STUDIES ON SODIUM AND POTASSIUM ION ACTIVATED ADENOSINE TRIPHOSPHATASE

BY

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ABSTRACT

The Na^+K^+-activated ATPase was obtained from the rectal glands of dog fish. Optimum conditions for extraction of the membrane bound enzyme into solution and stabilization by glycerol was investigated, followed by attempts to purify the enzyme by affinity chromatography using insolubilized Concanavalin A, wheat germ agglutinin and a modified ATP molecule as affinity ligands. The structure and size of the enzyme as a macromolecule was characterized by a combination of the results from velocity sedimentation in an isokinetic sucrose gradient and gel filtration with Sepharose 6B. The sedimentation coefficient was found to be 5.0 S by velocity sedimentation, and the Stokes' radius was found to be 114 Å by gel filtration. Using these data, the molecular weight of the intact enzyme was estimated to be 240,000. This is in good agreement with the molecular weight derived from sodium dodecyl sulfate gel electrophoresis obtained by other workers.

CONTENTS

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ABBREVIATIONS

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
NAD	Nicotinamide adenine dinucleotide
Na^+K^+-activated ATPase	Sodium and potassium ion activated adenosine triphosphatase
P	Inorganic orthophosphate
E-P	Phosphorylated sodium and potassium ion activated adenosine triphosphatase

INTRODUCTION

INTRODUCTION

PART I Characteristics of Na^+K^+-activated ATPase

Under normal physiological conditions, concentration gradients of Na^+ and K^+ exist across the cell membrane. $[Na^+] \approx 140$ mM and $[K^+] \approx 5$ mM in human blood plasma, compared to $[Na^+] \approx 10$ mM and $[K^+] \approx 150$ mM in the cytoplasm, depending on the type of the cell. These cation gradients are required for a variety of functions of the cell. The high K^+ concentration inside the cell is required for protein synthesis by ribosomes and also for maximal activity of pyruvate kinase. The Na^+ concentration gradient provides the energy required for the active transport of amino acids and sugars into the cell. Moreover the combined cation gradients generate an electrochemical potential difference which may be as high as 100 mV in muscle and nerve cells. The potential difference, which can actually be measured in giant squid axons, plays an important part in the transmission of nerve impules (1).

The existence of such cation gradients implies that there is a 'cation pump', presumable suituated in the cell membrane, which functions to transport Na^+ out of and K^+ into the cell. Experiments with resealed red blood cell ghosts provide evidence that ATP is the energy source (2). In 1957 an important breakthrough opened the molecular basis for the understanding of the cation pump to biochemical experimentation. Skou (3) discovered that a fraction of homogenized crab nerve known

to contain cell membrane fragments exhibited Mg^+-dependent ATPase activity that was greatly stimulated when both K^+ and Na^+ were added. Skou suggested that this cell membrane fraction contained an enzyme which is reponsible for maintaining the Na^+ and K^+ concentration gradients. His suggestion is strongly supported by the fact that both cation transport and the enzyme activity require ATP, and inhibition of cation transport also led to inactivation of enzyme activity (4,5).

This enzyme, referred to as Na^+K^+-activated ATPase, is ubiquitous. It has been found in many tissues of mammals, such as brain, muscle and red blood cells in different amounts (6). However, the richest sources of Na^+K^+-activated ATPase are specialized organs, either for generation of electrical potential, such as the electroplax of electric eels, or for the extrusion of excess Na^+, such as the kidney cortex of mammals (7), the salt gland of sea gulls (8), and the rectal gland of dog fish (9), which is the source used in this study. The rectal gland of dog fish has been shown to secrete a fluid into the fish gut with a sodium concentration as high as 540 mM, 100 mM higher than that of sea water (10). Its function is to excrete excess sodium chloride accumulated through ingestion.

To date, the research on Na^+K^+-activated ATPase has been concentrated in the following five main areas:

(1) Mechanism of catalysis and cation transport

(2) Interaction with cardioactive steroids

(3) Purification

(4) Subunit structure and size

(5) Lipid requirement

(1) Mechanism of catalysis and cation transport

It is generally agreed that Na^+K^+-activated ATPase hydrolyses ATP in a stepwise fashion, each step requiring different cation(s).

$$E_1 + ATP \xrightleftharpoons{Mg^{2+}\ Na^+} E_1\text{-}P + ADP \qquad 1$$

$$E_1\text{-}P \xrightleftharpoons{Mg^{2+}} E_2\text{-}P \qquad 2$$

$$E_2\text{-}P + H_2O \rightleftharpoons E_2 + P \qquad 3$$

$$E_2 \rightleftharpoons E_1 \qquad 4$$

More elaborate and sophisticated schemes have been proposed (19), but they are essentially modification and refinement of the above. E-P represents the phosphorylated enzyme. E_1 and E_2 represent a lower and a higher energy conformation of the enzyme molecule, respectively (20,81). These partial reactions were established by binding studies and substantiated by kinetic data (20,21). It was found that ^{32}P was incorporated from $[\gamma^{32}P]ATP$ into Na^+K^+-activated ATPase preparation in the presence of Na^+ and Mg^{2+}, this corresponds to partial reaction 1 and 2. If K^+ was added to the enzyme system after Na^+, the ^{32}P in the enzyme was discharged, this corresponds to partial reaction 3 (22,23). The eventual goal of these studies is to elucidate the mechanism of cation transport based on the partial reactions. At the moment, the mechanism of cation transport is poorly understood.

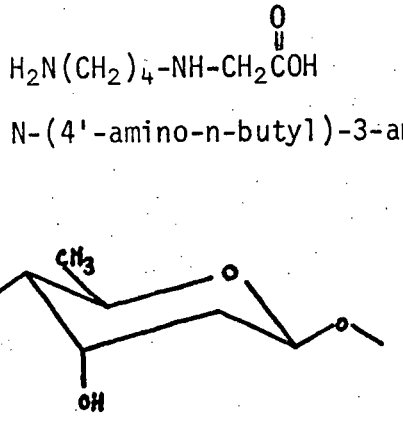
Recently, magnetic resonance techniques were applied to deal with this problem. Noggle et. al found that the spin lattice relaxation

time T_1 of ^{23}Na in a solution containing the enzyme decreased upon the addition of ATP, which indicates the binding of Na^+. When K^+ was added, T_1 increased, which indicates the displacement of Na^+ by K^+ (25). Mildvan et. al, using electron paramagnetic resonance and water proton relaxation spectra, showed that Mn^{2+} can substitute for Mg^{2+} at one tight binding site on the enzyme. When inorganic phosphate was added in the presence of Na^+, the relaxation time T_1 for water proton was increased. It was concluded that this increase was due to the displacement of a rapidly exchanging water proton from the coordination sphere of Mn^{2+} by the binding of phosphate to the active site. Furthermore, this increase in T_1 was almost abolished by changing the pH from 6.1 through the second ionization of inorganic phosphate to 7.5. This suggested the binding of phosphate in the presence of Na^+ is much more favored when it is a monoanion. The opposite effect was observed for K^+, which suggested the binding of phosphate in the presence of K^+ is more favored when it is a dianion. Based on these data, a transport mechanism was postulated in which Na^+ and K^+ bind to the same site, i.e. the acylphosphate of the enzyme. The binding of one excludes the binding of the other. Na^+ is transported out of the cell as a protein complex, stabilized by the phosphate monoanion, then K^+ is transported into the cell as a protein complex, stabilized by the phosphate dianion (24). Coordination of the metal ion by phosphate occurs in both cases, but via different modes. This postulate requires that the cation binding site be very close to the site for ATP hydrolysis. This was substantiated by further experiments in which the T_1 of ^{205}Tl, which as Tl^+, substitutes

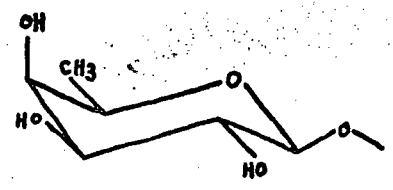
for K^+, was decreased by the paramagnetic relaxation of Mn^{2+}, but only in the presence of the enzyme (46).

(2) Interaction with cardioactive steroids

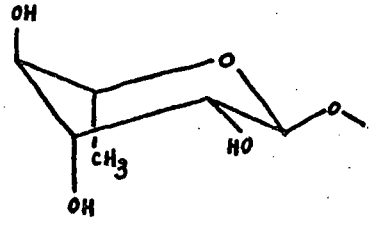
The cardioactive steroids are a class of compound useful for treatment of heart failure. Their structures are shown in Fig. 1. Much research has been directed to studying the binding and inhibition of Na^+K^+-activated ATPase by cardioactive steroids because of these pharmaceutical implications. The inhibition is highly specific, and no other enzyme systems have been found to be sensitive to these steroids. This is very important in the search for Na^+K^+-activated ATPase activity. If the enzyme activity is inhibited by a cardioactive steroid, then the activity is due to Na^+K^+-activated ATPase, otherwise it is due to other ATPase activity, such as Ca^+-activated ATPase. This specificity led to the speculation that Na^+K^+-activated ATPase is the pharmacological receptor for these drugs and that their effect on the enzyme are responsible for their therapeutic and toxic effects (74). The steroid moiety is classified into four types: digitoxigenin, digoxigenin, ouabagenin and strophanthidin as shown in Fig. 1. A cardioactive glycoside is formed when one or more sugar residue is linked to the 3β hydroxyl group of the steroid. ATPase inhibition is essentially irreversible at physiological pH (26). As shown in Table I, the dissociation constant of the enzyme-inhibitor complex is approximately 10^{-9} M but



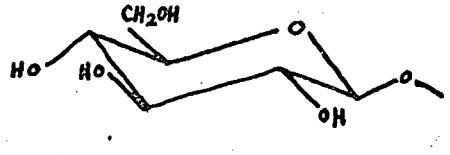
Digitoxide



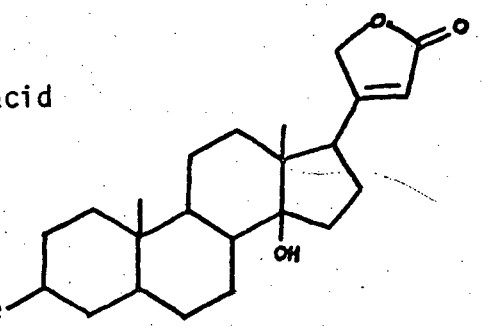
D-fucoside



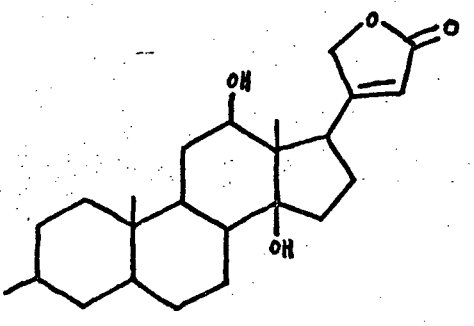
L-rhamnoside



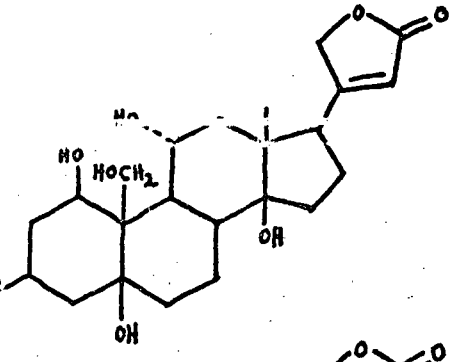
D-glucoside



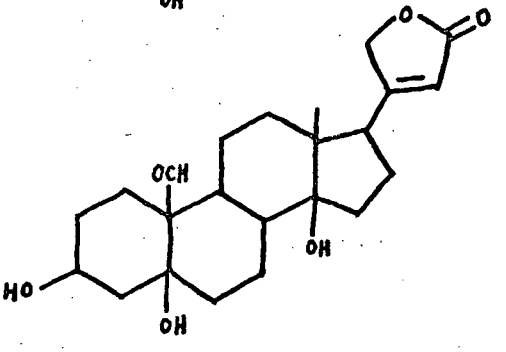
Digitoxin 1



Digoxin 2



Oubain 3



Strophanthidin 4

Fig. 1 Structure of cardioactive glycosides

the actual value depends on the concentrations of Na^+, K^+, and ATP. A stoichiometry of close to 1 : 1 binding was reported in most cases.

TABLE I

Source	Inhibitor	Apparent Dissociation Constant	Stoichiometr Inhibitor : enzyme
Outer medulla of dog kidney cortex (27)	N-(4'-amino-n-butyl)-3-amino acetyl-strophanthidin 4	1.92×10^{-9}M	0.8-1.2
Calf heart (28)	digoxin 2	-	0.9
Electroplax of electric eels (26)	ouabain 3	-	0.5
Cat brain (26)	ouabain 3	-	1.0

Both the rate and equilibrium constant of binding are increased by Na^+ and decreased by K^+. Although K^+ retards the binding, it cannot, nevertheless, reverse it (26,27,28). The mechanism of binding is still not understood. It has also been shown that ouabain inhibits transport (and by inference the Na^+K^+-activated ATPase) only by binding ATPase exposed on the external surface of the cell membrance (29).

