on F_1 was also observed under similar conditions of incubation (60,61). However, whether this third nucleotide binding site is a catalytic site or a low affinity exchangeable site which serves a regulatory role was not clearly distinguished (104). One possibility involving three sites and the binding of a second ATP molecule is shown in Figure 28. Evidence has been presented for three separate interacting subunits per molecule of F_1 (48,98,99). Figure 11 and Table III show that the amount of ATP bound by F_1 increases as the ATP concentration increases for low concentrations of ATP. Figure 15 and Table V show that the amount of ATP bound increases until a maximum value is reached, and that it is possible for F_1 to bind more than one ATP molecule at high nucleotide concentration (100 μM). Note that in the experiments described in Figures 3 and 4, 10 to 100 and 0.1 to 25 mM ATP concentrations, respectively, were used, thus the possibility of F_1 binding more than one ATP molecule is likely. However, as pointed out by Grubmeyer and Penefsky (61,62), it is not necessary to invoke a third site to explain promotion of the release of hydrolyzed nucleotides.

Figure 28

P_i release from F_1 on exposure to high ATP concentrations. (For explanation of symbols, see Figure 26.)


Pi
2ATP
>Pi
ATP•
ATP•
>Pi
Conformational
Change
2ADP
slow
2Pi
ATP>
ATP>
•Pi
fast
Pi
2HOH
ADP
Pi
ADP
Pi
ADP>
Pi>
ADP>
Pi>
ATP>
ATP>

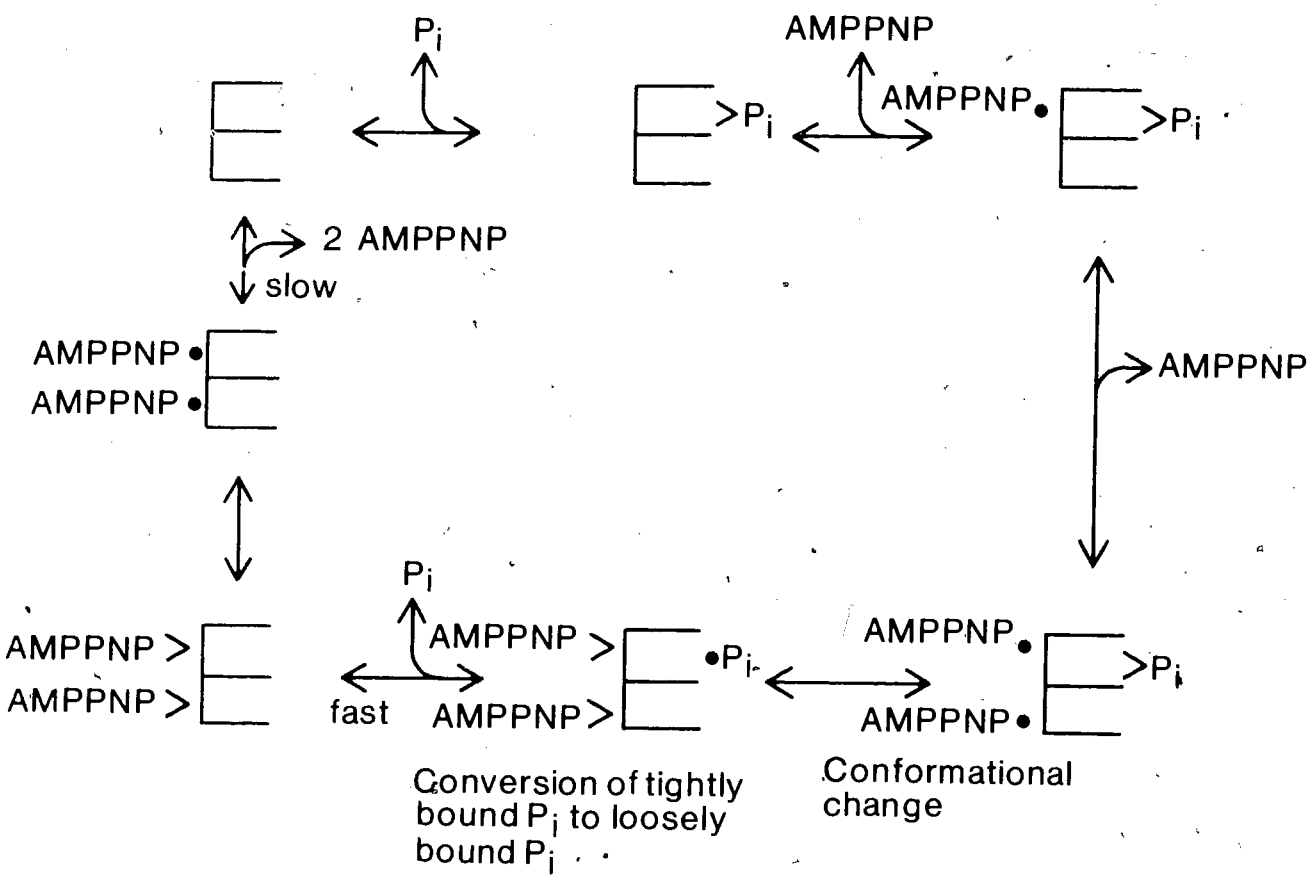
The effect observed with high concentrations of ADP (Figure 5) can be accounted for if a small amount of ($\sim$1.4%) of ATP is present in these ADP preparations (103,123). This very small percentage ($\sim$1.4%) of ATP in the 0.1 to 10 mM ADP would yield a range of about 1.4 to 140 μM ATP concentrations, which would be sufficient to effect the release of P_i from F_1 (Figure 5). This explanation, in addition to the longer time of incubation of $F_1 \cdot P_i$ with nucleotide, may account for the effect of ADP on the accelerated release of P_i from F_1 observed by Hutton and Boyer (96). In their studies about 0.28 μM ATP may have been present in their 20 μM ADP preparation in addition to their longer incubation time of about a minute. This longer time of incubation would allow ADP to bind to F_1. Grubmeyer et. al. found that the rate of ADP binding to F_1 was 10^3 $M^{-1} \cdot s^{-1}$ (103). Penefsky (73) has also observed that high concentrations (30-120 μM) of ATP and ADP (Figure 9 of Ref. 73) inhibited the binding of P_i to about the same extent. Penefsky's ADP preparations in the range studied may have contained about 0.42 to 1.68 μM ATP, and in addition he used a long incubation period of 30 minutes. The results reported here (Figures 1-5) show that the release of P_i is more sensitive to ATP than to ADP, and that the P_i release observed with high ADP concentrations can be accounted for by the presence of trace amounts ($\sim$1.4%) of ATP in the ADP preparations. High nucleotide concentrations

(Figures 3-5) enabled the release of the P_i ($\sim$30%) which remained bound at low ATP concentrations. One possible explanation is that high nucleotide concentrations resulted in higher ionic strength and this effect alone caused the release of bound P_i, which was insensitive to lower ATP concentrations. Kasahara and Penefsky found that salts inhibited P_i binding to F_1 which was attributed to their effects on the enzyme and on the ionic strength. It is also possible that at the low ATP concentrations used, approximately 60% of the F_1 is not active, but this fraction can become active upon longer exposure to ATP or upon brief exposure to high ATP concentrations.

In the experiment described in Figure 1, a higher concentration of AMPPNP was used than in that described in Figure 2. The nucleotides ATP and AMPPNP show basically the same pattern of P_i release from F_1 (Figure 1 and Ref. 123), however the sensitivity of P_i release to ATP is greater (Figure 1). Figure 15 and Table VI show that the amount of AMPPNP bound by F_1 increases as the nucleotide concentration in the column increases. At higher AMPPNP concentrations (i.e. > 1 μM), the release of P_i from F_1 is observed, thus the conformational change which results in the release of P_i has been achieved. This can be explained if it is assumed that more than one AMPPNP molecule binds to F_1 (Figure 29). It was shown that ATP and AMPPNP bind to F_1 equally well (c.f. Tables III and VI, and see

Figure 29

P_i release from F_1 on exposure to high AMPPNP concentrations.


P_i
AMPPNP
AMPPNP
P_i
P_i
2 AMPPNP
slow
AMPPNP
AMPPNP
AMPPNP
AMPPNP
AMPPNP
P_i
AMPPNP
AMPPNP
fast
AMPPNP
P_i
AMPPNP
AMPPNP
P_i
Conversion of tightly bound P_i to loosely bound P_i
Conformational change

Ref. 124), and that AMPPNP did not cause a release greater than 30% of the bound AMPPNP from F_1 (Fig. 10), thus the net effect is that AMPPNP accumulates on F_1 in the presence of higher AMPPNP concentrations. There is no hydrolysis of AMPPNP so it remains bound to F_1 (125,126). Cross and Nalin (48) have demonstrated the presence of three readily exchangeable AMPPNP binding sites that are distinct from three very slowly exchangeable AMPPNP binding sites on F_1, so the $F_1 \cdot P_i$ complex can bind a maximum of two AMPPNP molecules at the catalytic sites. The second AMPPNP molecule binding at the third site would be doing so with a lower affinity; Cross and Nalin found one high affinity site, K_d = 18 nM, and two lower affinity sites, K_d = 1.0 μM (48). Penefsky's observation that AMPPNP was more effective than ATP in inhibiting P_i binding to F_1 (Figure 9 of Ref. 73) can be explained as outlined below. AMPPNP is not very effective in promoting the release of bound AMPPNP from F_1 (Figure 10), whereas ATP is very effective in promoting the release of bound ATP (i.e. hydrolysed ATP) from F_1 (Figure 13). Therefore, when F_1 is incubated with AMPPNP fewer binding sites are available for P_i binding (see Figure 15, and Tables V and VI). The order of effectiveness of the nucleotides in promoting the release of P_i from F_1 is ATP > AMPPNP >> ADP.

The finding that ATP quenches the aurovertin fluorescence, whereas AMPPNP (a strong, competitive inhibitor of ATPase activity) does not (31,127) led Ferguson et. al. to suggest the possibility that aurovertin fluorescence quenching may represent something more than mere ATP binding. They suggested that subunit-subunit interactions may be occurring. This proposal may well explain why ATP is more effective than AMPPNP in promoting the release of P_i from F_1. (In the preincubation mode, the slopes of the P_i release curves are $140\%.\mu M^{-1}$ (Figure 6), and $13\%.\mu M^{-1}$ (Figure 7), with ATP and AMPPNP, respectively. Thus ATP is about 10 times more effective than AMPPNP in promoting P_i release from F_1.)

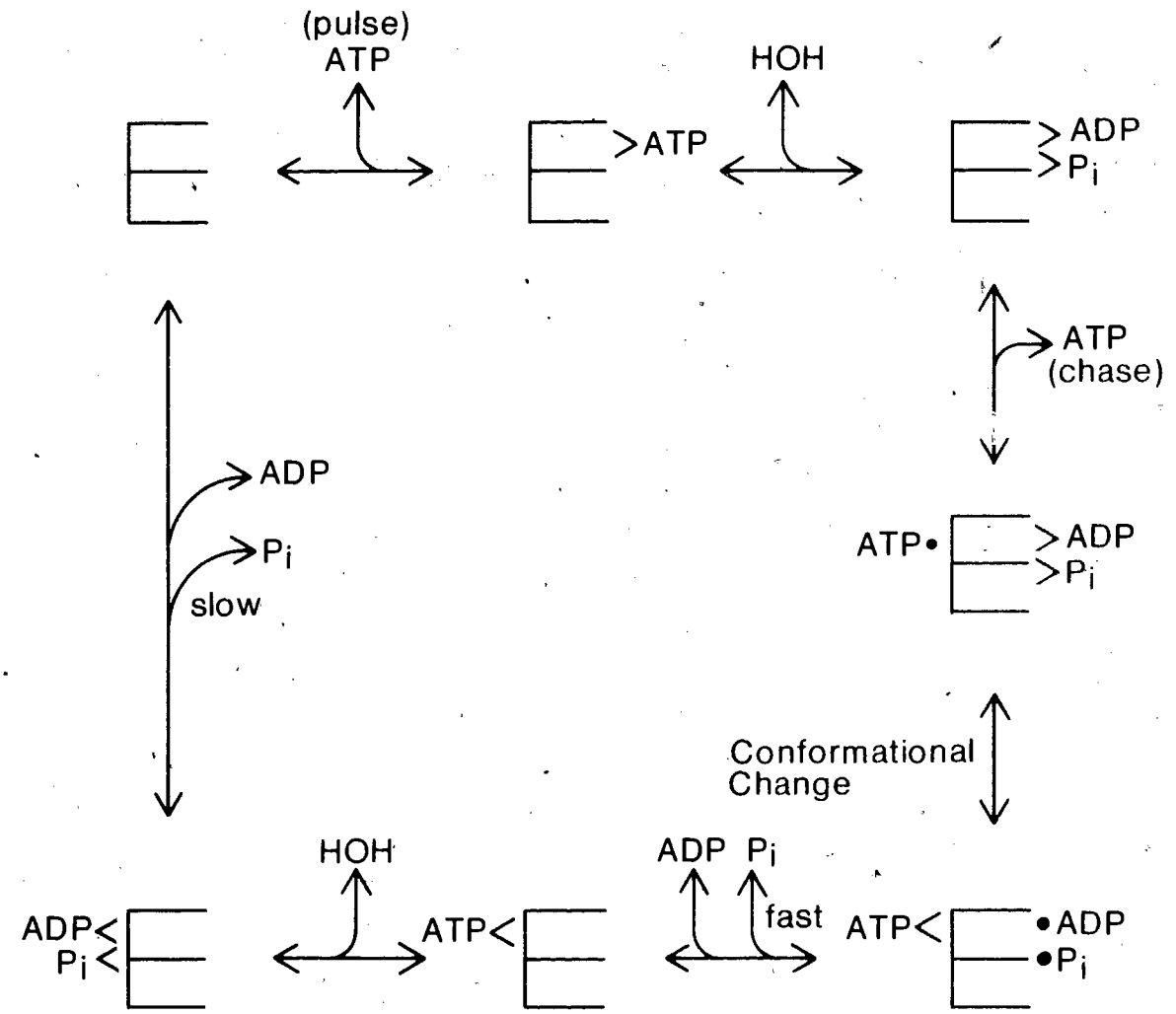
Release of P_i from F_1: Pulse-Chase Method

The pulse-chase method offered the opportunity to study the effect of the nucleotides on the release of P_i from F_1 when ADP was also present at the catalytic site. Chase-ATP was effective in achieving the conformational change which resulted in the expulsion of P_i and ADP from F_1 (Figures 6,8,9). Figure 30 shows a schematic representation of the process that may be occurring: the pulse-ATP (at equilibrium with ADP,P_i) is bound at one site, and the chase-ATP binds at another site (61,62,103,104); this latter event then effects the release of hydrolysed pulse-ATP (i.e. ADP,P_i) from F_1. Thus the presence of ADP at the same site with P_i did not prevent P_i release nor ATP binding at an alternate site.

The results obtained with chase-ADP are explained, as for the preincubation studies discussed above, by the failure of F_1 to bind ADP under these experimental conditions (Figure 23). The small P_i released observed (Figure 8) can be accounted for by the presence of trace amounts of ATP in these ADP preparations (e.g. 100 μM ADP solution may contain about 1.4% ATP, i.e. 1.4 μM ATP, which accounts for the results shown in Figure 8) (103,123).

Figure 30

P_i and ADP release from F_1 on the binding of chase-ATP.



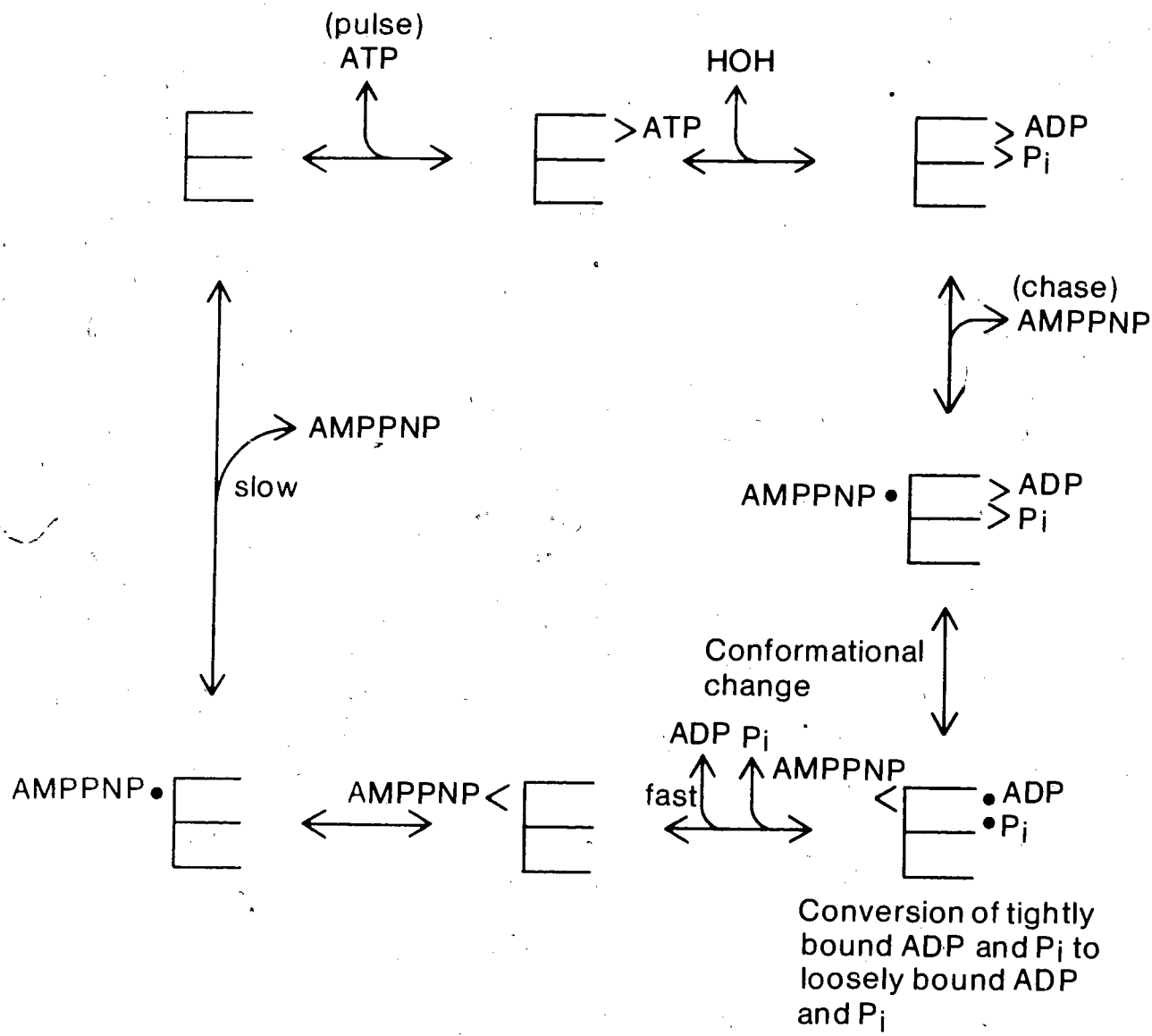
Conversion of tightly bound ADP and P_i to loosely bound ADP and P_i

Figures 7 and 8 show that AMPPNP was able to effect the release of P_i from F_1 in the presence of ADP at the catalytic site. In addition, AMPPNP was about equally effective as ATP in promoting the release of P_i from F_1 in the presence of ADP (Figure 8). Figure 31 illustrates the process that may be occurring on the binding of AMPPNP. The F_1 now has two nucleotide-binding sites filled, one with ADP plus P_i at equilibrium with ATP (61,62), and the other with AMPPNP (an analog of ATP); therefore the conformational change is achieved as when chase-ATP is bound (c.f. Figures 30 and 31). Since the effects of ATP and AMPPNP on the release of hydrolyzed ATP were similar (Figures 6-8), hydrolysis of chase-ATP (or nucleotide) is not necessary for the release of hydrolyzed pulse-ATP. What is necessary is for a second ATP-binding site to be filled. Thus binding of ATP or AMPPNP (i.e. substrate or substrate analog) at another site is sufficient to effect the conformational change which results in the release of P_i from another site on F_1 which also contains bound ADP. In the presence of ADP at the catalytic site, the order of effectiveness of the nucleotides in promoting the release of P_i from F_1 is ATP $\approx$ AMPPNP $>>$ ADP.

In Figures 6 and 13, the slopes (46 and 45%.μM^{-1}, respectively) of the ADP/P_i release curves of the ATP-chase experi-

Figure 31

P_i and ADP release from F_1 on the binding of chase AMPPNP.


(pulse)
ATP
HOH
ATP
ADP
Pi
(chase)
AMPPNP
AMPPNP
slow
AMPPNP •
ADP
Pi
Conformational
change
ADP Pi
AMPPNP
fast
AMPPNP •
AMPPNP
AMPPNP <
ADP
•Pi
Conversion of tightly
bound ADP and Pi to
loosely bound ADP
and Pi

ments were approximately equal; and in addition they were also equal to the slopes (45%.μM^{-1} in both cases) of the ADP/P_i release curves of the AMPPNP-chase experiments shown in Figures 7 and 14. This demonstrates that equivalent concentrations of ATP and AMPPNP in the chase had the same effect (Figure 8). Whereas, a comparison of the slopes (120 and 170%.μM^{-1}, respectively) of the chase-ATP binding curve (Figure 13) and the chase-AMPPNP binding curve (Figure 14), indicates that more AMPPNP than ATP is binding per mole of F_1 at an equivalent nucleotide concentration (see also Tables V and VI). However, the ratio of the slopes of chase-ATP to chase-AMPPNP binding (120/170 or 0.7) is not of such a magnitude to suggest that labelled F_1 is binding more than one AMPPNP molecule at these nucleotide concentrations. Since the ratio of the slopes of the chase-ATP binding curve to that of the ATP (i.e. ADP,P_i)-release curve (Figure 13) is approximately 2.8 (i.e. 120%.μM^{-1} ÷ 43%.μM^{-1})), and not 1, it must be that chase-ATP molecules are binding to F_1 without pulse-ATP molecules.

Grubmeyer and Penefsky (61,62) have found that hydrolyzable nucleotides such as ATP, GTP, and ITP are excellent promoters of hydrolysis or release of previously bound TNP-[γ-^{32}P]ATP, whereas non-hydrolyzable nucleotides such as TNP-ADP, ADP, and AMPPNP give lower rates and extents of hydrolysis. In the experiments

reported here, however, the non-hydrolyzable nucleotide, AMPPNP, which bound to F_1 was almost as effective as the hydrolyzable nucleotide, ATP, in promoting the release of previously bound P_i and ADP at equilibrium with ATP. Grubmeyer and Penefsky (62) were using higher concentrations of nucleotides (e.g. 100 μM AMPPNP, 1 mM ADP, and 140 μM ATP-ADP (see Figure 5 of Ref. 62)) in their experiments. (In the pulse-chase studies reported here, lower nucleotide concentrations (below 2 μM) were able to promote the release of P_i and ADP (Figures 6-8)). It is also possible that their observations are equivalent to the observations made in the less sensitive phase of release seen in both preincubation and pulse-chase modes of the studies reported herein.

Comparison of the Results of Preincubation and Pulse-Chase Studies

It appears that ATP was more effective in promoting P_i release from F_1 in preincubation experiments than in pulse-chase experiments (Figure 6), and a schematic diagram of each process is shown in Figures 26 and 30, respectively. The two processes are similar except for the presence of ADP at the P_i binding site in the pulse-chase experiments. Since the sensitivity of the P_i release to ATP is apparently greater in the preincubation than in the pulse-chase experiments, i.e. P_i release is greater in the absence than in the presence of ADP at the same catalytic site, it appears that ADP at the same catalytic site with P_i is influencing the P_i release reaction.

In Figure 6, the slopes of the P_i release curves are $140\%.\mu M^{-1}$ (Preincubation Mode) and $45\%.\mu M^{-1}$ (Pulse-chase Mode), respectively; and the ratio of the slopes (Preincubation/Pulse-chase) is approximately 3 ($140\%.\mu M^{-1} \div 45\%.\mu M^{-1}$). [N.B. In the preincubation mode, the mole of label bound/mole of F_1 is approximately 0.2 to 0.3; whereas in the pulse-chase mode, the mole of label bound/mole of F_1 is approximately 0.1, before exposure to nucleotide.] One likely explanation which may account for the less effective release of P_i in the pulse-chase experiments than in preincubation experiments is

competition between labelled and unlabelled F_1 for the chase-ATP. That there is competition between labelled and unlabelled F_1 for chase-ATP is substantiated by Figure 13 and Table V. The slope of the chase-ATP binding curve is $120\%.\mu M^{-1}$, and that of the P_i release curve is $43\%.\mu M^{-1}$; thus the ratio of ATP binding to P_i release is approximately 3 ($120\%.\mu M^{-1} \div 43\%.\mu M^{-1}$), and therefore chase-ATP is very likely also binding to unlabelled F_1.