The dissociation rate constant of the enzyme-inhibitor complex depends on the conditions for binding. It was found that the complex formed in the presence of Na^+, Mg^{2+} and ATP (type I) dissociates faster than the corresponding complex formed in the presence of Mg^{2+} and phosphate (type II). The dissociation rate constants of various type I complexes with different steroid moiety indicates that the stability of type I complex increases in the order : digitoxigenin glycoside <

strophanthidin glycoside < digoxigenin glycoside and strophanthidin glycoside < ouabagenin glycoside. If the steroid moiety is the same, the stability of the glycoside increases in the order digitoxide ≃ 6-deoxyglucoside ≃ fucoside < rhamnoside. It was concluded that the 2'α and 3'α or β-hydroxyl groups of the sugar moiety stabilize the binding through hydrogen bonding (47).

(3) Purification

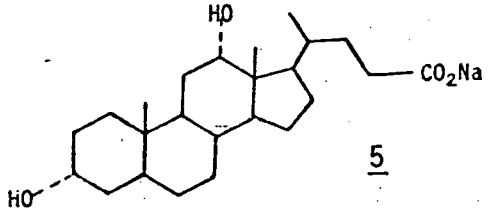
Several workers have purified the enzyme to different extents from a variety of sources. Table II summarizes some examples. All of them utilize classical protein purification techniques. The complete purification takes from four days to one week, depending on the method. Most procedures start with differential centrifugation. Thus centrifugation of a suspension of homogenized tissue at increasing speed will sediment membrane fragments of decreasing density. Membrane fragments of different density may contain different amounts of the enzyme, and this allows those membrane fragments richest in Na^+K^+-activated ATPase activity be selected. Sodium or potassium iodide treatment is also commonly used. Such treatment is called chaotropic extraction and results in the removal of loosely bound membrane proteins from the membrane, leaving the membrane relatively richer in the more strongly bound Na^+K^+-activated ATPase.

The Na^+K^+-activated ATPase is very tightly bound to the membrane. Since most purification techniques are designed to be carried out in

TABLE II

Source	Procedure	Final yield mg of protein/ g of wet tissue	Specific Activity µmoles P/ mg protein/h
Outer medulla of dog kidney cortex (7)	(i) Differential centrifugation 'microsomes' collected at 50,000 x g (ii) Microsomes washed with .06% deoxycholate at low ionic strength (iii) KI treatment of microsome (iv) Microsomes washed with .06% deoxycholate at 160 mM KCl (v) Microsomes extracted with .6% deoxycholate at 267 mM KCl (vi) Sepharose 2B gel filtration enzyme collected in void volume	0.46/150	800
Outer medulla of dog kidney cortex (13)	(i) Differential centrifugation 'microsomes' collected at 30,000 x g (ii) NaI treatment of microsomes (iii) Solubulization with .35% deoxycholate (iv) Glycerol precipitation (v) Solubulization with .35% mixture of 3:1 deoxycholate-cholate (vi) Ammonium sulfate precipitation	17/100	1,552
Rectal gland of dog fish (11)	(i) Differential centrifugation 'membranes' collected at 23,500 x g (ii) Membranes extracted with 1.6% Lubrol WX (iii) Zonal centrigugation with 2 to 22% linear sucrose gradient (iv) Ammonium sulfate precipitation	19.3/15	1,510
Electroplax of electric eels (16)	Essentially the same as above 1% Lubrol was used instead of 1.6%	24.5/405	1,260

aqueous phase, an important step in any purification is the extraction of the enzyme from the membrane into aqueous solution by a detergent. Two detergents frequently employed are sodium deoxycholate 5 and Lubrol WX.



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Sodium deoxycholate is a steroid secreted from the bile for emulsification of ingested lipids to facilitate absorption. Lubrol WX, a commercial detergent, is a polyoxyethylene ether of the general formula $R(OCH_2CH_2)_nOH$ where n averages 6. Once detergent extracted, the enzyme is very unstable and quickly loses its activity. Precipitation by ammonium sulfate as the final step not only improves the purification but also allows the enzyme to be stored in a physical state in which it is more stable. One criterion for assessing the purity of a preparation of the enzyme is the specific activity, expressed as the number of moles of phosphate produced by the hydrolysis of ATP per milligrams of total protein per hour. As shown in the last column of Table II, the specific activity of the highly purified enzyme, irrespective of the source or method of purification, is approximately 1,500 μmoles/mg/hr.

(4) Subunit structure and size

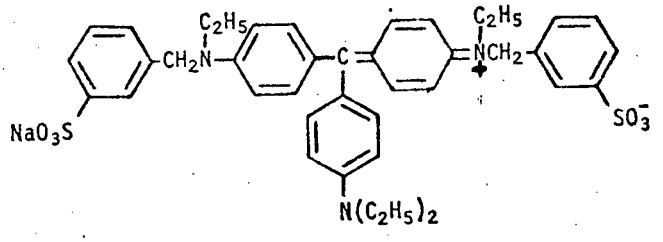
The subunit structure of purified Na^+K^+-activated ATPase has been investigated by many workers, most of them relying on the results

from sodium dodecyl sulfate gel electrophoresis. In this method, proteins are separated into their subunits by reduction of any intramolecular disulfide bonds using β-mercaptoethanol or dithioerythritol followed by denaturation by coating the protein with the anionic detergent sodium dodecyl sulfate. The proteins are then electrophoresed in a polyacrylamide gel. The R_f value of the subunit has a linear relationship with the molecular weight (14). With the highly purified Na^+K^+-activated ATPase, two bands are always found, regardless of the source. This indicates that the enzyme consists of two different subunits with different molecular weights. The actual values of the subunit molecular weight depend on the source, but are consistently found to be approximately 100,000 and 50,000, as can be seen in Table III

TABLE III

Source	Molecular Weights Large subunit(α)	Small subunit(β)	Molar ratio	Subunit Structure
Outer medulla of dog kidney cortex (15)	84,000	57,000	1 : 1	αβ
Outer medulla of dog kidney cortex (13)	89,000	56,000	1 : 1	αβ
Rectal gland of dog fish (11,48)	97,000	55,000	2 : 1	$\alpha_2\beta$
Electroplax of electric eel (16,48)	93,500	47,000	2 : 1	$\alpha_2\beta$
Ox brain (12)	94,000	53,000	-	-

The larger subunit was found to be the one that binds cardioactive steroid (17) and also contains the active site for phosphorylation by ATP (12,18). The smaller subunit was found to be a glycoprotein (11,13,15). The molar ratio for the large and small subunit was also estimated. The ratio of the intensity of the two stained bands in the gel electrophoresis experiment was taken as the mass ratio, this combined with the molecular weights, gave the molar ratio as shown in Table III. There is a clear discrepancy in this molar ratio (large subunit to small subunit) between results obtained from different sources. The discrepancy is open to several interpretations: (i) The glycoprotein, or the small subunit, may not be an integral part of the enzyme. This is unlikely because none of the different purification methods applied on Na^+K^+-activated ATPase from different sources could eliminate it. (ii) The estimation of the relative intensity of the two stained bands may not be quantitative enough. Staining after electrophoresis of the gels is accomplished with a dye known as coomassie brilliant blue 6 .



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In slightly acidic media, the dye-anion is electrostatically attracted to the charged cationic groups of the protein. The protein-dye complex has an absorption maximum at 549 nm. Background staining of the blank gel is high, and has to be removed with destaining solutions. Then the gels are scanned for the absorbance of the dye. Protein bands appear as peaks in a plot of absorbance verses distance along the gel. Integration

of the areas under the peaks then yields the mass ratio. Fazekas et. al have carefully analysed the quantitative aspect of the staining process (49). Deviation from Beer's Law was observed when absorbance was above 0.8 . Since the amount of staining depends on the number of exposed cationic groups, different peptides containing different amounts of cationic residues will be stained to different extends even when Beer's Law is obeyed. This was indeed observed. For equal weights of five different proteins, the absorbances deviated as much as ±21 % of the mean. Furthermore, the coomassie blue-protein complex dissociated appreciable during destaining even under carefully controlled conditions. This leads to an uncertain systemic error over and above the random deviation due to the difference in the distribution of cationic residues. Although this analysis was made on cellulose acetate electrophoretic strips, similar deviation has been reported for polyacrylamide gel electrophoresis (50). It has also been observed that the presence of fatty acids in a preparation of membrane protein can influence the intensity of staining (51). Base on these considerations, the ratio of the intensity of the coomassie blue stain of the large subunit to small subunit is at best a measure of the ratio of cationic amino acids of the two subunits, rather than their mass ratio. (iii) It is also possible that the discrepancy is real, due to species difference, but there is not enough data on other species to substantiate this.

If the small subunit is an integral part of the enzyme, then the minimum molecular weight would be 150,000 for an $\alpha\beta$ enzyme, and 250,000 for an $\alpha_2\beta$ enzyme.

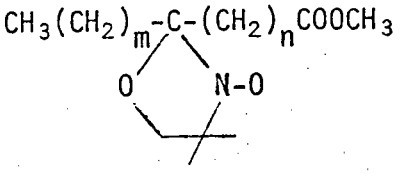
The possibility of the large and small subunits acting as an ionophore has also been investigated. An ionophore was defined as the material which caused an increase in the conductance of a black lipid membrane (52,53), and consequently centres our attention on the mechanism of cation translocation. In the experiments either a tryptic digest of the small subunit or a mixture of the large and small subunit were able to act as a Na^+ specific ionophore. The conductance of the black lipid membrane doped with the protein being highest when the molar ratio of the large subunit to the small subunit was 1 : 2. The results were interpreted to mean that the Na^+ specific site is in the small subunit and the ratio of the subunits in the intact enzyme is that which maximized the conductance of the black membrane, ie. $\alpha\beta_2$ (54). However, it should be noted that the subunits used have been denatured by sodium dodecyl sulfate and were no longer active enzymatically.

(5) Lipid requirement

Lipids were found to be required for Na^+K^+-activated ATPase activity. Removal of lipids with organic solvent leads to inactivation, but activity can be restored by adding back the extracted lipids, or new lipids with cholesterol (30). Treatment with phospholipases also leads to inactivation. A variety of phospholipids, including phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine can reactive the enzyme. It is believed that

phosphatidylserine is the phospholipid required for activity under physiological condition (31,32,33).

Using a nitroxyl spin labelled fatty acid ester 7, Barnett (55)



7 n = 3,4,5,6 or 10
m+n = 14 or 15

correlated the enzyme activity with the mobility of the membrane phospholipid. Arrhenius plots of both mobility and enzyme activity changed slope at the same "critical" temperature of 20°C. This was interpreted to indicate that enzyme activity increases with increasing lipid mobility. At the critical temperature, the lipid undergoes a phase transition to a more fluid state which lowers the activition energy for enzyme catalysis, probably by allowing more freedom for the enzyme to achieve the necessary conformation change. Papahadjopoulos (56) observed that activation by phosphatidylglycerol depends on the fatty acid side chains to which the phosphatidylglycerol was esterified. Both the critical temperature obtained from the Arrhenius plot of enzyme activity, and the phase transition temperature of phosphatidylglycerols (from differential scanning calorimetry) increase with increasing chain length of the phosphatidylglycerol, as expected. However, although the critical temperature and the transition temperature are similar, they are not exactly equal, and one can be higher or lower than the other, depending on the particular phosphatidylglycerol. It appears that the relationship between critical temperature as detected

by the Arrhenius plot of enzyme activity and the phase transition temperature detected by calorimetry is not straightforward. During phase transition, lipids may be separated into different domains with different extents of fluidity. Calorimetry measures the 'average' phase transition temperature of the bulk sample, while the enzyme may be concentrated in a domain with a higher or lower degree of disorder.

PART II Affinity Chromatography

The purification of Na^+K^+-activated ATPase by conventional methods has been described in Part I of this introduction. These procedures are lengthly and often involve the use of techniques requiring expensive instrumentation such as zonal centrifugation. This drawback is not unique, it is a common problem in many purification methods. The reason for this is the lack of specificity of the technique for the protein to be purified.

Affinity chromatography is a more modern purification technique which is designed to be highly specific for the protein to be purified. It makes use of the specific interaction of the protein with some other molecule. These specific interactions include such examples as the affinity between enzyme and substrate, enzyme and inhibitor, receptor and toxin, and protein and carbohydrate (82,83). The protein to be purified is passed through a column containing a cross linked polymer or gel to which a ligand molecule specific for the protein has been covalently attached. All proteins without substantial affinity for the bound molecule will pass directly through the column, whereas one that recognizes the bound molecule will be retarded in proportion to its affinity constant. Elution of the bound protein is achieved by changing the salt concentration or pH, or by elution with another bindable molecule to

dissociate the protein from the ligand. The successful application of this method depends on a number of factors : (1) A suitable stationary phase or matrix, (2) a ligand molecule which can specifically bind the protein, (3) a method to covalently link the ligand to the matrix, (4) condition of binding of protein to affinity column and (5) a method to elute the bound protein.