Figure 7 shows that AMPPNP was more effective in facilitating the release of P_i in the pulse-chase mode than in the preincubation mode, this in spite of the greater competition by unlabelled F_1 for chase-AMPPNP in the pulse-chase experiment. In the preincubation experiments, relatively low amounts of AMPPNP were not effective in promoting P_i release from F_1 (Figure 2), whereas higher amounts of AMPPNP were effective (Figure 1). These results are diagramed in Figures 27 and 29, respectively, and the effect of AMPPNP in pulse-chase experiments is diagramed in Figure 31. A comparison of Figures 29 and 31 reveals that the two processes are the same except that in one case (preincubation mode) two AMPPNP molecules are required; whereas in the other case (pulse-chase mode) one AMPPNP molecule is required; and in addition, ADP is absent at the P_i binding site in Figure 29 and present at the P_i binding site in Figure 31. Thus the difference shown by the two processes may be attributed to the ADP at the P_i binding site. In Figure 7,

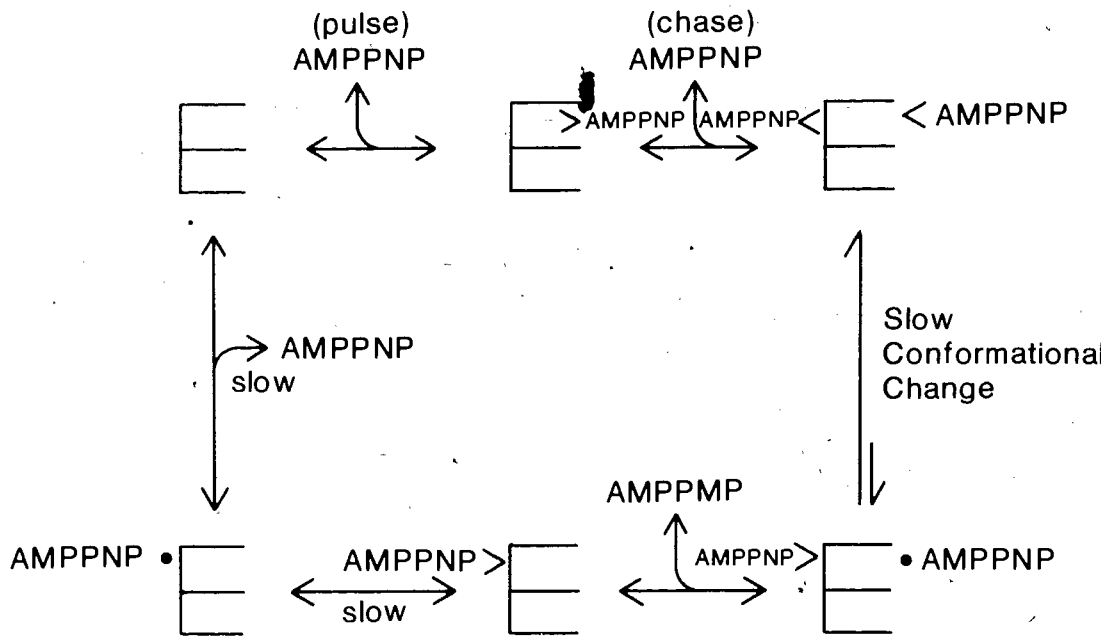
the slopes of the P_i release curves of the preincubation and pulse-chase modes are 13 and 45%.μM^{-1}, respectively; hence from the ratio of the slopes (Pulse-chase:Preincubation, i.e. 43:13), the release is about 3 times greater in the pulse-chase mode than in the preincubation mode. It is therefore easier for AMPPNP to effect the conformational change (which results in the release of P_i), if another nucleotide (i.e. ADP) is present at the P_i binding site.

Release of AMPPNP from F_1: Pulse-Chase Methods

Chase-AMPPNP was not very effective in expelling the bound pulse-AMPPNP (< 30%) from F_1 (Figure 10). AMPPNP is not hydrolyzed by F_1, hence all the AMPPNP bound in both pulse and chase accumulates on F_1. (As mentioned before, F_1 can contain a maximum of three AMPPNP molecules at the exchangeable nucleotide binding sites (48)). Table VI supports the hypothesis that chase-AMPPNP is bound and accumulated on F_1 as the chase-AMPPNP concentration increases. Figure 32 offers a scheme for the non-release of pulse-AMPPNP from F_1 on the binding of chase-AMPPNP: the pulse-AMPPNP is not hydrolyzed and it is not readily changed from being tightly bound to loosely-bound (under these experimental conditions). This relative inability of AMPPNP to promote the rapid dissociation of AMPPNP from F_1•AMPPNP complex was also found by Nalin and Cross (102). This finding lends support to the proposal of Ferguson et. al. (127) that the binding of AMPPNP is different from that of ATP in that ATP is more readily able to effect subunit-subunit interactions.

Figure 32

The binding of pulse-AMPPNP and chase-AMPPNP to F_1.


(pulse)
AMPPNP
(chase)
AMPPNP
AMPPNP
AMPPNP
AMPPNP
AMPPNP
slow
Slow
Conformational
Change
AMPPMP
AMPPNP
AMPPNP
AMPPNP
slow
AMPPNP

Conclusions

Several conclusions were made from this work. These included: (1) The order of effectiveness of the nucleotides (ATP, ADP, and AMPPNP) in promoting the release of approximately 70% (i.e., the steep phase of the biphasic release) of the bound P_i (0.2 mole P_i/mole F_1) from F_1 was ATP > AMPPNP >> ADP. (ii) Only binding of ATP or AMPPNP (substrate or substrate analog) at an alternative site (hydrolysis of incoming nucleotide was not necessary) was essential to produce the conformational change which resulted in the release of bound P_i from F_1. (iii) The ratio of mole of P_i released from F_1 to mole of ATP bound to F_1 was approximately one, thus one molecule of incoming ATP was able to effect the release of one molecule of bound P_i from F_1. This observation implied that the P_i was probably binding at a catalytic site. (iv) More than one molecule of AMPPNP had to bind to F_1 in order to effect the release of bound P_i from F_1. This observation implied that the binding of AMPPNP was somehow different from the binding of ATP to the $F_1 \cdot P_i$ complex. (v) Both ATP and AMPPNP effected the release of bound P_i in the presence of ADP, with equal sensitivity in the steep phase of the biphasic release of P_i from F_1. (vi) Bound P_i and ADP were probably released with equal sensitivity from the $F_1 \cdot ADP \cdot P_i$ complex by incoming ATP. (vii)

The results were consistent with the binding-change mechanism for the F_1 catalysed hydrolysis of ATP. (viii) Lastly, the modified Sephadex centrifuge column technique developed here (both preincubation and pulse-chase modes) could be used in the investigation of other complex multisubunit enzymes (110,111) which have tightly bound molecules and in which subunit-subunit interactions are thought to be involved in the catalytic process.

FUTURE WORK

1. Since AMPPNP can effect the release of hydrolyzed ATP (ADP,P_i) from F_1 (Figure 7), it is desirable to know whether the reverse is also true. This would indicate whether the bound nucleotide does or does not have to be hydrolyzed before being released from F_1 in the presence of incoming ATP. In addition, it was shown that AMPPNP did not effect the release of bound AMPPNP from F_1 as well as it did effect the release of hydrolyzed ATP (c.f. Figures 7 and 10). Thus it is also desirable to compare the effects of ATP and AMPPNP on the release of AMPPNP from F_1. This may lend support to the proposal of Ferguson et. al. (127) that the binding of AMPPNP is different from that of ATP in that ATP is more readily able to effect subunit-subunit interactions. These proposals can be investigated by using both pulse-chase and preincubation type experiments outlined in this study (see Materials and Methods). In the pulse-chase mode of the Sephadex centrifuge column technique, labelled AMPPNP ([^{3}H]-AMPPNP) will be used in the pulse-section, and labelled ATP ([γ-^{32}P]ATP) will be used in the chase section. In the preincubation mode, the F_1 will be preincubated with [^{3}H]AMPPNP before being applied to the column with [γ-^{32}P]ATP in the middle section of the column. In these experiments, the F_1.[^{3}H]AMPPNP complex is exposed to incoming [γ-^{32}P]ATP of various concentrations. The amount of each type of label bound is monitored,

thus the effect of ATP on bound AMPPNP can be determined. If as the ATP concentration increases, less [^{3}H]AMPPNP label is found on F_1 this would imply that ATP effects the release of previously bound AMPPNP from F_1. On the other hand, if as the ATP concentration increases, the amount of [^{3}H]AMPPNP label on F_1 remains unchanged, this would imply that ATP does not effect the release of previously bound AMPPNP from F_1. If ATP can effect the release of AMPPNP from F_1, whereas AMPPNP cannot do so (Figure 10), this would indicate that the binding of ATP is different from that of AMPPNP. An interesting question that may be raised in these investigations is whether the incoming ATP would be hydrolyzed and released in preference to the release of the previously bound non-hydrolyzable AMPPNP. This may be looked at by doing the appropriate controls and/or tests, e.g., running columns without AMPPNP in the pulse for each ATP concentration used to see how much ATP is bound, and examining the bottom part of the column for [^{32}P]P_i and [γ-^{32}P]ATP (see Methods and Ref. 114). Alternatively, comparable quantities of labelled AMPPNP can be placed in the chase (unlabelled AMPPNP in the pulse) to determine how much AMPPNP is bound under these conditions. Assuming that ATP and AMPPNP bind equally well to F_1, it can be determined how much ATP should bind in the chase. Thus if less ATP than expected is found on F_1, it can be assumed that the ATP was bound but was hydrolysed and released preferentially to bound AMPPNP.

2. The sensitivity of release of P_i and ADP (both from hydrolyzed ATP) from F_1 by incoming ATP were shown to be similar in these studies (Figure 9). However, the rate of release of bound ADP (the other product of ATP hydrolysis by F_1) was not extensively studied as was done with bound P_i. Thus it is proposed to investigate the release of bound ADP (i.e. in the absence of P_i) from F_1 on exposure to ATP and AMPPNP. In preincubation studies: the F_1 will be preincubated with labelled ADP ([^{3}H]ADP) before application to columns with ATP, or AMPPNP in the middle sections (cf. Figures 1-5). (Precautions will be taken to ensure that the small amount of ATP is removed from the ADP solutions (31)). The results of these findings will be compared with those illustrated in Figures 1-5. If the results are similar, this would indicate that ADP and P_i do bind at the same site, since the conformational change effected by the incoming nucleotide should be similar in both cases.

These results would also tell of the relative effectiveness of the two nucleotides (ATP and AMPPNP) in promoting the release of ADP from F_1. It was proposed in Figure 31 that AMPPNP was just as effective as ATP in promoting the release of P_i from F_1 in the pulse-chase mode (Figures 6 and 7) because ADP (another nucleotide) was present on F_1. Therefore in these experiments, it is reasonable to expect that AMPPNP be just as effective as ATP in promoting the release of ADP from F_1. Thus

the proposed mechanism of P_i release in the presence of ADP by AMPPNP (Figure 31) can be further enhanced or be called into question.

Using pulse-chase experiments, the role of P_i on the release of ADP in the presence of incoming nucleotides will be investigated. The F_1 will be preincubated with labelled ADP, i.e. [^{3}H]ADP and applied to columns containing labelled P_i (i.e. [^{32}P]P_i) of varied concentrations in the pulse sections, and either ATP or AMPPNP (fixed concentration) in the chase sections. If, as the P_i concentration in the pulse increases, the release of labelled ADP on exposure to chase nucleotide decreases, this would suggest that the P_i is binding at a catalytic site and thus preventing the binding of chase nucleotide. The amount of nucleotide bound in the chase in the absence and presence of the P_i pulse can be determined by doing the appropriate control experiments, i.e. F_1 is not preincubated with labelled ADP, but labelled chase nucleotides ([^{3}H]ATP or [^{3}H]AMPPNP) are used. Thus the experiments outlined in this section should help to clarify the role of ADP at the catalytic sites and what influences its release from F_1.

3. To show that catalytic sites are involved in the binding and release of nucleotides to and from F_1, pulse-chase experiments will be performed as outlined below. The F_1 will be

preincubated with labelled ADP ([^{3}H]ADP) and then applied to columns which contain labelled ATP ([γ-^{32}P]ATP) (fixed concentration) in the pulse-section and unlabelled ATP (variable concentration) in the chase section. The amount of each type of label on the F_1 will be monitored. The pulse-ATP is expected to effect the release of labelled ADP; and the chase-ATP in turn is expected to promote the release of pulse-ATP (as well as labelled ADP). This experiment would demonstrate that what got on the enzyme can get off, i.e. the pulse-ATP, which effected the release of previously bound ADP, can in turn be released (as ADP and P_i) from its binding site by chase-ATP. Thus if chase-ATP can effect the release of pulse-ATP, therefore most likely binding of pulse-ATP and release of hydrolyzed pulse-ATP (ADP and P_i) is occurring at the same site, i.e. a catalytic site.

4. Similar studies as those performed here (both preincubation and pulse-chase modes) can be carried out with chloroplast, bacterial, other mitochondrial ATPases, and other ATPases. Since these enzymes are similar or similar mechanisms may be involved, the studies will be helpful in revealing similarities or differences. Other complex multisubunit enzymes in which subunit-subunit interactions are thought to be involved in the catalytic process may be similarly investigated. Examples

of such enzymes include Alkaline Phosphatase, Alcohol Dehydrogenase, Succinyl-CoA Synthetase, Glyceraldehyde-3-Phosphatase Dehydrogenase, and Malate Dehydrogenase (110,111).

APPENDIX I

Development of the Modified Sephadex Centrifuge Column Technique

Introduction

In an investigation of the effect of nucleotides (ATP and ADP) on the release of bound P_i from F_1, the experiment of Hutton and Boyer was repeated. The results shown in Figure 25 were similar to theirs (Fig. 2 of Ref. 95). It was concluded that both ATP and ADP facilitated the dissociation of P_i from F_1, and that the effects of ATP and ADP were not distinguishable below 15 seconds. Penefsky had previously found that P_i binds reversibly to F_1 with a half-life of about 2 minutes (73), whereas the half-life for release of P_i in the presence of nucleotides is shorter (Ref. 96 and Fig. 25).

Thus in order to distinguish the effects of ATP and ADP on the release of P_i from F_1, another approach was used. Some of the experiments performed in the modification of the conventional Sephadex centrifuge column technique are reported here.

Results and Discussion

The Effect of ATP-Containing Gels in the Middle of the Columns

Figure 1A outlines the preparation of a column with an ATP-containing section in the centre. The rationale was that the F_1 in moving through the column during centrifugation would have

Figure 1A

Outline of the modified Sephadex centrifuge column technique:-

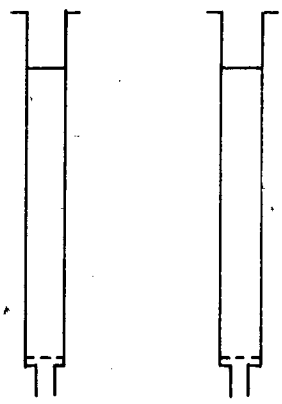
(a) Preparation of the gels. Two sets of Sephadex columns were prepared (28,73). In one set the equilibration buffer of the Sephadex contained: 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 47 μM P_i. The other set had ATP added to the equilibration buffer.

(b) Removal of gels. The gels were removed from the column barrel by decanting gently. The gel without ATP was cut into two halves (~1.5 cm), and from the ATP-containing gel was cut a measured length.

(c) Assembly of column. One of the buffer-equilibrated gels was placed inside the column barrel. The ATP-containing gel was placed on top of the previously inserted gel. Lastly, the other buffer equilibrated gel was placed on top of the ATP-containing gel.

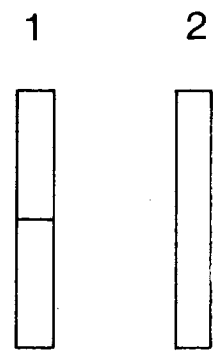
(d) Column assembly. The reassembled column was placed in a 15 mL conical centrifuge tube, and was then ready for use.

(a)



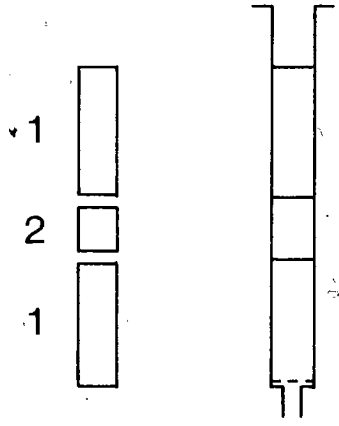
Column 1 is equilibrated with the appropriate buffer
Column 2 is equilibrated with nucleotide in buffer

(b)



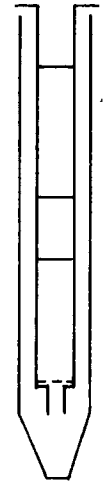
Gels are removed from columns
Gel 1 is cut into halves
From gel 2, x cm is cut

(c)



The pieces of gels are arranged as shown above and then reassembled in a column

(d)



The reassembled column is placed in a centrifuge tube

only brief contact with the ATP of the ATP-containing gel. It was expected that this relatively brief exposure would be enough for the ATP to exert its effect (see Fig. 25).

Figure 2A shows that as the concentration of ATP in the 1.0 cm middle ATP-containing section of the column increased, so did the release of bound P_i from F_1. This clearly demonstrated that the F_1 had access to the ATP of the ATP-containing middle section. However, all of the bound P_i was not removed from F_1 despite the high concentration (400 mM) of ATP used.

Figure 3A shows that on increasing the length of the ATP-containing middle section, the release of bound P_i from F_1 also increased. From the results shown in Figures 2A and 3A, it was inferred that the removal of all bound P_i from F_1 might be possible if (i) the amount of ATP in the middle section was higher, or (ii) the ATP-containing middle gel was longer, or (iii) the time of contact between F_1 and the ATP was longer.

Experiments were performed with the reassembled columns containing various fixed lengths of middle gels, with each fixed length having been equilibrated with buffer containing different concentrations of ATP. Figure 4A shows the results of the first such experiment, in which a 1.5 cm ATP-containing middle section was used. It should be noted that a very high concentration (458 mM) of ATP was used; and that it was impracticable to use a higher concentration in the equilibration buffer since the maxi-

Figure 2A

The effect of ATP on the release of bound P_i from F_1. The reassembled columns were prepared as described in Figure 1A. The concentration of ATP in the equilibration buffer used to prepare the 1.0 cm middle ATP-containing gels was varied (0, 100, and 400 mM). 80 µL aliquots of a reaction mixture containing: 3.7 µM F_1, 90 mM Tris-acetate, pH 7.5, 1.6 mM $MgSO_4$, and 47 µM P_i with $[^{32}P]P_i$ (1.3×10^6 cpm/nmole), which had been allowed to stand for 30 minutes at 23°C, was added to the reassembled column. Centrifugation (1050 × g) was carried out immediately for 2 minutes. For each ATP concentration a control, i.e. no ATP in the middle gel, was also run simultaneously. The 100% P_i bound corresponded to a ratio of 0.35 mole P_i/mole F_1. Each point is the average of duplicate experiments.

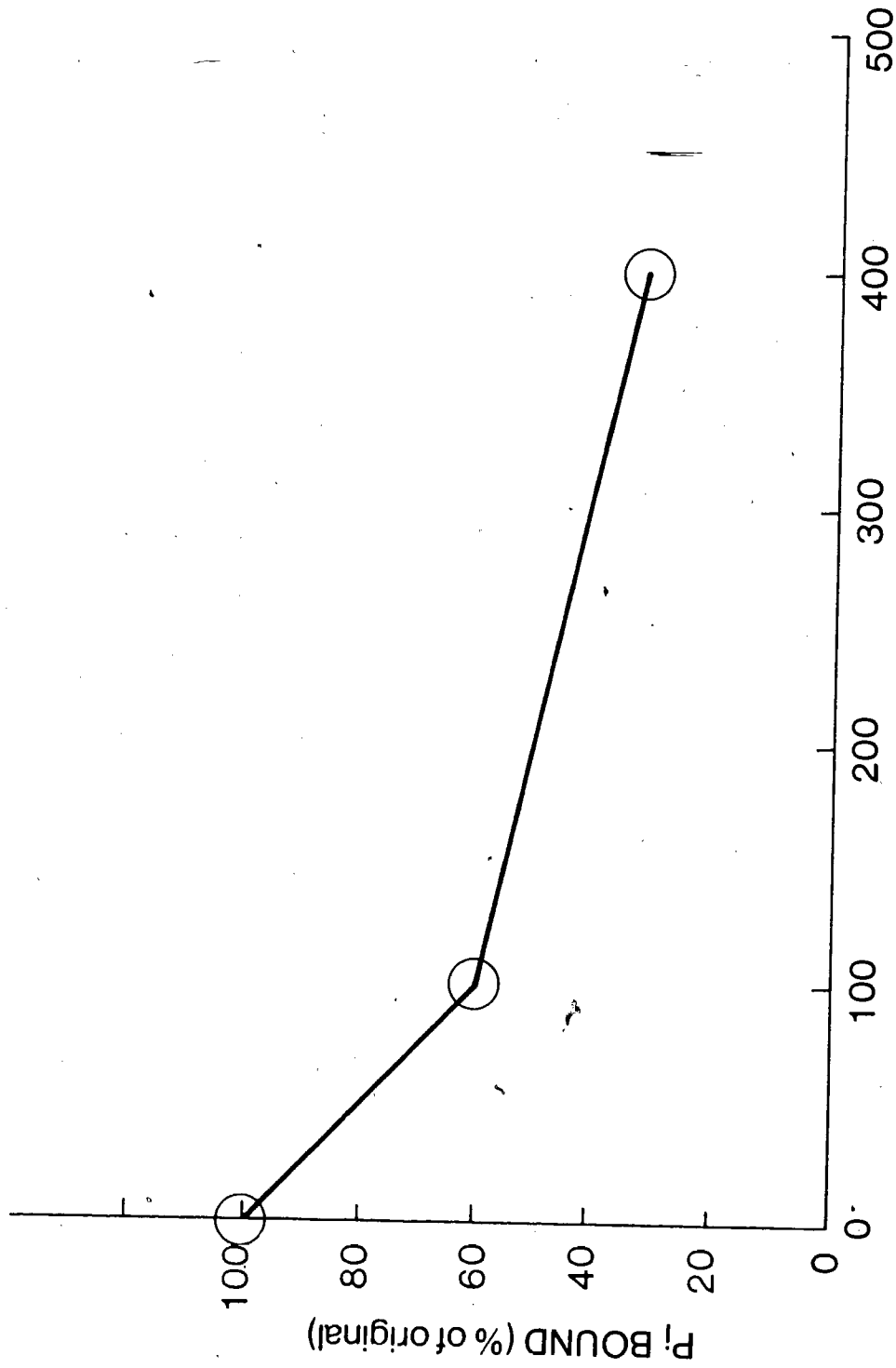

Pi BOUND (% of original)
100
80
60
40
20
0
0
100
200
300
400
500
[ATP],mM

Figure 3A

The effect of ATP on the release of bound P_i from F_1. The reassembled columns were prepared as described in Figure 1A. The conditions and concentrations were the same as described in Figure 2A, except that a concentration of 100 mM ATP was in the equilibration buffer used to prepare the different lengths of the ATP-containing middle gels. The specific activity of the $[^{32}P]P_i$ added to the reaction mixture was 1.2 $\times 10^6$ cpm/nmole. For each length a control, i.e. a column with no ATP In the middle gel, was also run simultaneously. The 100% P_i bound corresponded to a ratio of 0.17 mole P_i/mole F_1. Each point is the average of duplicate experiments.