(1) Stationary matrix

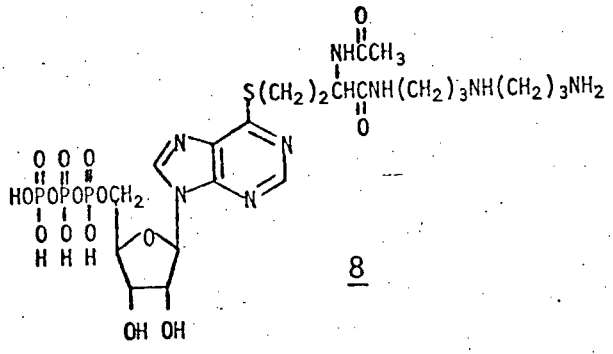
This should be an inert material which shows minimum interaction with proteins in general; it must form a loose, porous network that permits easy entry and exit of macromolecules. Its chemical structure must permit extensive covalent attachment of the specific ligand. Agarose gel, polyacrylamide beads and glass beads have been used for this purpose (57,58). An agarose gel in common use is Sepharose, a commercially available gel containing 2-6 % agarose.

(2) Ligand

The ligand molecule may be an inhibitor, a substrate analog, a cofactor for an enzyme, an agonist or antagonist for a receptor protein or it can be a haemagglutinating agent for a glycoprotein (64,70). Table IV summarizes some examples.

TABLE IV

Protein to be purified	Ligand	Method of elution
Nicotinic acetylcholine receptor (59)	Cobratoxin	hexamethonium chloride
Acetylcholine receptor (60)	$-NH(CH_2)_5-CONH(CH_2)_3N(CH_3)_3$	NaCl gradient
Acetylcholine esterase (63)	1-methyl-9-[N^{α}-(ε-amino-caproyl)-γ-aminopropyl-amino]acridinum bromide hydrobromide 9	decamethomium
Na^+K^+-activated ATPase (62)	6-(purine 5'ribosyl-triphosphate)-4-(1,3 dinitrophenyl)thioester (ATP analog) 8	ATP
Myosin (84)	sebacic acid hydrazide-ATP 10	KCl gradient
Glycerol kinase and lactate dehydrogenase (68,85,86)	N^6-(6-aminohexyl)-5'-AMP 11	KCl gradient
Glyceraldehyde 3-phosphate dehydrogenase (87)	NAD	NAD
Rhodopsin (61)	Concanavalin A	D-glucose



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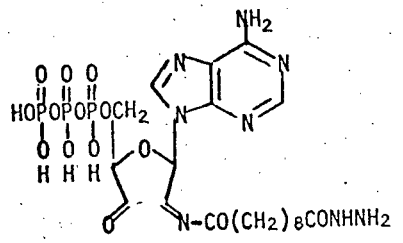

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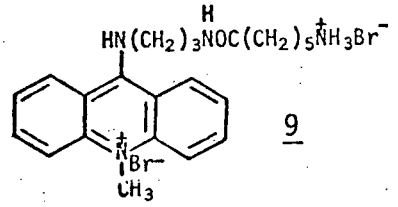

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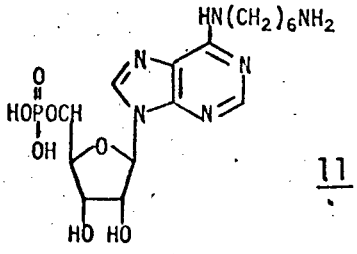

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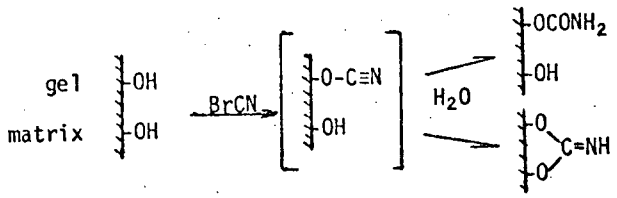
Fig. 2 Affinity Ligands

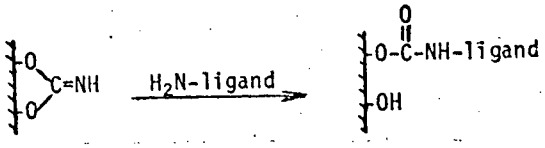
The ligand should have a high and specific affinity for the protein. For example, the dissociation constant of nicotinic receptor-cobra toxin complex is 1.13×10^{-9}M (59). Likewise that of Rhodopsin-Concanavalin A complex is 2×10^{-7} M (61). Often a hydrocarbon backbone inserted between the ligand and the gel matrix was found to be necessary in cases where the binding between the ligand and the protein is not very strong ($K_D > 10^{-3}$ M) (57). The effect of increasing distance may be partly explained by relief of steric hindrance imposed by the matrix backbone, and perhaps by the increased flexibility and mobility of the ligand as it protude further into the solvent (91).

(3) Covalent coupling to the matrix

An efficient technique has been developed to covalently link the ligand to an agarose gel matrix. It makes use of the terminal primary amino group of proteins or synthetic ligands, and the hydroxyl groups of agarose. The vicinal hydroxyl groups are activated by cyanogen bromide to yield either an inert carbamate or active imidocarbamate.



The active imidocarbamate then reacts with the terminal NH_2 of the ligand (65,66,67).



(4) Binding of protein to affinity column

Strong binding between the protein and ligand is obviously necessary for a good purification, hence experimental conditions such as temperature (88), pH (89), metal ion concentrations etc. have to be optimized to favor binding. For example, the binding of myosin to ATP required the presence of Ca^{2+} or Mg^{2+} (84). The concentration of ligand in the gel matrix is also important. Raftery and Schmidt (60) found that increasing the ligand concentration improved the binding capacity, but it also caused the ligand to act as a nonspecific ion exchanger and reduced the selectivity. Generally speaking, binding should be allowed to take place under physiological conditions when the protein is most active.

(5) Elution of the bound protein

After the bound protein is washed free of other proteins, it has to be dissociated from the ligand. In some cases where the affinity of the protein for the ligand is affected by salt concentration, the elution can be achieved by changing the salt concentration (60,68,84). In most cases the protein is dissociated and eluted with a solution containing a molecule to compete with the ligand for the enzyme (59,62), or vice versa (61,69).

Affinity chromatography is not limited to the use of 'physiological'

ligands such as inhibitor or substrate analog. Hydrophobic interaction has also been used to design an affinity ligand. In aqeous solution, hydrophobic bonds may be the most important interaction between a hydrophobic site or "pocket" of a protein and a small molecule having the optimum dimension to fit the pocket (71). This concept was applied to separate a mixture of glycogen phosphorylase and glycogen synthtase. Both enzymes have hydrophobic pockets which will bind the ligand $-NH(CH_2)_nNH_2$, but since glycogen phosphorylase has a larger hydrophobic pocket, it will bind only when $n > 4$, whereas glycogen synthetase which has a smaller hydrophobic pocket will bind the ligand when $n \leq 4$. Using a ligand of appropiate length, the two enzymes could be separated and purified (72).

PART III Theory of Velocity Sedimentation & Gel Filtration

(1) Velocity sedimentation

All particles in solution have a tendency to sediment under the influence of gravity and a tendency to disperse randomly in solution via diffusion. A small molecule which has a small mass does not sediment because the gravitational force is too small to overcome diffusion. For macromolecules, the gravitational force is considerable larger but still not enough. The force necessary for sedimentation can be increased by centrifugation. When the centrifugal force is larger than approximately 200,000 x g, sedimentation of protein molecules becomes observable on a time scale of tens of minutes. A particle subjected to centrifugation experiences two opposing forces, the centrifugal force and the friction between the particle and the solvent. Taking these two forces into consideration, Svedberg derived an equation governing the motion of any macromolecule in any medium in a centrifugal force field (42) :

$$\frac{1}{\omega^2 x}\frac{dx}{dt} = \frac{M(1-\bar{v}\rho)D}{RT} \qquad [1]$$

ω = angular velocity

x = distance of macromolecule from center of rotation

t = time

M = molecular weight

$\bar{v}$ = partial specific volume or inverse density of macromolecule

ρ = density of solution

D = diffusion coefficient

R = gas constant

T = absolute temperature

It can be seen that the right side of equation [1] is a constant for a particular experiment, and the left side contains the variables to be measured. The sedimentation coefficient s is defined as follows

$$s = \frac{1}{\omega^2 x} \frac{dx}{dt} \qquad [2]$$

Using this definition and rearranging equation [1] :

$$M = \frac{RT}{(1-\bar{v}\rho)D} s \qquad [3]$$

When more than one type of macromolecule is present, each will sediment past a given point at an instantaneous velocity proportional to its sedimentation coefficient. In other words, after a given length of time of centrifugation, the macromolecules will be separated, having travelled different distances along the length of the centrifuge tube.

Those with a larger sedimentation coefficient will be further away from the axis of rotation. Thus the distance from the center of rotation can be calibrated with macromolecules of known sedimentation coefficient, and the sedimentation coefficient of the macromolecule of interest can be found by interpolation.

The centrifugal force is proportional to $x^2\omega$. This means that the macromolecule will experience a larger centrifugal force when it is further down the centrifuge tube. If the macromolecule were in a homogeneous medium, it will accelerate due to the increasing centrifugal force. Consequently, the velocity of sedimentation will not be directly proportional to the sedimentation coefficient at all times during the experiment. This difficulty can be overcome by having an increasing viscosity and density gradient along the length of the centrifuge tube. Thus, the macromolecule, sedimenting through, say, an increasing concentration of sucrose solution, will experience an increasing friction and an increasing buoyancy which combine to compensate for the increasing centrifugal force. Under such condition, the macromolecule will sediment with a constant velocity proportional to its sedimentation coefficient. This is the underlying principle of constant velocity sedimentation.

The theory of velocity sedimentation in a sucrose concentration gradient was developed by Hans Noll (43). The desired gradient will depend on the temperature and on the dimension of the rotor being used. If the dimension of the rotor is known, a function relating the sucrose concentration and thus viscosity, and the distance from the axis of rotation can be calculated. This is known as an isokinetic sucrose gradient.

(2) Gel filtration

Gel filtration, also known as molecular sieve chromatography, or gel permeation chromatography, is a type of partition chromatography which separates molecules according to their sizes. The gel is made of porous material and acts as the stationary phase. A variety of gels are available for fractionation of molecules of different size (M.W.) ranges. The one used in this study is Sepharose 6B (Pharmacia), which consists of 6% agarose.

During chromatography, the solution containing the molecules to be fractionated are passed through the gel column. The solute molecules partition themselves between the stationary phase (the gel), and the mobile phase (the solvent). Molecules with a small size will be able to enter most of the pores of the gel and spend a comparatively longer time inside the gel before eluted out of the column, whereas molecules with a large size will be excluded from entering most of the pores and hence eluted faster. In the extreme case when a molecule is large enough to be excluded from all the pores, it will be eluted out of the column after a volume of solvent equal to the interstitual space between the gel beads has passed through. This is called the void volume, V_0 . In the other extreme when a molecule is small enough to enter all the pores, it will be eluted out of the column after a volume of solvent equal to $V_t - V_0$ has passed through the column, where V_t is the volumn of the gel bed.

An important paramenter in gel filtration is the partition coefficient K_{av} . The partition coefficient for a molecule is defined as

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \qquad [4]$$

where V_e is the volume of solvent which has passed through when that molecule is eluted out of the column.

The partition coefficient is a measure of how fast a molecule travels through the column compared to the solvent, or the relative amount of time that the molecule spends in the gel phase. The partition coefficient is independent of the compactness of the gel and the solvent used within experimental error (73). A molecule which is neither totally excluded nor able to enter all the pores of the gel phase has a partition coefficient between 0 and 1. From the definition of V_0, a totally excluded molecule has a K_{av} equals to 0. Conversely, a molecule small enough to enter pores of all sizes has a K_{av} equals to 1.

Gel filtration has been used to estimate molecular weights of proteins by assuming a linear relationship between the logarithm of molecular weight and partition coefficient (75). A column can be calibrated by measuring the K_{av} for several proteins with known molecular weight, then the molecular weight of the protein of interested can be found by interpolation in a plot of molecular weight verses K_{av} . This calibration is only valid for symmetric, or globular proteins. Large proteins with molecular weight larger than 10^5 are often asymmetric. For these asymmetric proteins, the calibration in terms of molecular weight is no longer valid. For example, it was observed that fibrinogen which has a molecular weight of 330,000 eluted earlier than urease which has a higher molecular weight of 482,700 ; also aldolase with

a molecular weight of 149,000 eluted earlier than alcohol dehydrogenase with a molecular weight of 160,000 (76). Since it is expected that larger protein should elute earlier (smaller K_{av}), these results indicated that for asymetric proteins, molecular weight is not necessary proportional to the "effective size". The effective size is better expressed in terms of Stokes' radius R_e . The Stokes' radius of an asymmetric macromolecule is defined as the radius of a rigid sphere which has the same diffusion coefficient as that macromolecule. This definition is expressed by the Stokes-Einstein Equation.

$$R_e = \frac{kT}{6\pi\eta D} \qquad [5]$$

Several equations have been proposed to relate Stokes' radius and the partition coefficient (73,77,78,79). Demassieux and Lachance selected nine proteins with different asymmetry and found that the logarithm of their Stokes' radii have a linear relationship with the partition coefficients (78). Thus a gel filtration column calibrated by standard proteins with known Stokes' radii or diffusion coefficients can be used to find the Stokes' radius of the protein being studied. The diffusion coefficient can then be calculated from equation [5].