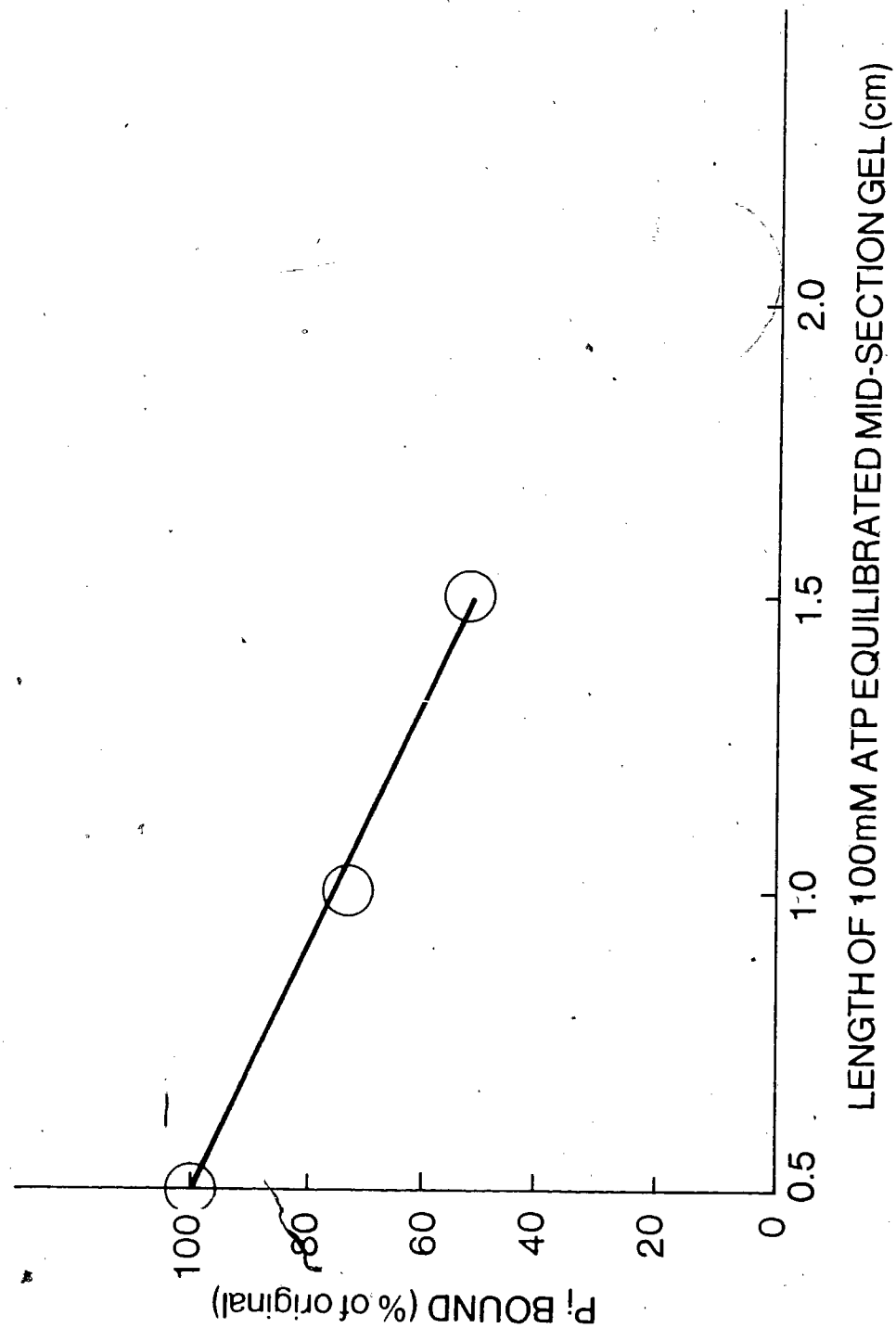
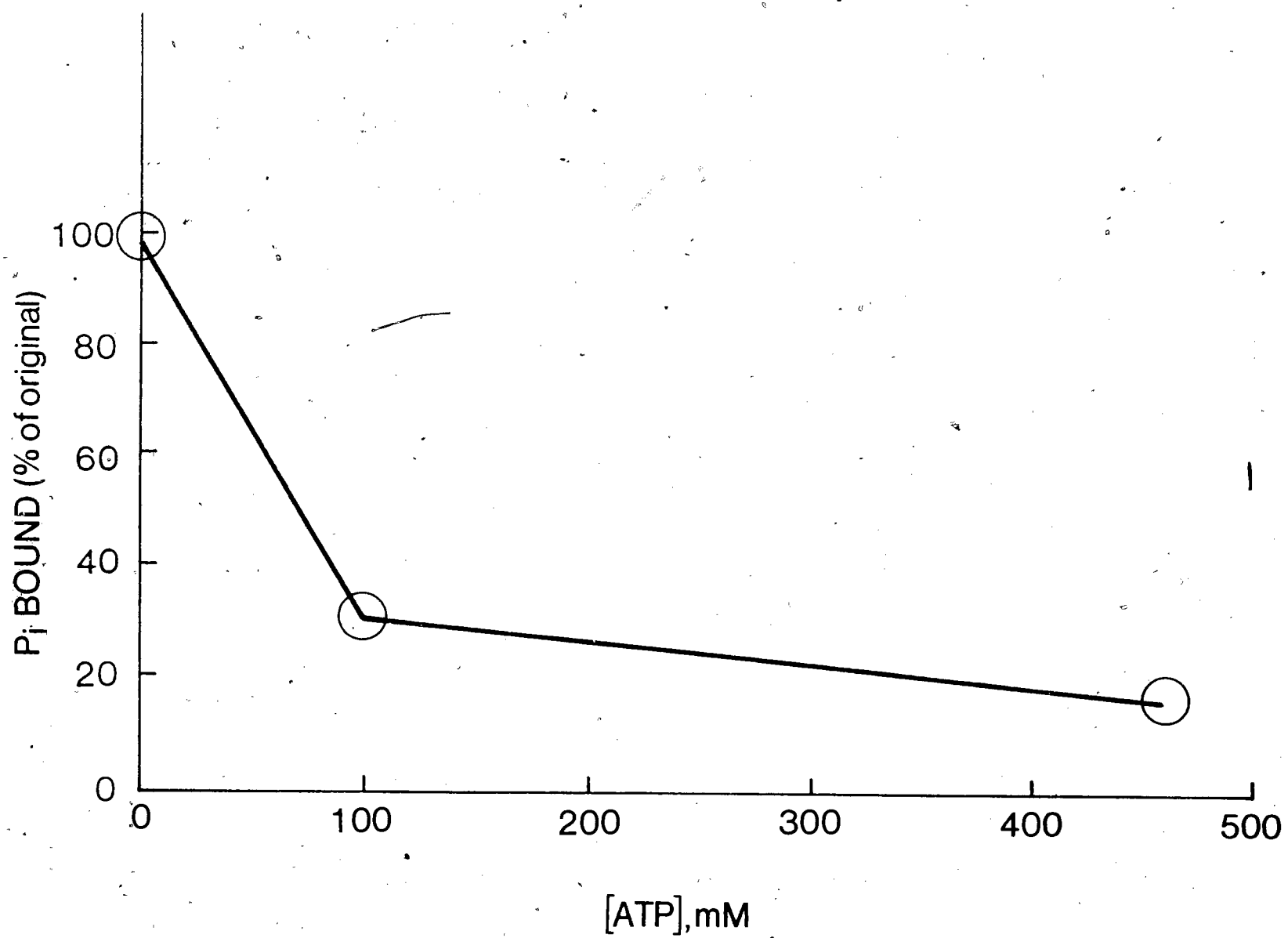

Pi BOUND (% of original)
100
80
60
40
20
0
0.5
1.0
1.5
2.0
LENGTH OF 100mM ATP EQUILIBRATED MID-SECTION GEL (cm)

Figure 4A

The effect of ATP on the release of bound P_i from F_1. The reassembled columns were prepared as described in Figure 1A. The conditions and concentrations were the same as described in Figure 2A, except that the length of the middle section was kept constant at 1.5 cm, and the ATP concentration was varied (0, 100, 458 mM) in the equilibration buffer of the middle gels. The specific activity of the $[^{32}P]P_i$ in the reaction mixture was 1.23×10^6 cpm/nmole. 150 µL aliquots of the reaction mixture were added to the reassembled columns. The 100% Pi bound corresponded to 0.18 mole Pi/mole F_1. Each point is the average of duplicate experiments.


100
80
60
40
20
0
P_i BOUND (% of original)
0
100
200
300
400
500
[ATP],mM

mum solubility of ATP was being approached. A comparison of Figures 2A and 4A shows that as the length of the ATP-containing middle section increased, so did the amount of P_i released from F_1.

Experiments with 2.0 cm ATP-containing middle sections gave results (not shown) similar to those obtained previously (Fig. 4A). Here even with the incresed length (2.0 cm) of the ATP-containing middle gel (300 mM ATP in the equilibration buffer), not all the bound P_i was released from F_1. Experiments were also performed with a longer ATP-containing mid-section gel (3.0 cm, 300 mM ATP in the equilibration buffer), and again not all the bound P_i was removed from F_1 equilibrated with F_1.

The reassembled columns were subjected to various centrifugal forces (from 420 × g to 1050 × g, i.e., Setting No. 2 to No. 5, I.E.C. Clinical Centrifuge, Rotor 221). The objective of this experiment was to allow longer exposure of the F_1 to ATP in the middle section. The experimental conditions and procedures were as described in Figures 1A and 2A, except that the 1.0 cm mid-section gel was equilibrated in buffer containing 100 mM ATP. For each speed of centrifugation investigated, the time of centrifugation was kept constant (2 minutes). It was found that centrifugations at 420 × g, 630 × g, and 840 × g produced gels which were difficult to handle, i.e., these gels were difficult to remove and reassemble without being broken. Centrifugations

of reassembled columns at 630 × g and 840 × g gave negligible amounts of centrifugate. The centrifugations at 1050 × g produced gels which were easy to work with, and with the reassembled columns gave enough centrifugate. Thus all centrifugations (preparation of gels and investigations with F_1) were at 1050 × g, hence speed of centrifugation was eliminated as a variable to increase the contact time between F_1 and ATP of the mid-section gel.

Determination of the Optimum Lengths of Gels for the Top and Bottom Sections of the Reassembled Columns

The top section of the reassembled column is to ensure that only P_i bound to F_1 reaches the nucleotide-containing mid-section gel. Figure 5A shows the results of an experiment with various lengths of buffer-equilibrated gels alone in the column (bottom gel and nucleotide-containing middle gel were not used). Evidently, as the lengths of the gels increased, the amount of label in the centrifugate decreased. Thus the length of the top section influenced the removal of free, loosely, and non-specifically bound label from the applied sample. It was concluded that a top gel of at least 2.5 cm was required. Table IA shows that the $[^{32}P]P_i$-equilibrated F_1 samples gave centrifugates with higher amounts of label than the $[^{32}P]P_i$-equilibrated buffer without F_1. The difference was due to the $[^{32}P]P_i$ bound tightly to F_1 which was not removed by the gel.

Figure 5A

The effect of different lengths of buffer-equilibrated gels on the removal of label from the reaction mixtures. The experimental conditions and procedures were the same as described in Figures 1A and 2A, except that no bottom gel nor ATP-containing gel was used. .80 μL aliquots of each reaction mixture (i.e. with and without F_1) was applied to each column. The specific activity of the $[^{32}P]P_i$ in each reaction mixture was 1.4×10^6 cpm/nmole, and the concentration of F_1 was 1.85 μM. A 25 μL aliquot of each centrifugate was counted to determine the amount of label that passed through the column. The 100% cpm of $[^{32}P]P_i$ that passed through the column corresponded to the amount of label in 25 μL of reaction mixture. Each experiment was performed in duplicate. (○ $[^{32}P]P_i$ equilibrated buffer, and □ $[^{32}P]P_i$ F_1-reaction mixture).