To summarise, independent experiments on velocity sedimentation and gel filtration can be used to estimate sedimentation coefficient and diffusion coefficient respectivley, and the data can be combined in equation [3] to give an estimation of the molecular weight.

MATERIALS

Rectal glands of dog fish were kindly supplied as a gift by Mr. Martin Handel of Sea Fresh Pacific Products, Ladysmith, B. C.

Purified chicken egg white lysozyme was kindly supplied by Ms. Kathleen Chance.

All inorganic and organic chemicals used were of reagent grade.

Dodecylamine, methylbromide, imidazole, cyanogen bromide and diethyladipic acid were obtained from Eastman Kodak Co., Rochester, New York.

Potassium bicarbonate, sodium EDTA, sodium carbonate, glycerol, sodium potassium tartrate and sodium metaperiodate were obtained from Fischer Scientific Co., Fair Lawn, New Jersey.

Acetonitrile, dioxane, glycerol and sucrose were obtained from Mallinckrodt Chemical Co., St. Louis, Mo.

Lubrol WX, bovine serum albumin, ATP (sodium salt), ouabain, Triton X-100, Concanavalin A (Jack bean), cacodylic acid (sodium salt). 2,4,6-trinitrobenzene 1-sulfonate acid, N-acetylglucosamine, α-methyl-D-mannoside, alcohol dehydrogenase, catalase, Trisma base, NAD, Micrococus lysodeikticus and bovine fibrinogen were obtained from Sigma Chemical Co. St. Louis, Mo.

Folin reagent was obtained from Harleco Co. Phila., Pa.

Fluorescamine (4-phenylspiro [furan-2(3H),1'-phthalan]-3-3'dione)

was obtained from Hoffman-La Roche Ltd., Quebec.

Phosphatidyl-L-serine (bovine brain) was obtained from Koch-Light Lab. Ltd., England.

Ammonium molybdate tetrahydrate and disodium hydrogen orthophosphate were obtained from The British Drug House Ltd., England.

Sepharose 4B, Sepharose 6B and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Hydrazine hydrate (99-100%) was obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Horse spleen apoferritin, deoxycholic acid (sodium salt) were obtained from Calbiochem, San Diego, Calif.

Wheat germ agglutinin was obtained from Miles-Yeda Ltd., Rehovoth, Israel.

Amicon CF50A membrane ultrafilters were obtained from Amicon Corp., Lexington, Mass.

Millipore filters were obtained from Millipore Corp., Bedford, Mass.

METHODS

(1) Synthesis of trimethyldodecylammonium bromide

Dodecylamine (30.0g, 0.162 mole), methyl bromide (39.3ml, 0.694 mole) and potassium bicarbonate (64.8g, 0.647 mole) were refluxed in acetonitrile (70 ml) for 12 hrs. Product was extracted from the mixture with hot ethanol. It was recrystallized once from benzene-ether and once from acetone-ether, decomposition temperature: 228°C. Microanalyses for C, H, N and Br confirmed that it was the desired product.

	% by weight			
Element	C	H	N	Br
Expected	58.39	11.12	4.54	25.92
Found	58.66	11.29	4.55	26.10

(2) Collection of rectal glands of dog fish

Dog fish were obtained in the summer of 1973. Rectal glands were removed from the fish on the same day they were caught, wrapped in aluminum foil and stored in dry ice. They were transferred to a -70°C freezer for storage after being shipped back to the laboratory.

(3) Lyophilization of tissues

Tissues (42.8 g, 30 glands) were homogenized in a Model 23 Virtis homogenizer (The Virtis Co., Gardiner, N.Y.) at maximum speed in 440 ml of water at 0-5°C for 2 min., frozen in a dry ice-acetone mixture, and then lyophilized. 1 g dry weight of lyophilized tissue is equivalent to 6.7 g of the wet tissue.

(4) Extraction of Na^+K^+-activated ATPase

Either the lyophilized or the frozen tissue can be extracted for Na^+K^+-activated ATPase. All procedures were carried out at 0-5°C. In a typical experiment, 0.5 g of lyophilized tissue was resuspended in 50 ml of buffer, or 2 glands (approximately 3 g) was homogenized in 45 ml. The amount of lyophilized or frozen tissue can be scaled up or down to suit a particular experiment, with the proportional increase or decrease in the amount of buffer. The buffer contains 30 mM imidazole, 100 mM NaCl, 1 mM sodium EDTA, titrated to pH 7.0 with concentrated hydrochloric acid. This tissue homogenate was centrifuged in a SS-34 rotor (Ivan Sorvall Inc., Norwalk, Connecticut) at 14,000 r.p.m. (max. 23,600 x g) for 30 min. The supernatant was discarded. The pellet was homogenized in 45 ml of the same buffer. This pellet homogenate was again centrifuged at 14,000 r.p.m. for 30 min. The supernatant was discarded. The pellet was homogenized in 12.5 ml of the same buffer

plus 10% (v/v) glycerol. To this pellet homogenate was added 1.3 ml of a 16% (w/v) Lubrol WX solution, perviously titrated with concentrated hydrochloric acid to pH 7.0 and filtered through a 0.45 μ Millipore filter. The mixture was stirred for 15 min., and then centrifuged in a Beckman 42.1 rotor (Beckman Instruments Inc., Palo Alto, Calif.) at 42,000 r.p.m. (max. 195,000 x g) for 3 hrs. The pellet was discarded. The resulting supernatant was filtered through a Millipore filter. This is called the "enzyme extract".

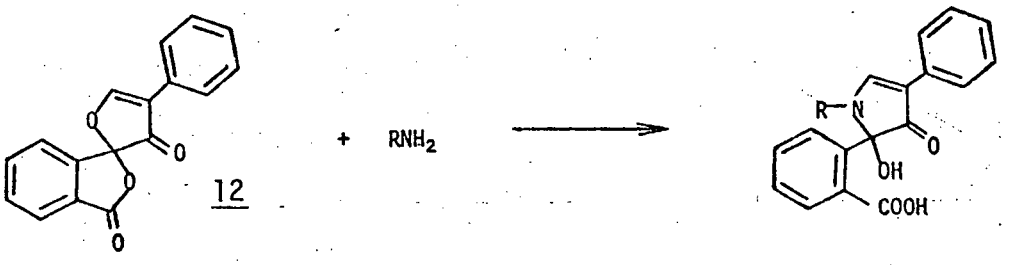
(5) Protein determination

Total protein in a sample was assayed by either one of two methods. The method of Lowry, Rosenbrough, Farr and Randall (34) was used in samples where Lubrol WX concentration was lower than 0.2 % (w/v). A calibration was prepared using bovine serum albumin as the standard. Absorbance was measured at 650 nm on a Carl Zeiss PMQII spectrophotometer.

When the Lubrol WX concentration is higher than 0.2%, interference will occur with Lowry's method. A yellow precipitate would be formed upon addition of the Folin reagent and the subsequent extinction would be too low. Hence, for sample with high Lubrol WX concentration, so high that dilution to 0.2% was not practical, a fluorescence method was used.

The fluorescence method is based on the reaction of 4-phenyl-spiro[furan-2(3H),1'-phthalan]-3-3'dione 12, known as fluorescamine,

with primary amines.



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The product of the reaction fluoresces intensely, whereas fluorescamine itself is nonfluorescent, and excess reagent is hydrolysed to form nonfluorescent, water soluble products (35,36).

This fluorescamine will likewise react with the N-terminal and any exposed lysine residue of a peptide, providing a convenient assay for small quantities of protein. 100 µl sample containing 10 to 100 µg of protein was mixed with 2 ml of a buffer containing 0.01 M sodium carbonate and 0.04 M sodium bicarbonate, pH 9.4, 0.5 ml of 0.015% (w/v) fluorescamine in acetone was added while mixing. Relative fluorescence was measured against a blank with the buffer in which Na^+K^+-activated ATPase was routinely extracted, with emission wavelength at 480 nm and excitation wavelength at 390 nm. Bovine serum albumin was used as the standard for calibration.

(6) Na^+K^+-activated ATPase activity assay

The appropiate amount of enzyme was incubated at 37°C in a pH 7 medium containing 30 mM imidazole-HCl, 120 mM sodium chloride, 20 mM potassium chloride, 7 mM magnesium chloride, 4 mM ATP (sodium salt),

0.006% (w/v) phosphatidylserine in a final volume of 0.5 or 1.0 ml. A control, containing all the above plus 10^{-4} M ouabain, was incubated simultaneously with every assay. The duration of incubation was between 5 and 30 min., depending on the activity of the enzyme preparation being used. Reaction was started by adding the enzyme, and terminated by the addition of 0.5 or 1.0 ml of 3% (w/v) perchloric acid. The resulting solution was assayed for the amount of inorganic phosphate liberated by hydrolysis of ATP, using the method of See and Fitt (37). Absorbance was measured at 650 nm. A calibration was prepared using disodium hydrogen orthophosphate as the standard. Na^+K^+-activated ATPase activity is defined as the difference in activity in the absence and presence of ouabain.

(7) Preparation of affinity resins

(a) Cyanogen bromide activation of Sepharose 4B

10 ml of Sepharose 4B was washed thoroughly with water, suspended in an equal amount of water and cooled in ice for the rest of the procedure. The activation was done in the fumehood. Cyanogen bromide (250 mg) was dissolved in a minimum volume of dioxane and added to the Sepharose with stirring while the pH was maintained at 10.5 ± 0.1 with 0.4 N NaOH. When very little change of pH with time was detected (after about 20 min.), the Sepharose was washed with cold water, and then with a pH 6.8 sodium acetate buffer on a sintered glass funnel. The method used

is essentially the same as described by Cuatrecasas (38). The activated Sepharose has to be coupled with the desired affinity ligand immediately.

(b) Coupling of Concanavalin A to Sepharose 4B

The activated Sepharose 4B as prepared from part (a) was added to a solution of 15 mg of Concanavalin A in 7.5 ml of the pH 6.8 sodium acetate buffer. The mixture was gently stirred for 12 hrs at 4°C. The unbound Concanavalin A was removed by washing the Sepharose with a solution containing 0.5 M sodium chloride, 10^{-3} M calcium chloride and 10^{-3} M manganese chloride.

The absorbance of the washing solution containing the unbound Concanavalin A was measured at 280 nm. By subtracting the total amount of protein in the washing solution from the original amount of Concanavalin A used, the concentration of Concanavalin A covalently coupled was estimated to be 1.8 mg per ml of Sepharose 4B.

(c) Coupling of Wheat germ agglutinin to Sepharose 4B

The method used was essentially the same as that for Concanavalin A. 1.35 mg of wheat germ agglutinin in 3 ml of water was added to the activated Sepharose 4B. A 0.05 M sodium cacodylate buffer, pH 7.2, was used to wash the coupled Sepharose, instead of sodium acetate. The concentration of coupled wheat germ agglutinin was 0.88 mg per ml of Sepharose 4B.

(d) Synthesis of hexanedioic acid (adipic acid) dihydrazide

Hexanedioic acid diethyl ester (100 ml, 0.497 mole), hydrazine hydrate (99-100%, 200 ml, 4.04 moles) and ethanol (100%, 200 ml) were refluxed for 3 hrs in a one litre flask. The resulting solids were washed thoroughly with hot ethanol (100%), recrystallized from a water-ethanol mixture, and then recrystallized again from water. The colorless crystals were dried under vaccum with calcium sulfate for 3 hrs. The m.p. (180-2°C) and microanalyses for C, H and N confirmed that the crystals were the desired product.

	% by weight		
Element	C	H	N
Expected	41.37	8.10	32.17
Found	41.67	8.06	31.86

(e) Coupling of hexanedioic acid dihydrazide to Sepharose 4B

Sepharose 4B was activated as in part (a). A pH 9.4 buffer containing 0.01 M sodium carbonate and 0.04 M sodium bicarbonate was used to wash the activated Sepharose instead of the sodium acetate buffer. 10 ml of a 38 g/l hexanedioic acid dihydrazide was added. The mixture was stirred for 12 hrs at 4°C. It was then washed with water and 0.2 M sodium chloride. The washing solution was monitored for unbound hexanedioic acid dihydrazide by the 2,4,6-trinitrobenzene 1-sulfonic acid test (39). To a 0.5 ml sample containing 0.05 to 0.4 µmole of hexanedioic acid dihydrazide was added 0.5 ml of 4% (w/v) sodium bicarbonate and 0.5 ml of 0.1% (w/v) 2,4,6-trinitrobenzene 1-sulfonic

acid. The mixture was kept in the dark at 40°C for 2 hrs. It was then diluted with 1 ml of water and the absorbance was measured at 340 nm. The amount of hexanedioic acid dihydrazide coupled was 70 µmole per ml of Sepharose.