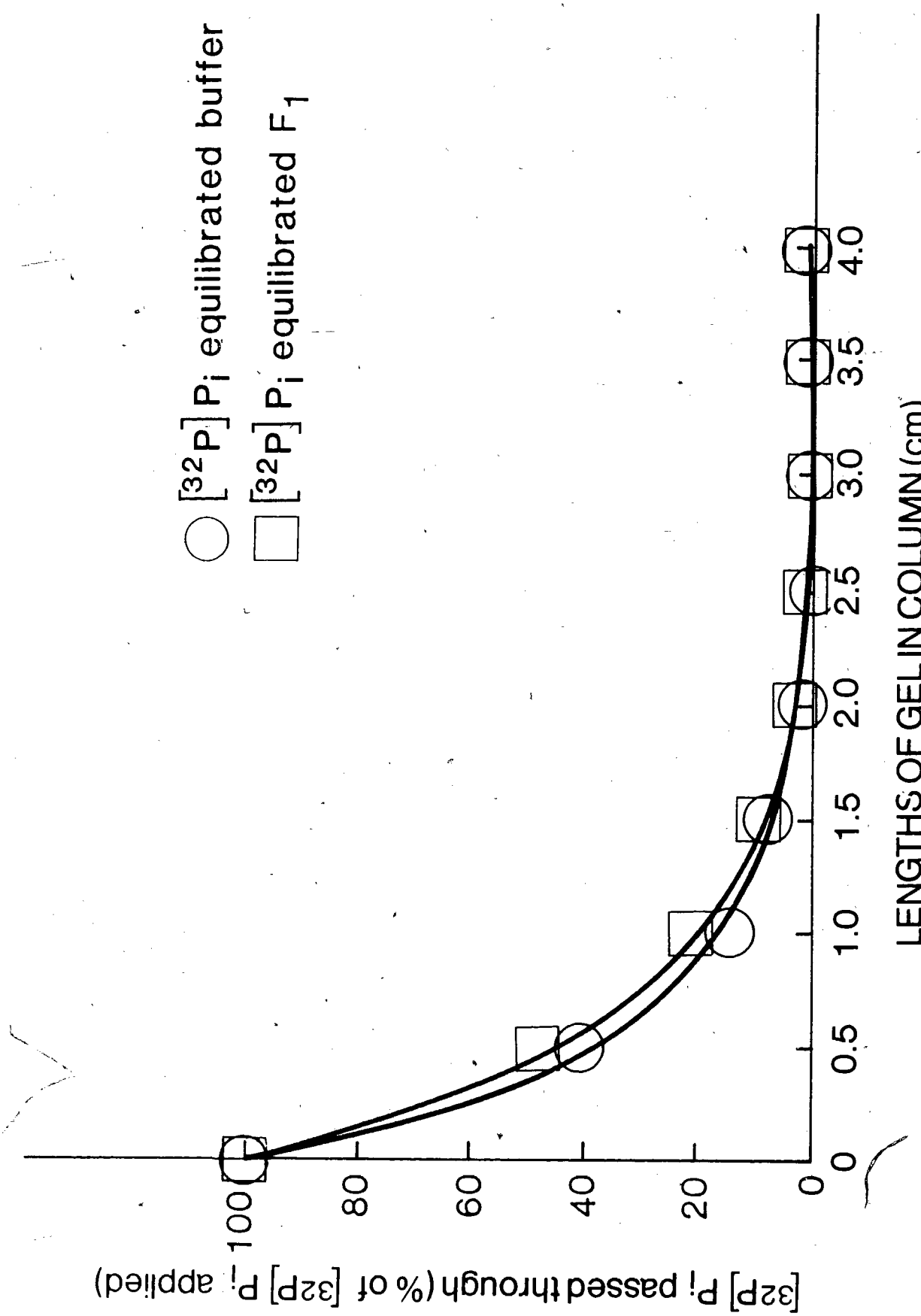

[32P] Pi equilibrated buffer
[32P] Pi equilibrated F1
[32P] Pi passed through (% of [32P] Pi applied)
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
LENGTHS OF GEL IN COLUMN (cm)

Table IA

The effect of different lengths of buffer-equilibrated gels on the removal of label from the reaction mixtures.

Length of Gel in Column (cm)	Sample Applied to Column	% cpm passed through column (25 μL counted)
0	P_i	100
0.5	P_i	40.48
	$P_i + F_l$	47.68
1.0	P_i	13.92
	$P_i + F_l$	20.9
1.5	P_i	7.48
	$P_i + F_l$	8.42
2.0	P_i	1.85
	$P_i + F_l$	2.02
2.5	P_i	0.38
	$P_i + F_l$	0.83
3.0	P_i	0.32
	$P_i + F_l$	0.65
3.5	P_i	0.13
	$P_i + F_l$	0.51
4.0	P_i	0.2
	$P_i + F_l$	0.48

Experimental conditions and procedures were as described in Figure 5A.

The bottom gel of the column is involved in the uptake of previously bound molecules which are released by F_1 during or after the binding of nucleotide from the middle section. Figure 6A and Table IIA show that as the total length of the gel in the column increased (i.e., as the length of the bottom section increased), the amount of P_i going through the column decreased. It was concluded that a minimum length of 2.0 cm was required for the bottom section, since with shorter bottom sections, the amount of label in the controls (i.e., samples without F_1) was high. Even with the maximum 3.0 cm bottom section, all the bound P_i was not removed from F_1; this suggested that a bottom section greater than 3.0 cm was needed to allow the complete removal of P_i from F_1. However, the length of the 1.0 mL tuberculin syringe which was used as the column barrel could not accommodate more than 6.5 cm total length of reassembled gels.

Use of a Longer Reassembled Column and the Determination of the Parameters under which the Column Functions Best

A longer column barrel was made (as described under Methods) to accommodate the optimum lengths of the three sections, viz.: (i) a 2.5 cm top gel, (ii) a 1.0 cm middle gel, and (iii) a 4.0 cm bottom gel. The 4.0 cm bottom section was the maximum that could be used without any alteration in the lengths of the other

Figure 6A

The effect of different lengths of bottom gels on the removal of label from the reaction mixtures. The experimental conditions and concentrations used were as described in Figure 3A, except that the lengths of the top and middle gels were 2.5 cm and 1.0 cm, respectively. The concentration of F_1 in the reaction mixture was 1.85 μM. For bottom sections 2.0 cm and shorter, the specific activity of the $[^{32}P]P_i$ in the reaction mixture was 1.42×10^6 cpm/nmole; and for bottom sections longer than 2.0 cm, the specific activity of the $[^{32}P]P_i$ in the reaction mixture was 1.35×10^6 cpm/nmole. Each point is the average of duplicate experiments. [○ % $[^{32}P]P_i$ in buffer which passed through, □ % $[^{32}P]P_i$ in F_1-reaction mixture which passed through, and △ ratio of mole P_i/mole F_1].

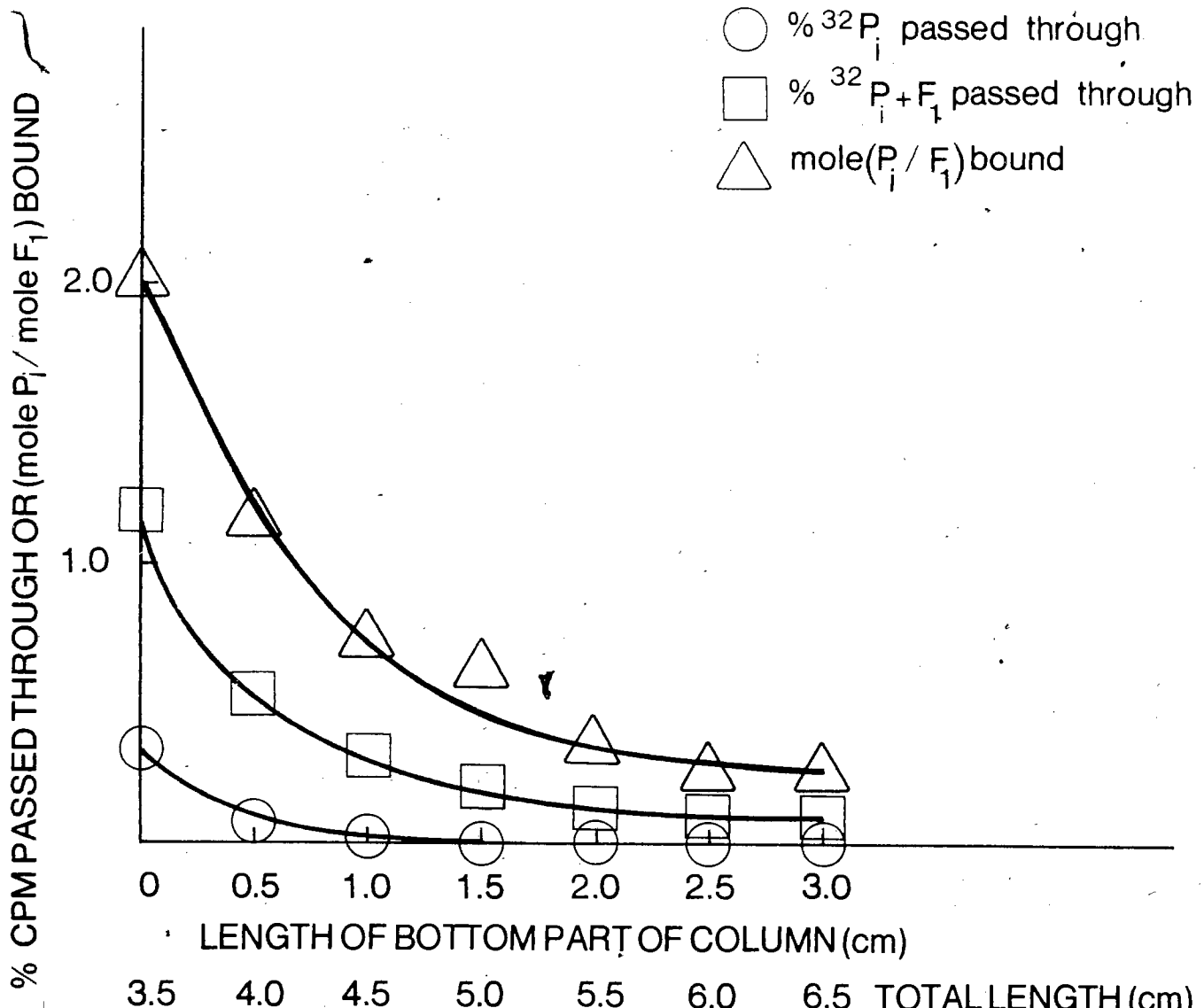

% $^{32}P_i$ passed through
% $^{32}P_i + F_1$ passed through
mole (P_i / F_1) bound
% CPM PASSED THROUGH OR (mole P_i / mole F_1) BOUND
2.0
1.0
0
0.5
1.0
1.5
2.0
2.5
3.0
LENGTH OF BOTTOM PART OF COLUMN (cm)
3.5
4.0
4.5
5.0
5.5
6.0
6.5
TOTAL LENGTH (cm)

Table IIA

The effect of the total length of the column on the removal of label from the reaction mixture.

Total Length of Column	Length of Bottom Section (cm)	Sample Applied to Column	Label in 25 μL Centrifugate (cpm)	% of label* in 25 μL Centrifugate	mole P_i / mole F_l
3.5	0	$^{32}P_i$	5716	0.34	
		$^{32}P_i+F_l$	19994	1.20	0.21
4.0	0.5	$^{32}P_i$	1115	0.07	
		$^{32}P_i+F_l$	8619	0.52	0.12
4.5	1.0	$^{32}P_i$	344	0.02	
		$^{32}P_i+F_l$	5184	0.03	0.07
5.0	1.5	$^{32}P_i$	188	0.01	
		$^{32}P_i+F_l$	3546	0.02	0.05
5.5	2.0	$^{32}P_i$	64	0.00	
		$^{32}P_i+F_l$	2209	0.13	0.03
6.0	2.5	$^{32}P_i$	24	0.00	
		$^{32}P_i+F_l$	1530	0.10	0.02
6.5	3.0	$^{32}P_i$	26	0.00	
		$^{32}P_i+F_l$	1661	0.10	0.02

* 100% label was the amount of label in 25 μL reaction mixture.

Experimental conditions and procedures were as described in Figure 6A.

two sections, because some space in the column barrel above the top gel was required to allow for the insertion of a cut 1.0 mL pipette tip. Through the tip the measured volume of the particular reaction mixture was added. It was found that adding the sample of the reaction mixture directly to the gel (immediately before centrifugation) caused the results to be somewhat less reproducible. Thus using the cut tip on top of the column, besides giving more reproducible results, allowed six columns to be prepared for simultaneous centrifugation.

An experiment was performed with the optimum lengths (2.5, 1.0, and 4.0 cm) of the three sections in the longer column barrel. The reaction mixtures and procedures were the same as described in Figures 1A and 6A, except that the volume of the reaction mixture applied to each via the cut pipette tip was 100 μL. The concentration of ATP in the buffer used in the preparation of the middle gel was 50 mM; and the concentration of F_1 and specific activity of the $[^{32}P]P_i$ in the reaction mixture were 1.28 $mg.mL^{-1}$ (or 3.7 μM) and 1.0×10^6 cpm/nmole, respectively. Without ATP in the middle section of the column, the experiment gave a ratio of 0.187 mole P_i/mole F_1. It showed that it was possible to remove almost all of the bound P_i from F_1. This was similar to the results seen earlier when 2.5 and 3.0 cm bottom sections were used (Fig. 6A). It was decided to

use the lengthened column barrel in future investigations of the bound species of F_1.

However, with the increased length of the reassembled gels in the column, the other variables were investigated to determine whether other changes were required. The parameters that were considered previously included: (i) the total length of the reassembled column, (ii) the different lengths of the three sections, and (iii) the centrifugal force and the time it was applied. Additional factors that had to be considered included: (i) the amount of F_1 applied to the column, (ii) the volume of the reaction mixture applied to the column, (iii) the amount of nucleotide and/or P_i in the middle section, (iv) the specific activity of the radiolabelled compounds used, and (v) the ionic composition, pH, and temperature of the equilibration buffers and gels.

Table IIIA shows the effect of using varying amounts of F_1 in the modified Sephadex centrifuge column technique. The volume of the F_1 reaction mixture added to the column was kept constant, whilst the concentration (mg protein.mL^{-1}) was varied. With ATP in the columns, no great difference in the amount of [^{32}P]P_i bound to F_1 was observed with the different concentrations of F_1 in the reaction mixtures (Table IIIA). Values of the concentrations of F_1 lower than 0.63 mg protein.mL^{-1} did not give reproducible results (these are not shown in Table IIIA);

Table IIIA

The effect of ATP on the release of P_i from F_1 when different amounts of protein were used in the reaction mixture.