(f) Coupling of periodate oxidized ATP to Sepharose-hexanedioic acid dihydrazide (40,41)

5 ml of 0.02 M sodium metaperiodate was mixed with 5 ml of 0.02 M sodium ATP previously adjusted to pH 11 with 1 N NaOH. The mixture was allowed to stand at 0°C in the dark for 1 hr. A 7 ml aliquot was then taken and added to 18 ml of 0.1 M sodium acetate, pH 5 at 0°C. The mixture was stirred and immediately added to 10 ml of Sepharose-hexanedioic acid dihydrazide prepared as described in part (e) and equilibrated in the same pH 5 buffer at 4°C. The suspension was stirred for 3 hrs in the dark at 4°C. 75 ml of 2 M sodium chloride was added and stirring continued for 30 min. It was then washed free of unbound nucleotide by monitoring the absorbance at 260 nm. The amount of ATP bound was also estimated by acid hydrolysis of ATP to yield inorganic phosphate. 0.1 ml of the coupled Sepharose was added to 0.9 ml of 1 N hydrochloric acid and heated in a boiling water bath for 10 min. The sample was then assayed for inorganic phosphate by the method of See and Fitt (37).

The amount of ligand estimated by extinction at 260 nm and inorganic phosphate determination was 1.8 and 2.2 µmole per ml of Sepharose, respectively.

(8) Affinity Chromatography

In a typical experiment, the affinity column was prepared by packing 2 to 3 ml of the affinity resin in a pasteur pipet. All procedures were carried out at 0-5°C. The column was equilibrated and eluted with a buffer containing 30 mM imidazole-HCl, 100 mM sodium chloride, 10% glycerol, pH 7.0 . 0.5 ml of enzyme extract, prepared as described in page 33 to 34, was loaded on the column. Approximately 0.55 ml fractions were collected. When four bed volumes of buffer had passed through, another buffer, containing a competitor for the affinity ligand or Na^+K^+-activated ATPase but otherwise identical to the original buffer, was used for elution. This buffer contained saturated α-methyl-mannoside, or 5 mg/ml N-acetylglucosamine, or 12 mg/ml sodium ATP for the affinity ligands Concanavalin A, wheat germ agglutinin, or periodate oxidized ATP respectively. A further four bed volume of fractions were collected. These fractions were assayed for enzyme activity and total protein.

(9) Velocity sedimentation

(a) Calculation of sucrose concentration gradient

A sucrose concentration gradient was calculated for the Beckman SW 41 rotor which was used for this study.

The method of Hans Noll (43) was used with some modification. The equation 5 he derived is equivalent to equation [6].

$$\eta_{m(x)} = mx[\rho_p - \rho_{m(x)}] \qquad [6]$$

$$\text{where } m = \frac{\eta_t}{x_t(\rho_p - \rho_t)} \qquad [7]$$

η_t = viscosity at the beginning of the sucrose gradient

x_t = distance of the beginning of the sucrose gradient from the axis of rotation

ρ_t = density of sucrose solution at the beginning of the sucrose gradient

ρ_p = density of the macromolecule

x = distance from the axis of rotation

$\eta_{m(x)}$ = viscosity of the sucrose solution at x

$\rho_{m(x)}$ = density of sucrose solution at x

x_t is supplied by the manufacturer to be 7.0 cm. The concentration of sucrose at the beginning of the gradient was chosen to be 10% (w/v). At this concentration, the sucrose solution is denser than the buffer containing the proteins, so that minimum disturbance will occur when the sample is loaded on top of the sucrose gradient. The density and viscosity of a 10% (w/v) sucrose solution at 5°C are 1.041 g/cm^3 and 2.073 centipoise (44), respectively. ρ_p was chosen to be 1.343. Substituting these into equation [7],

$m = 0.979$

Substitution of m into equation [6], and using compiled data of

the viscosity and density of sucrose solution as a function of its concentration at 5°C (44), a relation between concentration $C_{m(x)}$ and the distance x can be calculated. The results are shown in Table V.

TABLE V

Sucrose Conc. % (w/v)	$\rho_{m(x)}$ (g/cm^3)	$\eta_{m(x)}$ (centipoise)	$\rho_p - \rho_{m(x)}$	x (cm)	$x - x_t$ (cm)	Volume (ml)
10	1.041	2.073	0.302	7.00	0.00	0.000
11	1.045	2.150	0.298	7.37	0.37	0.594
13	1.053	2.319	0.290	8.17	1.17	1.878
15	1.062	2.513	0.281	9.13	2.13	3.42
17	1.071	2.736	0.272	10.28	3.28	5.26
19	1.080	2.992	0.263	11.61	4.61	7.40
21	1.089	3.290	0.254	13.10	6.10	9.79
23	1.098	3.636	0.245	15.16	8.16	13.10

There is a direct relationship between the distance x and the volume in the centrifuge tube. The cross-sectional area of the centrifuge tube as supplied by the manufacturer is 1.605 cm^2, so that

$$\text{Volume} = 1.605\ (x - x_t)\ \text{ml}$$

or

$$V = 1.605\ (x - 7.0)\ \text{ml} \qquad [8]$$

This corresponding volumes are listed on the last column of Table V.

The solid line of Fig. 3 shows the shape of the sucrose concentration gradient as a function of the centrifuge tube volume.

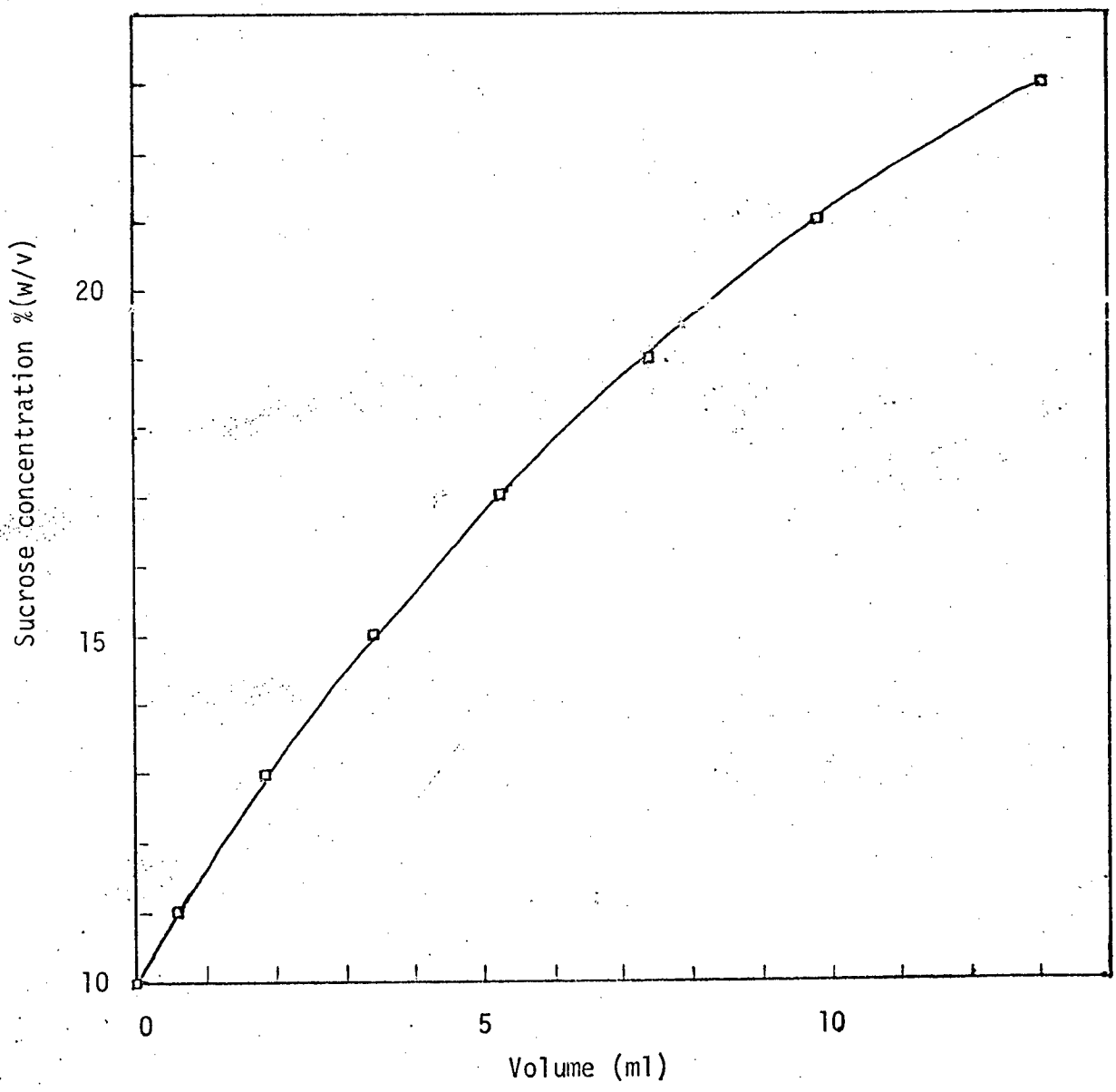

Fig. 3 Isokinetic gradient (for Beckman SW 41 rotor at 5°C)

——— $\eta_{m(x)} = 0.979x[1.34-\rho_{m(x)}]$ $V=1.61(x-7.0)$

□ □ □ $C = 29.3 - 19.3\exp(-V/11.7)$ simulation

(b) Generation of the sucrose concentration gradient

For the experimental application of the principle of constant velocity sedimentation, a method of preparing the calculated gradient is needed. The constant velocity gradient as shown in Fig. 3 exhibits a convex curvature. Noll (43) has demonstrated that this could be simulated by a convex exponential gradient. An exponential gradient maker designed for generation of such a gradient is shown in Fig. 4.

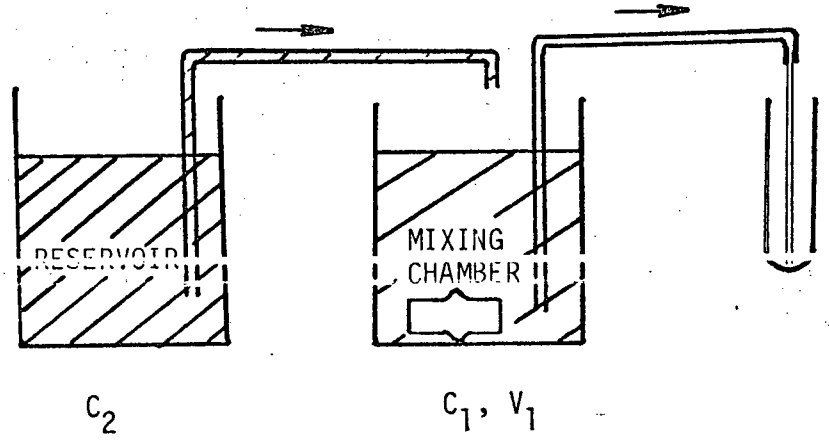

Fig. 4 Exponential gradient maker
Arrow indicates direction of flow of sucrose solution
C_1 = initial concentration in mixing chamber
V_1 = initial volume in mixing chamber
C_2 = concentration in reservoir

Sucrose solution is pumped from the mixing chamber and delivered to the centrifuge tube, while a more concentrated sucrose solution is being pumped into the mixing chamber. The solution in the mixing chamber is being stirred all the time. If C_2, the sucrose concentration in the reservoir is higher than the initial concentration C_1 in the mixing

chamber, then the sucrose solution being pumped into the centrifuge tube will have an increasing concentration. Low concentration sucrose solution introduced first is allowed to float to the top, as solution of increasing concentration is fed in. The two flow rates indicated by the arrows in Fig. 4 were made to be equal by the use of two identical channels of a peristaltic pump. More than one gradients can be made simultaneously using more connecting tubes, and the total number is limited only by the number of available channels in the pump.

The gradient produced is defined by equation [9] (42,45).

$$C = C_2 - (C_2 - C_1) \exp (V/V_1) \qquad [9]$$

where C is the concentration corresponding to the time when a volume V has been pump out of the mixing chamber. The parameters C_2, C_1 and V_1 determine the actual shape of the generated concentration gradient. These are to be adjusted so that equation [9] simulates the desired gradient as shown in Fig. 3. C_1 which becomes the concentration of the beginning of the gradient, should obviously be 10% (w/v). C_2 and V_1 were found by substituting the fifth and eigth row of data from Table V into equation [9]. This gives

$$17 = C_2 - (C_2 - 10) \exp (-5.26/V_1) \qquad [10]$$

$$23 = C_2 - (C_2 - 10) \exp (-13.10/V_1) \qquad [11]$$

Solving equation [10] and [11] by sucessive approximation :

$$C_2 = 29.3 \qquad V_1 = 11.7$$

Substituting these values into equation [9]

$$C = 29.3 - 19.3 \exp(-V/11.7) \qquad [12]$$

Equation [12] defines the gradient used in this work, made by putting 11.7 ml of 10% (w/v) sucrose solution in the mixing chamber per gradient made at one time, and 29.3% (w/v) sucrose solution in the reservoir.

The points in Fig. 3 were calculated from equation [12]. It can be seen that this gradient simulates the desired gradient to a good approximation.