F_1 ($mg.mL^{-1}$)	BSA ($mg.mL^{-1}$)	[ATP] in Column (mM)	Label in 20 μL Centrifugate (c.p.m.)	mole P_i / mole F_1
0	0	0	25	0
0.63	0	0	6600	0.18
0.63	0	50	70	0.002
0.63	0.63	50	300	0.008
0.95	0	0	9900	0.18
0.95	0	50	100	0.002
0.95	0.95	50	340	0.006
1.26	0	0	13000	0.18
1.26	0	50	80	0.001
1.26	1.26	50	200	0.003

The experimental conditions and procedures were as described in Figures 1A and 6A, except that in the reassembled column only optimum lengths (i.e., 2.5, 1.0, and 4.0 cm, respectively) were used. The concentration of ATP in the equilibration buffer of middle gel was 50 mM. The volume of reaction mixtures applied to the columns was 100 μL, and the volume of centrifugate counted was 20 μL. The specific activity of the $[^{32}P]P_i$ in the reaction mixtures was 1.0×10^6 cpm/nmole. The experiment was performed in duplicate.

whereas with 1.26 and 0.95 mg protein.mL^{-1} F_1 reaction mixtures, the results were somewhat more reproducible. It was decided that this lower concentration of F_1 (0.95 mg protein.mL^{-1}) would be used in subsequent studies. Table IIIA also shows the results when the total protein concentration (in each 100 μL volume applied to the column) was increased by the addition of the equivalent amount (mg protein.mL^{-1}) of bovine serum albumin (BSA). It was concluded that the BSA adsorbed variable and non-reproducible amounts of $[^{32}P]P_i$. Increased and reduced proportions (e.g. 10 and 0.1 fold, respectively) of BSA were added to the F_1 solutions with similar results (not shown). Thus the use of BSA in the F_1 reaction mixtures was avoided in subsequent studies.

An experiment similar to that described in Table IIIA was performed, except that no BSA was used and the concentration of F_1 in the reaction mixture used was 0.95 mg protein.mL^{-1}; and the specific activity of the $[^{32}P]P_i$ in the reaction mixtures was 4.3×10^5 cpm/nmole. In this case, however, different volumes (100, 125, 150, 175, and 200 μL) of the reaction mixtures (with and without F_1) were applied to the columns. It was found that 150 μL of reaction mixture was the minimum volume that gave adequate centrifugate for the analyses (bound label determination, assays for protein concentration and activity). In addition, 150 μL volume was easily accommodated by the cut 1.0 mL pipette tip ($\sim$200 μL capacity) placed on top of the column.

The assays for the concentration of protein and activity of F_1 in the reaction mixture and in the centrifugate gave similar values.

Conclusion

The results showed that an ATP-containing middle section in the column was able to influence the release of bound P_i from F_1. It was determined that the longer column with a 2.5 cm top, 1.0 cm middle, and a 4.0 cm bottom gel was the most appropriate. The most suitable volume of reaction mixture to be added to the column was 150 μL via a cut 1.0 mL pipette tip; and the protein concentration was about 1.0 $mg.mL^{-1}$, without the addition of BSA. All centrifugations with the longer column were performed at 1050 × g for 3 minutes at room temperature (20-23°C).

APPENDIX II

Adaptation of the Modified Sephadex Centrifuge Column Technique for Use in Pulse-Chase Experiments

The Arrangement of the Gels for Pulse-Chase Experiments

Figure 7A shows the arrangement of the reassembled gels in the pulse-chase mode of the modified Sephadex centrifuge column technique. 1.0 cm lengths were chosen for both pulse and chase sections, since this length provided sufficient time for F_1 to bind ATP from the 1.0 cm ATP-containing gel (see Appendix I and Results). 3.0 and 2.5 cm spacer or buffer-equilibrated gels were used after pulse and chase sections, respectively; and were selected since they were adequate to remove unbound label from F_1 (see Appendix I).

Determination of the Amount of ATP to Use in the Top or Pulse Section of the Column

The appropriate concentration of ATP to use in the equilibration buffer of the pulse gel was decided by the balance between the amount of ATP bound by F_1 from the pulse gel, and the amount of bound ATP that was removed from F_1 on exposure to chase ATP. The concentrations of ATP used in the chase gels were kept similar to those used in middle gels of the preincubation experiments in order to facilitate comparison of the results of the two modes of experimentation. Figure 8A shows the

Figure 7A

The arrangement of the gels in the modified Sephadex centrifuge column technique for pulse-chase experiments. The figure lists what occurs in each layer of the reassembled column. (Not drawn to scale.)

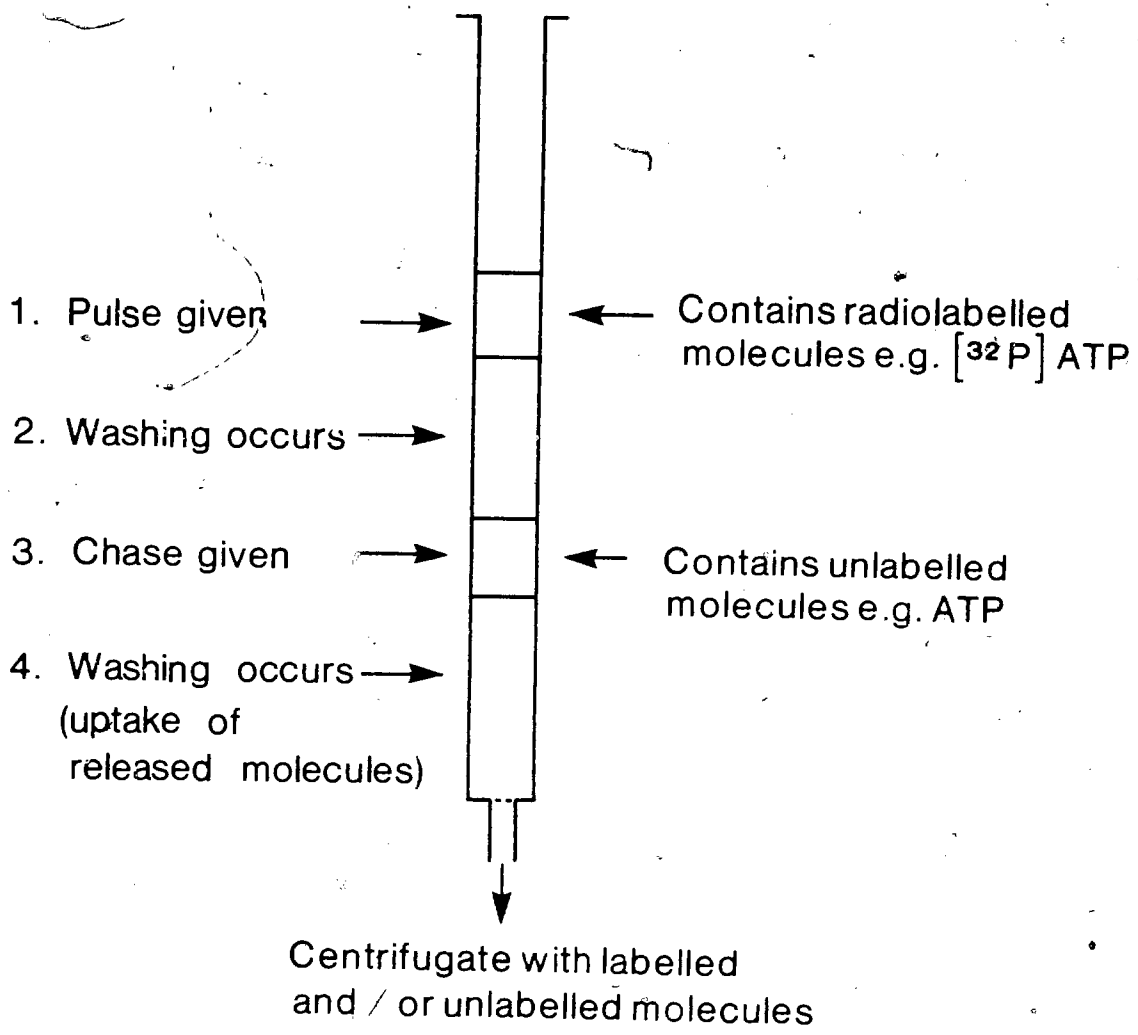
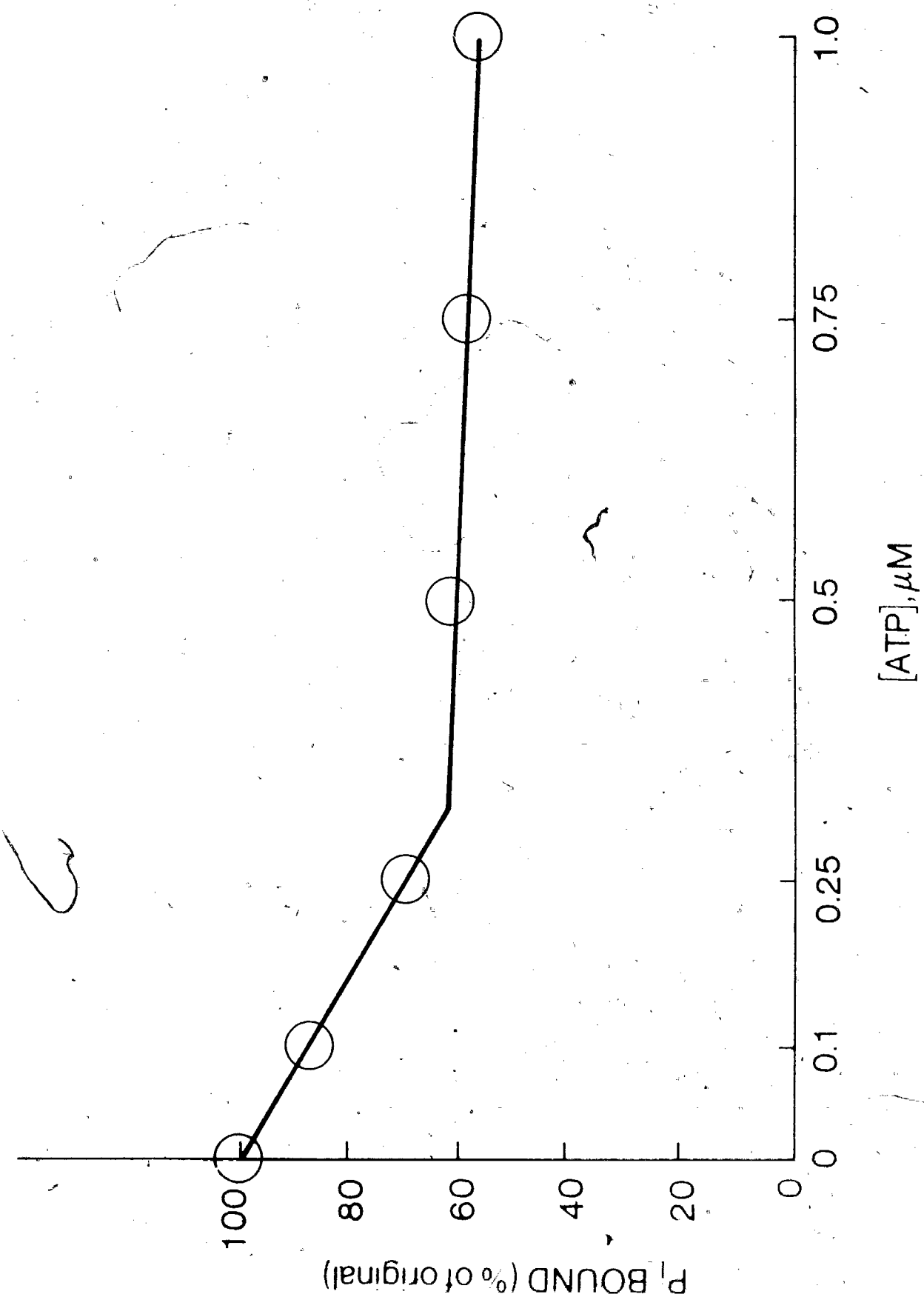

1. Pulse given
Contains radiolabelled molecules e.g. [32P] ATP
2. Washing occurs
3. Chase given
Contains unlabelled molecules e.g. ATP
4. Washing occurs (uptake of released molecules)
Centrifugate with labelled and / or unlabelled molecules

Figure 8A

The effect of chase ATP on the release of label bound when F_1 was passed through a 10 μM ATP pulse section. The experimental conditions and procedures were the same as those used in the modified Sephadex centrifuge column technique (Fig. 1), except that the gels were arranged as shown in Figure 7A. The lengths of the four sections, starting from the top of the column, were 1.0, 3.0, 1.0 and 2.5 cm, respectively. (Note that no $[^{32}P]P_i$ label was added to the reaction mixture.) The concentration of F_1 in the reaction mixture was 0.86 mg protein. mL^{-1} or 2.49 μM. The specific activity of the $[^{32}P]$ATP in the 10 μM ATP-containing buffer used to prepare the pulse gel was 3.4×10^4 cpm/nmole. The 100% P_i bound corresponded to a ratio of 0.15 mole P_i/mole F_1. The experiment was performed in triplicate.


P_i BOUND (% of original)
100
80
60
40
20
0
0
0.1
0.25
0.5
0.75
1.0
[ATP], μM

results of an experiment when 10 μM ATP was used in the equilibration buffer of the pulse gel. The results demonstrate that F_1 in passing through pulse gel bound ATP molecules, and that the release of these molecules (or hydrolysis products) was influenced by the chase ATP. However, less than half of the label bound in the pulse was released in the chase.

Figure 9A shows the results of an experiment in which the length of the chase section was 3.5 cm (i.e. the bottom 2.5 cm gel also contained ATP). The results were similar to those obtained previously (see Fig. 8A), thus even the increased number of chase ATP molecules and/or increased time of exposure to chase ATP molecules proved inadequate to remove all the label bound in the pulse. This prompted some experiments with different concentrations of ATP in the equilibration buffer of the pulse gel. The results of these experiments are summarized in Table IVA and plotted in Figure 10A. It was seen that with a 10 μM ATP pulse gel, the 1.0 μM ATP chase gel removed only about 42% of the bound ATP molecules. Secondly, with a 1.0 μM ATP pulse gel, the 1.0 μM ATP chase gel removed about 55% of the bound ATP. Lastly, with 0.1 and 0.5 μM ATP pulse gels, the amount of ATP bound in the pulse was too low to be useful in these pulse-chase studies. Thus, the best balance between the amount of ATP bound and ATP released was achieved whe 1.0 μM ATP equilibrated pulse gel was used.

Figure 9A

The effect of chase ATP on the release of label bound when F_1 was passed through a 10 μM ATP pulse section. The experimental conditions and procedures were the same as described in Figure 8A, except that the 2.5 cm bottom section also contained chase ATP (i.e. total ATP chase section was 3.5 cm). The concentration of F_1 in the reaction mixture was 1.16 mg protein. mL^{-1} or 3.36 μM. The specific activity of the [^{32}P]ATP in the 10 μM ATP-containing equilibration buffer of the pulse gel was 3.7×10^4 cpm/nmole. The 100% P_i bound corresponded to a ratio of 0.17 mole P_i/mole F_1. The experiment was performed in triplicate.

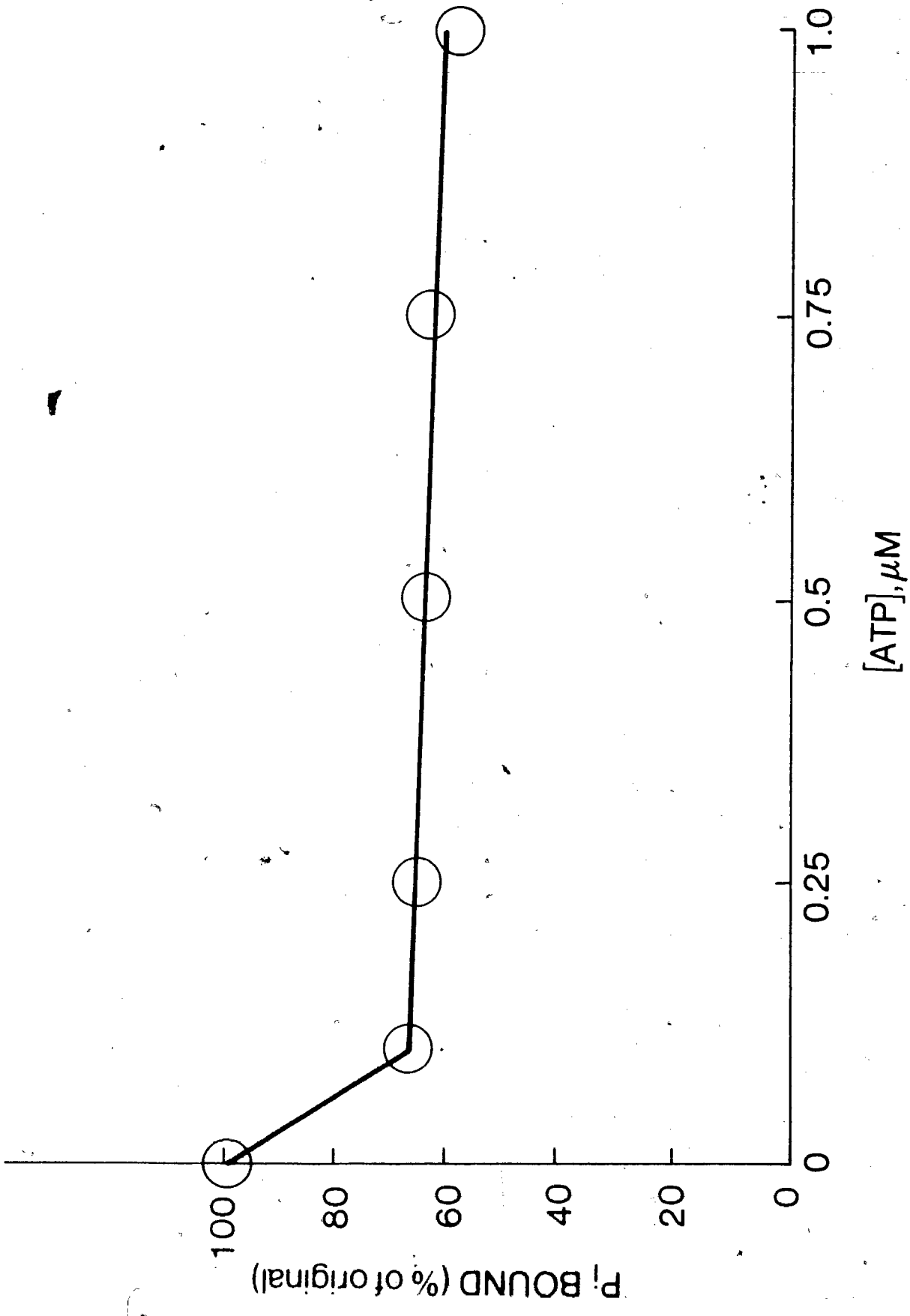

P_i BOUND (% of original)
100
80
60
40
20
0
0
0.25
0.5
0.75
1.0
[ATP], μM

Table IVA

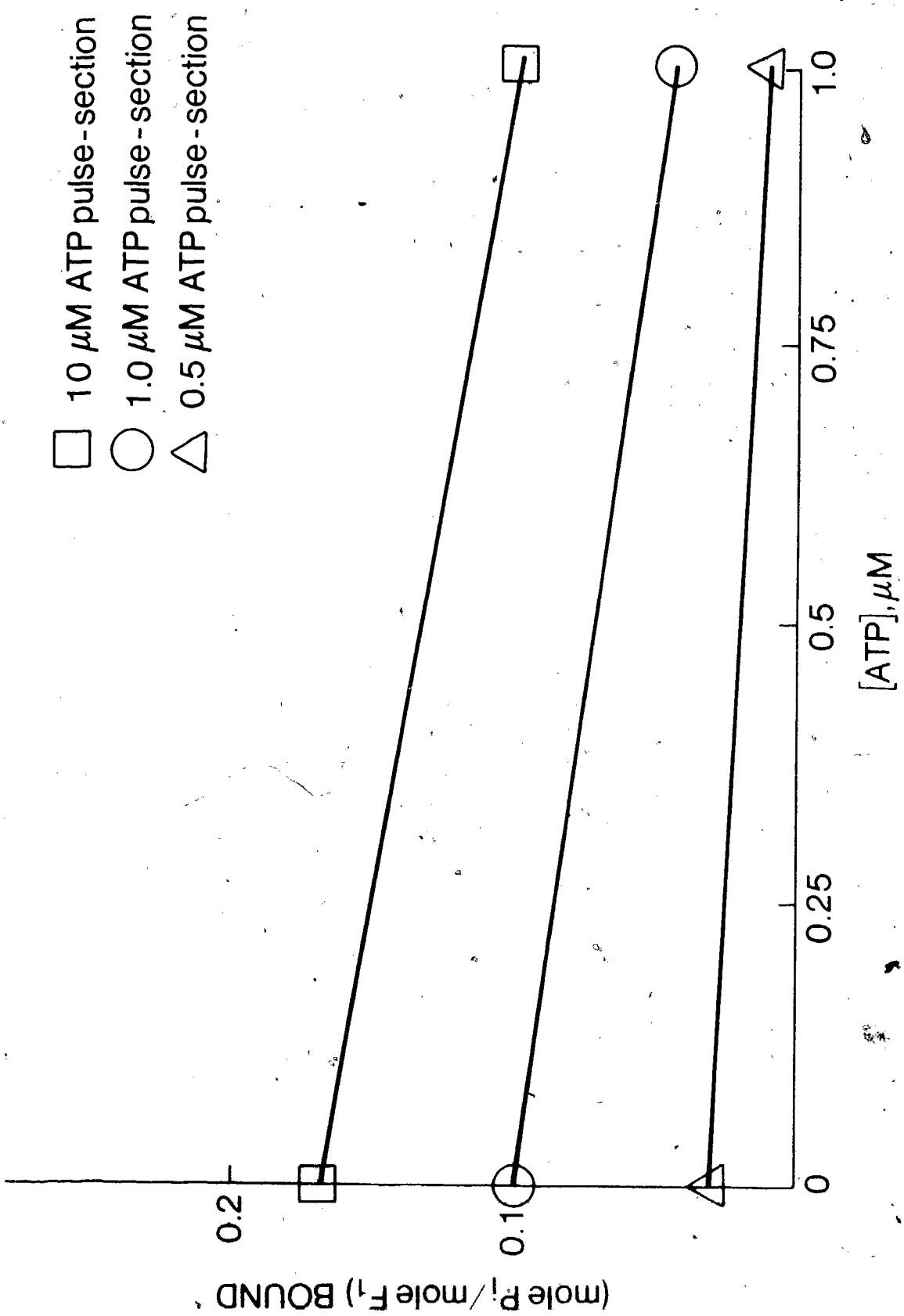
The effect of chase ATP on the release of label bound when F_1 was passed through pulse sections with different concentrations of ATP.

	Conc. of ATP in Buffer of Pulse Gel (μM)	Conc. of F_1 in Reaction Mixture ($mg.mL^{-1}$)	Specific Activity of [^{32}P]ATP in Reaction Mixture (cpm/nmole)	Conc. of ATP in Buffer of Chase Gel (μM)	mole P_i / mole F_1
1.	10	1.16	3.7×10^4	0	0.17
				1	0.10
2.	1	0.85	4.87×10^5	0	0.1
				1	0.04
3.	0.1	0.85	4.28×10^6	0	0.03
				0.1	0.03
4.	0.5	0.85	1.51×10^6	0	0.03
				1	0.01

The experimental conditions and procedures were the same as outlined in Figure 9A. The data are plotted in Figure 10A. Each experiment was performed in triplicate.

Figure 10A

The effect of chase ATP on the release of label bound when F_1 was passed through pulse sections with different concentrations of ATP. The experimental conditions and procedures were the same as described in Figure 9A. Other experimental details are given in Table IVA. (□ 10 μM ATP; ○ 1.0 μM ATP, and △ 0.5 μM ATP used in the buffer to prepare the pulse section.)


10 μM ATP pulse-section
1.0 μM ATP pulse-section
0.5 μM ATP pulse-section
(mole Pi/mole F1) BOUND
0.2
0.1
0
0.25
0.5
0.75
1.0
[ATP], μM

Conclusion

The rearrangement of the gels (1.0 cm pulse - 3.0 cm spacer - 1.0 cm chase - 2.5 cm bottom) in the column, and use of the appropriate concentration of ATP (1.0 μM) in the equilibration buffer of the pulse gel, allowed the adaptation of the modified Sephadex centrifuge column technique for pulse-chase studies. Note that except for the substitution of a 1.0 cm pulse section for the top 1.0 cm of the top spacer section, the experimental procedures and conditions were the same as those used in the preincubation mode.

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