(c) Calibration with standard proteins and determination of sedimentation coefficient

Four sucrose gradients were made in a 30 mM imidazole-HCl, pH 7.0 buffer as described in part (b) and cooled to 5°C. One rectal gland was used for extraction of Na^+K^+-activated ATPase, as outlined in page 33 to 34. The supernatant obtained after centrifugation at 42,000 r.p.m. was passed through a Millipore filter and concentrated to 3 ml on an Amicon CF50A membrane ultrafilter. 100 µl of this concentrated enzyme extract was loaded on each of two sucrose gradients. 1 mg each of alcohol dehy-

drogenase, catalase and lysozyme were dissolved in 0.5 ml of buffer and filtered through a Millipore filter. 100 µl of this standard protein solution was loaded on each of the two remaining sucrose gradients. Centrifugation was carried out in a Beckman L3-50 ultracentrifuge at 41,000 r.p.m. using a SW-41 rotor with the thermostat set at 5°C. The run was started as soon as the temperature was stabilized and the vacuum was down to 50 microns. It was stopped after 16.5 hrs.

Using a Desaga (W. Germany) peristaltic pump and a Gilson MF Mini-Escargot fractionator (Middleton, Wis.), the content of each tube was fractionated into 28 equal fractions with pump rate set on 1 and a time count of 1.7 min. for each fraction. These fractions were assayed for alcohol dehydrogenase, catalase, lysozyme and Na^+K^+-activated ATPase activity.

Alcohol dehydrogenase assay (79) : Samples of 25 to 100 µl were mixed with 3 ml of an assay medium which is a mixture of 4 ml of 1 M Tris-HCl (pH 7.6), 100 µl of 95% ethanol, 70 mg of NAD and 72 ml of water. The activity was followed at 340 nm.

Catalase was assayed by measuring directly the extinction at 405 nm.

Lysozyme assay (80) : 50 µl samples were mixed with 2.95 ml of an assay medium containing 0.25 mg of Micrococus lysodeikticus in a 0.05 M sodium chloride and 0.66 M phosphate buffer. The decrease in turbidity was immediately monitored at 540 nm, with a reference containing only the buffer solution. The activity of lysozyme was expressed as the decrease in absorbance per 100 seconds.

(10) Gel filtration of detergent solubilized Na^+K^+-activated ATPase and Stokes' radius calibration

157 ml of Sepharose 6B was packed into a K15/90 column (Pharmacia) and equilibrated at 4°C in a pH 7.0 buffer containing 30 mM imidazole-HCl, 100 mM NaCl and 10% (v/v) glycerol. The void volume was determined by loading 3 mg of Blue Dextran 2000 in 1.5 ml of the same buffer. 5 mg each of bovine fibrinogen and bovine serum albumin and 3 mg of horse spleen aproferritin were dissolved in 2 ml of the same buffer and centrifuged at 13,000 r.p.m. in a Sorval SS-34 rotor for 10 min. 1.5 ml of the supernatant was loaded on the column. 0.88 ml fractions were collected. The fractions were monitored for extinction at 280 nm until 1.5 litres of buffer had passed through. The column was then reequilibrated with the same buffer plus 1.5% (w/v) Lubrol WX, prepared from a stock solution of 10%(w/v) Lubrol WX filtered through Millipore. One rectal gland was used for extraction of Na^+K^+-activated ATPase, as outlined in page 33 to 34. The supernatant obtained after centrifugation at 42,000 r.p.m. was passed through a Millipore filter and concentrated to 3 ml on an Amicon CF50A membrane ultrafilter. 1.5 ml of this enzyme extract was loaded. 0.80 ml fractions were collected until 1.5 litres of buffer has passed through. These fractions were assayed for Na^+K^+-activated ATPase activity.

RESULTS

(1) Optimization of conditions for enzyme extraction

(a) Test of efficiency of extraction by different detergents

The efficiency of four detergents for the extraction of Na^+K^+-activated ATPase was tested at 0.1% (w/v) and 1.0% (w/v) detergent concentration. The results are summaried in Table VI.

TABLE VI

Detergent	Na^+K^+-activated ATPase activity (μmole/mg/hr)	
	0.1%(w/v)	1.0%(w/v)
Trimethyldodecylammonium bromide	0.03	0.00
Triton X-100	0.09	0.01
Sodium deoxycholate	0.15	0.00
Lubrol WX	0.34	0.96

(b) Extraction as a function of Lubrol WX concentration

The concentration of Lubrol WX was varied from 0.1 to 2.0% (w/v) in the presence of 0, 5, 10 or 20% (v/v) glycerol in the extraction medium. The results are shown in Fig. 5, 6, 7 and 8.

(c) Stability of Lubrol WX extracted Na^+K^+-activated ATPase

Enzyme extracts in the presence of 0, 5, 10 and 20% (v/v) glycerol were tested for their stabilities. The enzyme extracts were stored at 4°C and assayed for Na^+K^+-activated ATPase at approximately 24 hr intervals. The results are shown in Fig. 9. The Na^+K^+-activated ATPase activity is expressed as the percentage of the activity obtained immediately after extraction.

(2) Affinity chromatography

The procedure has been described under the 'Method' section in detail. A control, in which the Sepharose 4B was not linked to any affinity ligand, is shown in Fig. 10. The results of Concanavalin A, wheat germ agglutinin and periodate oxidized ATP used as affinity ligand are shown in Fig. 11, 12 and 13, respectively. In all cases enzyme activity and amount of protein were plotted against fraction number. Each fraction was approximately 1/2 to 3/4 ml.

(3) Velocity sedimentation

The procedure has been described under the 'Method' section. Fig. 14 shows the profile of the distribution of enzyme activities in the sucrose gradient after sedimentation. Since only the positions of

maximum enzyme activity were required for calculation, the enzyme activities were expressed as arbitrary absorbance units. Fig. 15 is a calibration of the isokinetic sucrose gradient. The sedimentation coefficients of the three standard proteins (44) : catalase, alcohol dehydrogenase and lysozyme were plotted against their relative distance of migration, taking the top of the sucrose gradient as origin. They are shown as the three data points in the plot. The position of Na^+K^+-activated ATPase relative to the standard is indicated by the arrow. From this calibration, the sedimentation coefficient of Na^+K^+-activated ATPase was estimated to be 5.0 S.

(4) Gel filtration of Na^+K^+-activated ATPase and Stokes' radius calibration

The procedure has been described under the 'Method' section. Fig. 16 shows how Blue Dextran 2000, Na^+K^+-activated ATPase, and the three standard proteins : fibrinogen, apoferritin and bovine serum albumin were fractionated by the column. The Blue Dextran peak indicates the void volume. The partition coefficients of Na^+K^+-activated ATPase and other proteins were calculated according to equation [4].

Fig. 17 is a calibration of the gel filtration column. The logarithm (base 10) of the Stokes' radius (R_e) of the three standard proteins (44) in Angstroms was plotted against their partition coefficients. They are shown as the three data points. The partition coeffi-

cient of Na^+K^+-activated ATPase is indicated by the arrow. From this calibration, the Stokes' radius of Na^+K^+-activated ATPase was estimated to be 114 Å .

(5) Estimation of the molecular weight of Na^+K^+-activated ATPase

Combining equation [3] and [5],

$$M = \frac{6sN\pi\eta R_e}{1 - \bar{v}\rho} \qquad [13]$$

Substituting the following into equation [13]

$s = 5.0 \times 10^{-13}$ sec

$\eta = 0.0102$ poise (viscosity of water at 20°C)

$\bar{v} = 0.73$ cm^3 g^{-1} (assumed)

$\rho = 1.00$ g cm^{-3}

$R_e = 1.14 \times 10^{-6}$ cm

gives $M = 240{,}000$

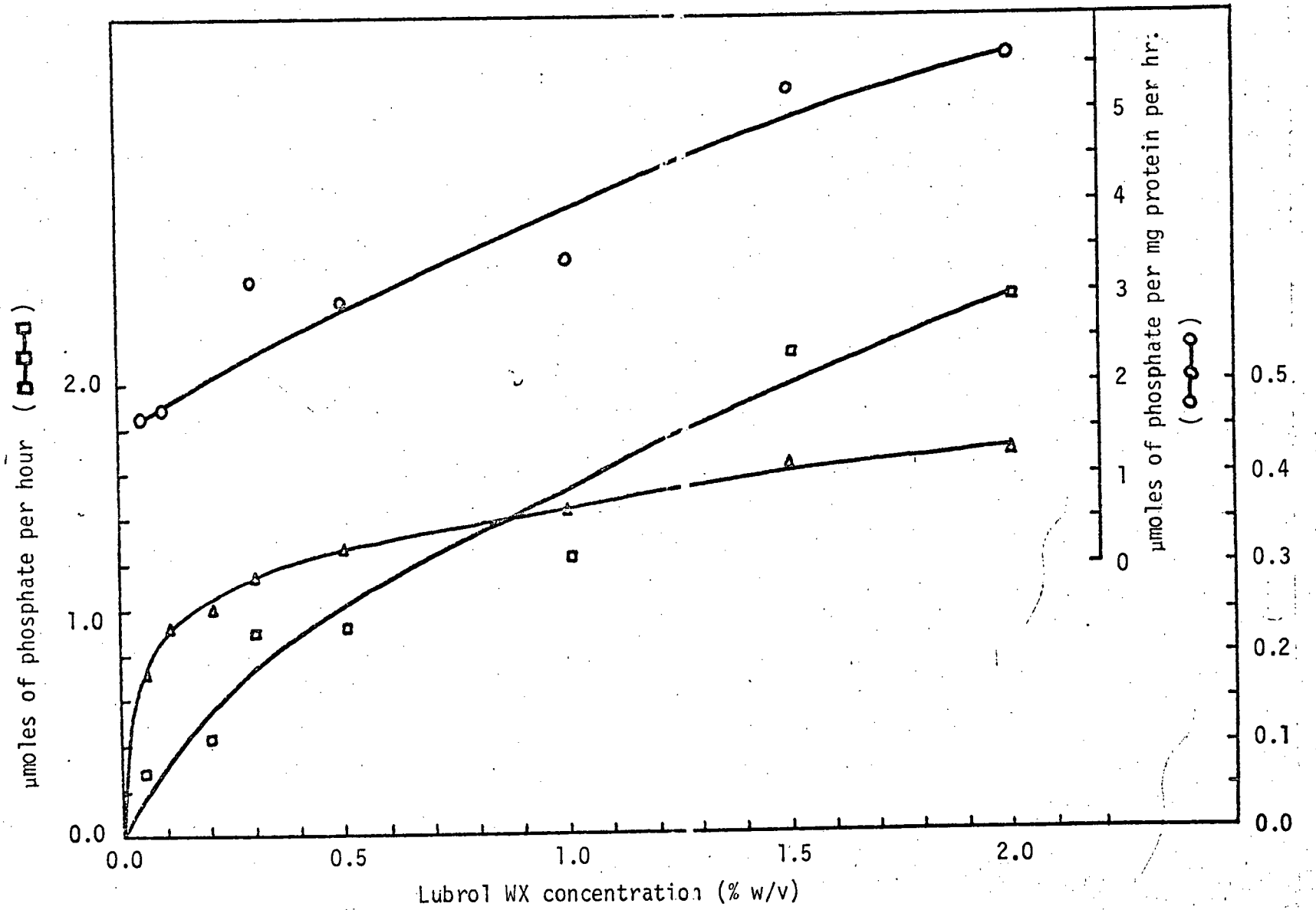

Fig. 5 Extraction as a function of Lubrol WX concentration
Na^+K^+-activated ATPase activity (□—□—□)
Protein (△—△—△)
Specific Activity (○—○—○)

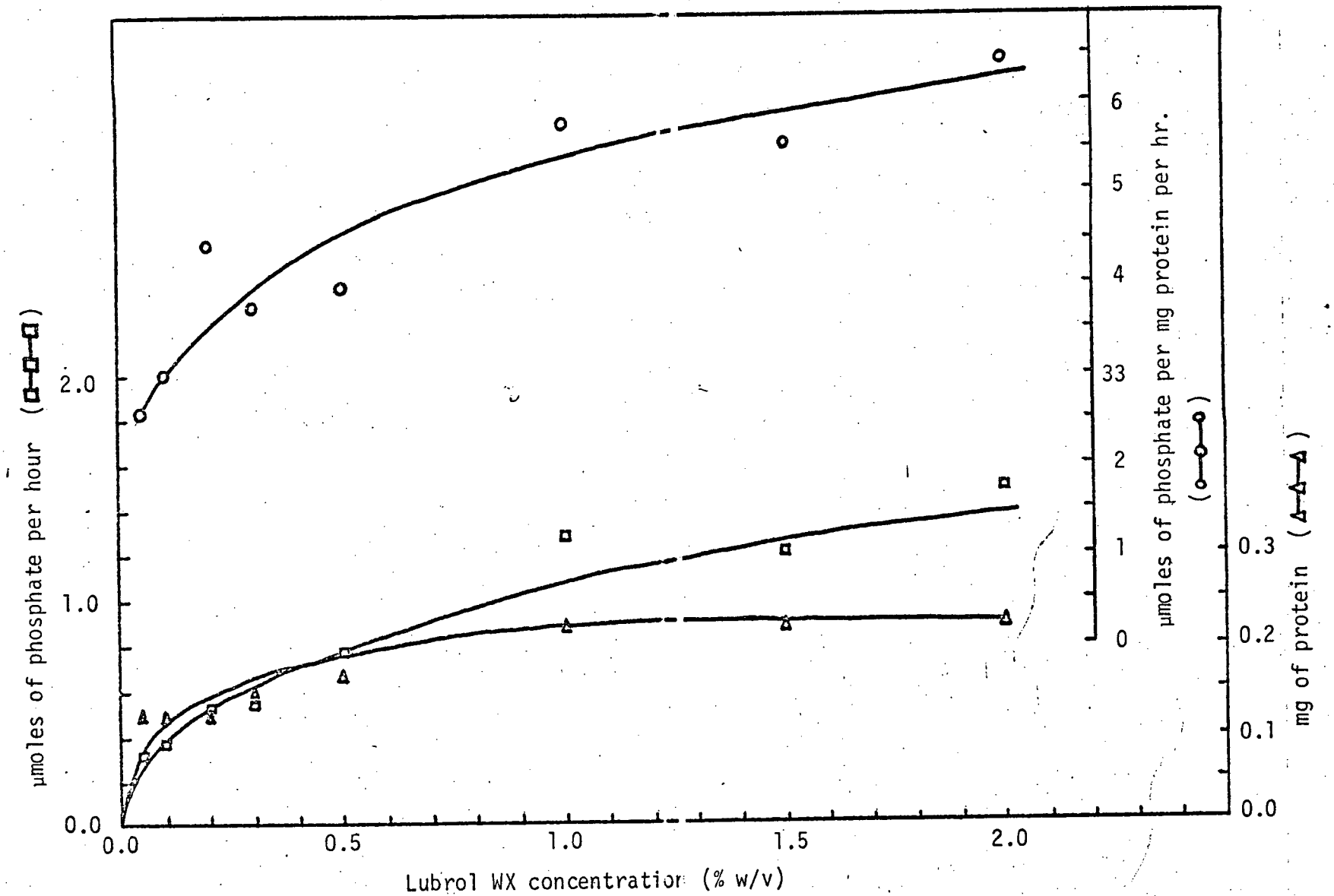

Fig. 6 Extraction as a function of Lubrol WX concentration in the presence of 5% (v/v) glycerol
Na^+K^+-activated ATPase activity
Protein
Specific Activity

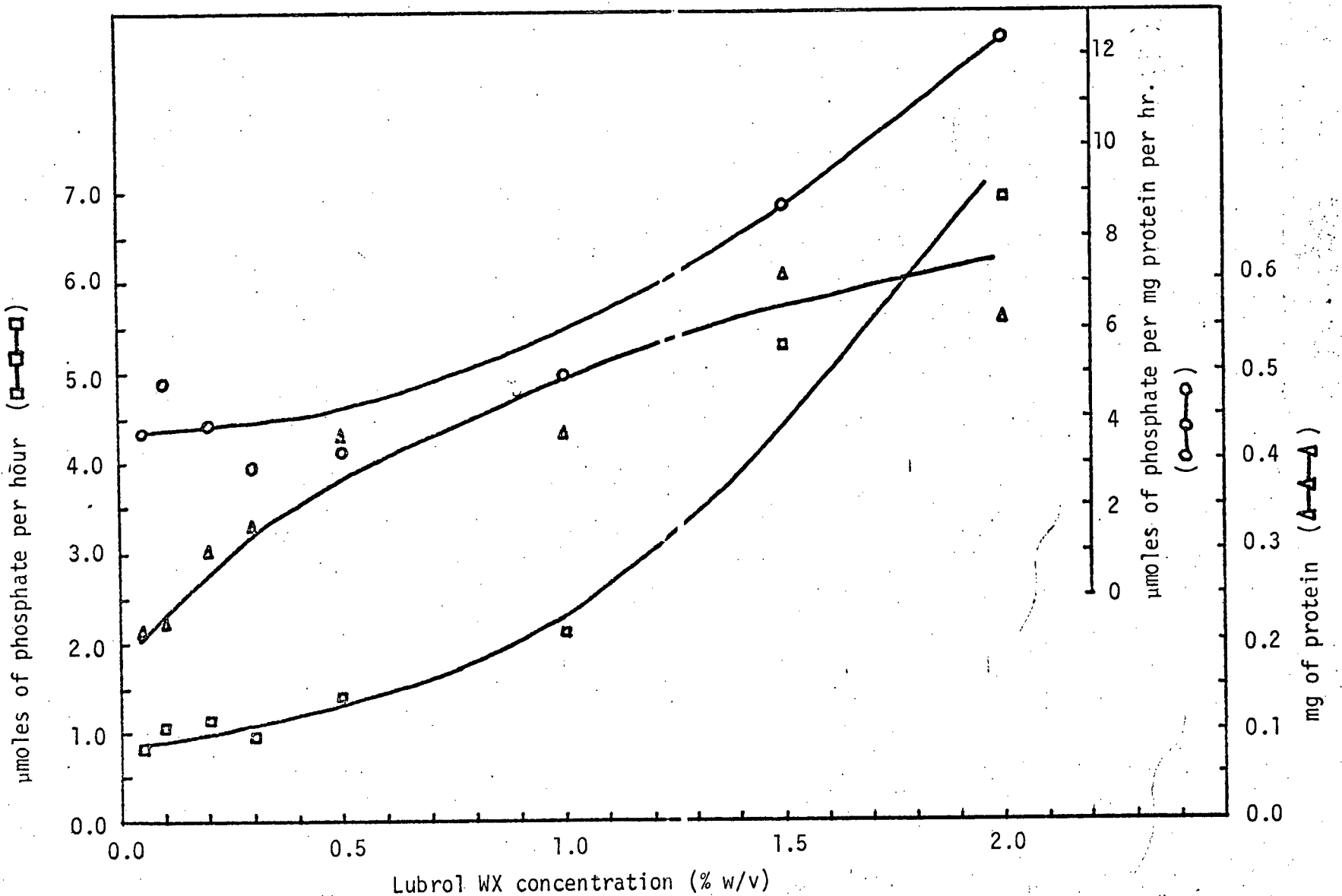

Fig. 7 Extraction as a function of Lubrol WX concentration in the presence of 10% (v/v) glycerol
Na^+K^+-activated ATPase activity (□—□)
Protein (△—△)
Specific Activity (○—○)

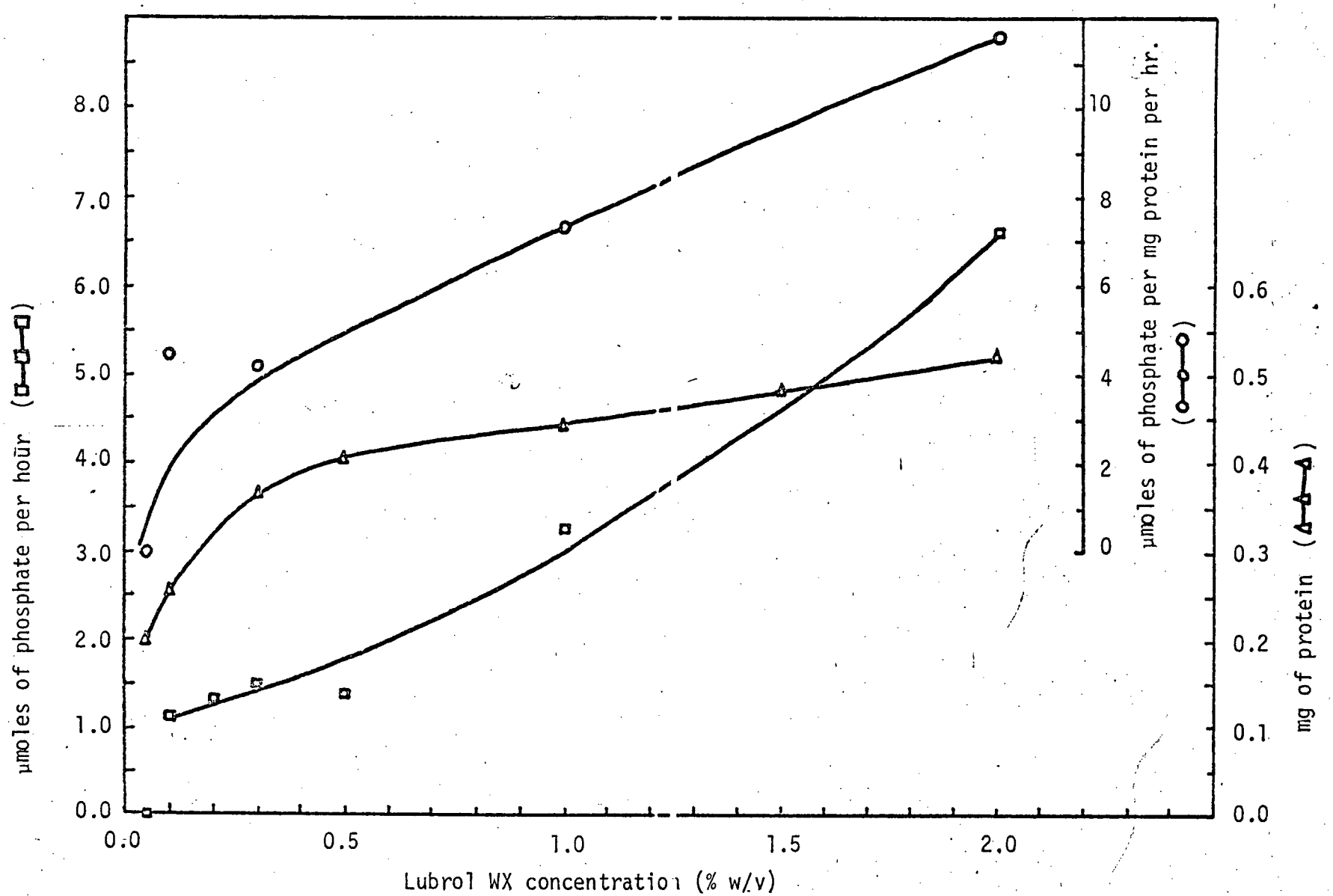

Fig. 8 Extraction as a function of Lubrol WX concentration in the presence of 20% (v/v) glycerol
Na^+K^+-activated ATPase activity
Protein
Specific Activity

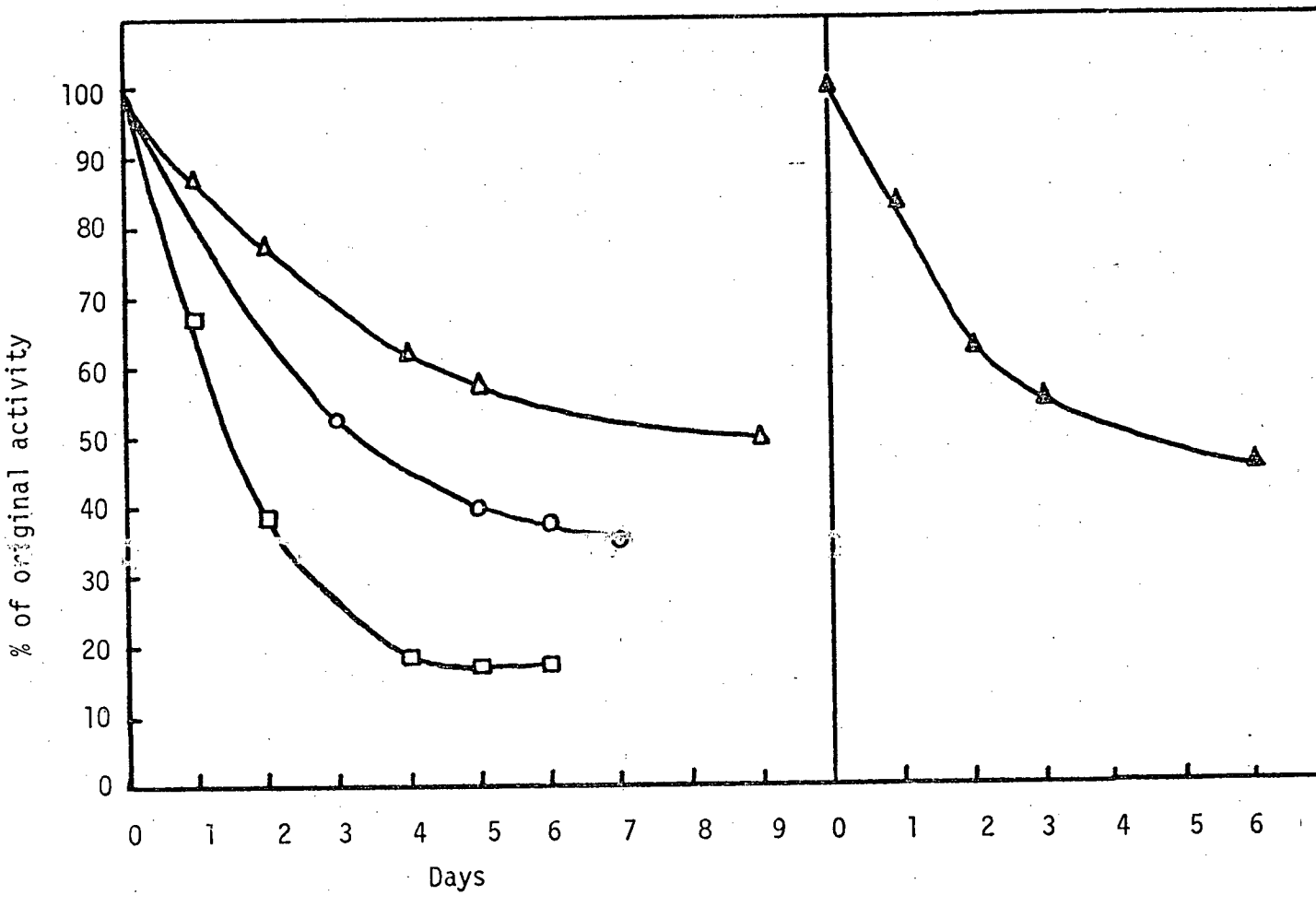

Fig. 9 Stability of Lubrol WX extracted Na^+K^+-activated ATPase

without glycerol (□—□—□)
5%(v/v) glycerol (○—○—○)
10%(v/v) glycerol (△—△—△)
20%(v/v) glycerol (▲—▲—▲)

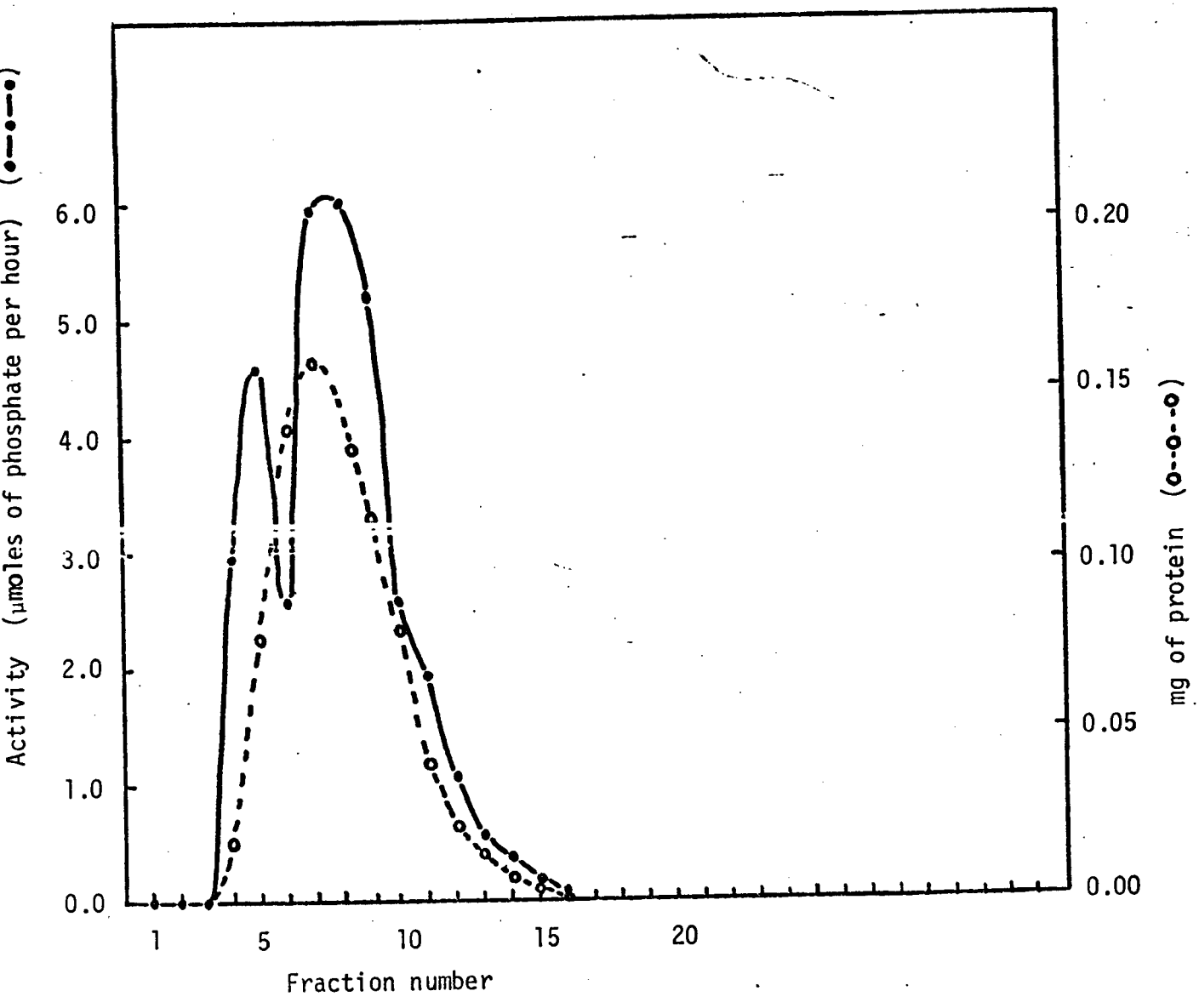

Fig. 10 Sepharose 4B filtration as control for affinity chromatography
$Na^{+}K^{+}$-activated ATPase activity (•—•—•)
Protein measured by fluorescence method (o--o--o)

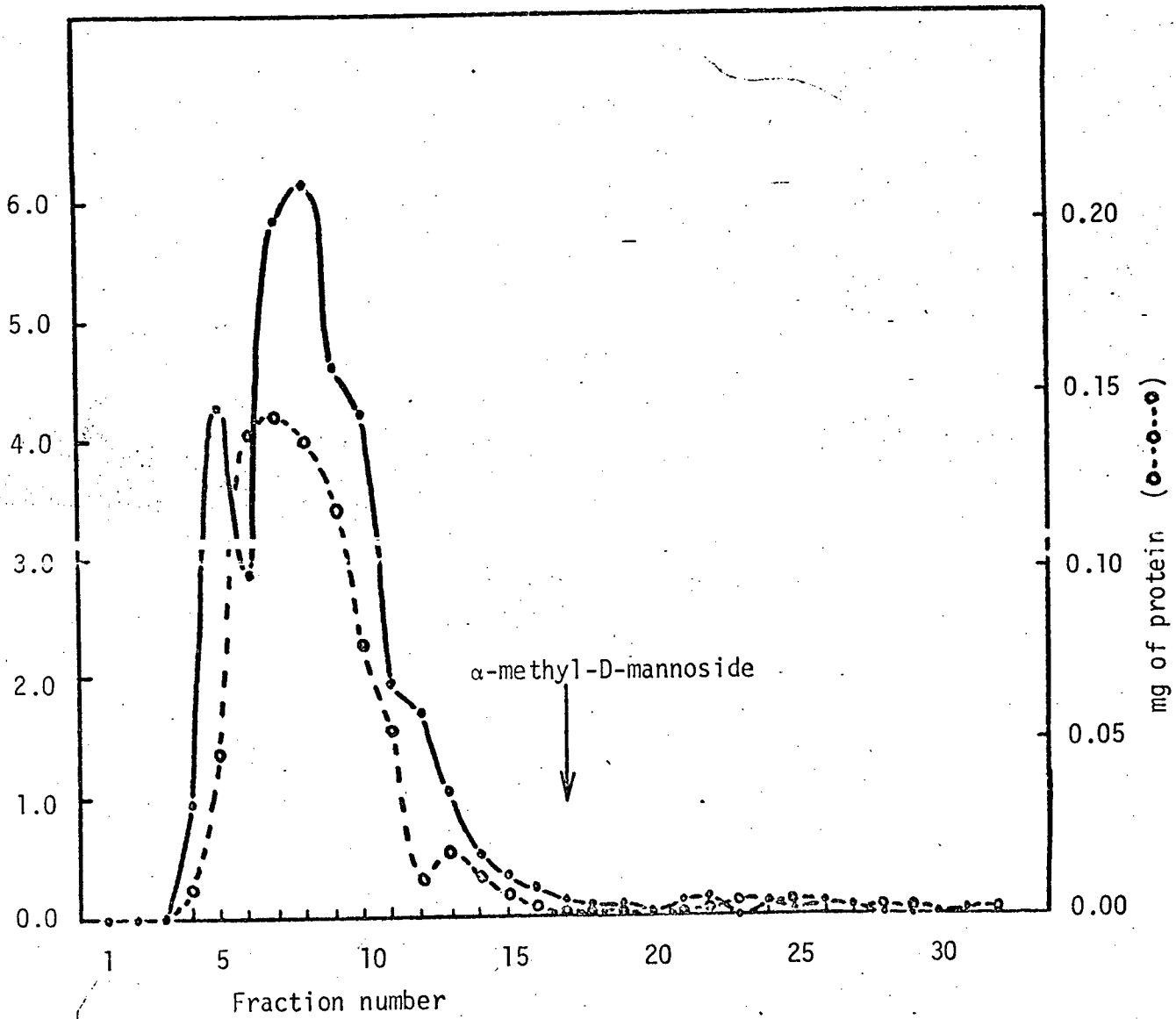

Fig. 11 Concanavalin A affinity chromatography
Na^+K^+-activated ATPase activity (o—o—o)
Protein measured by fluorescence method (o--o--o)

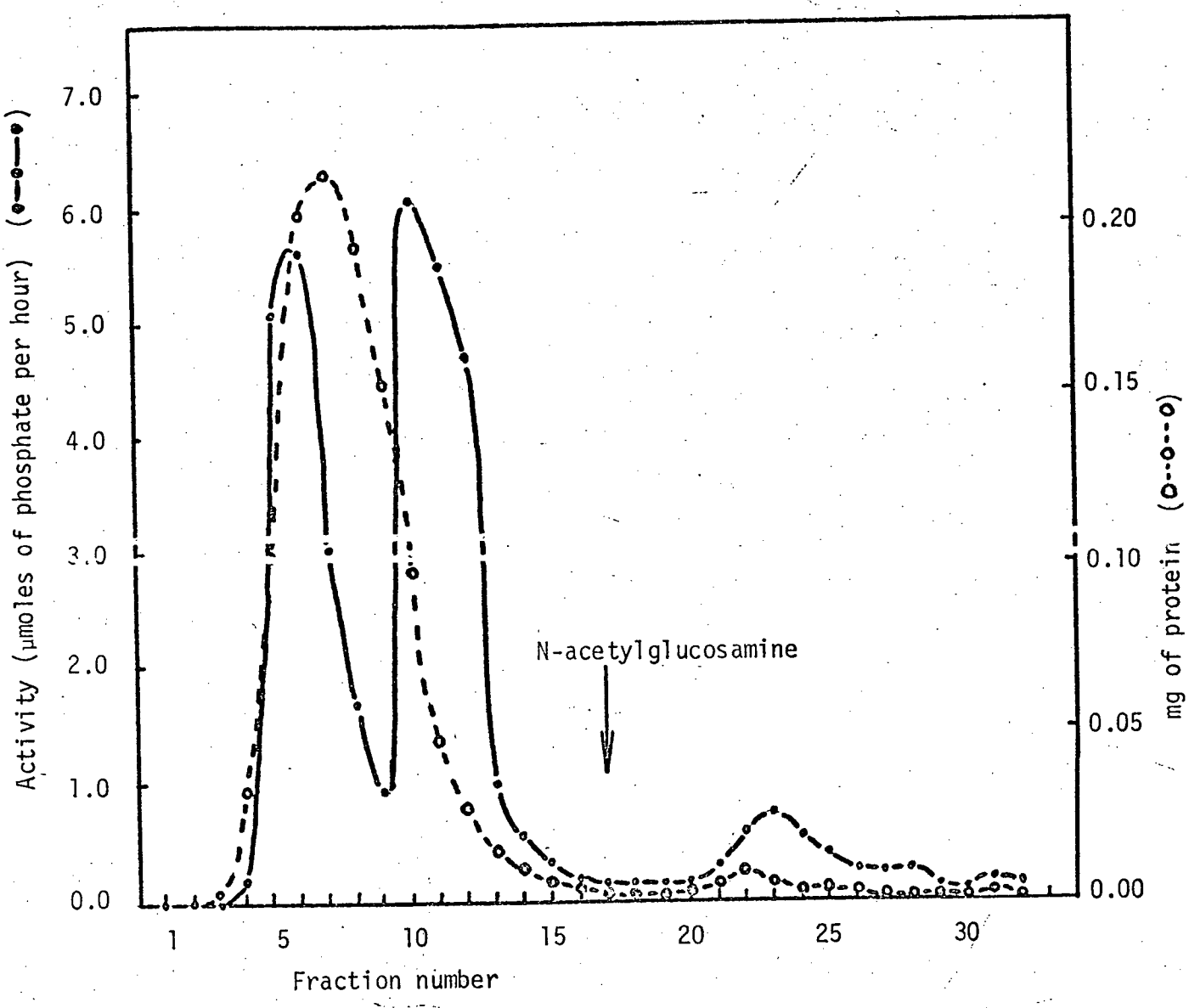

Fig. 12 Wheat germ agglutinin affinity chromatography
Na^+K^+-activated ATPase activity (o—o—o)
Protein measured by fluorescence method (o--o--o)

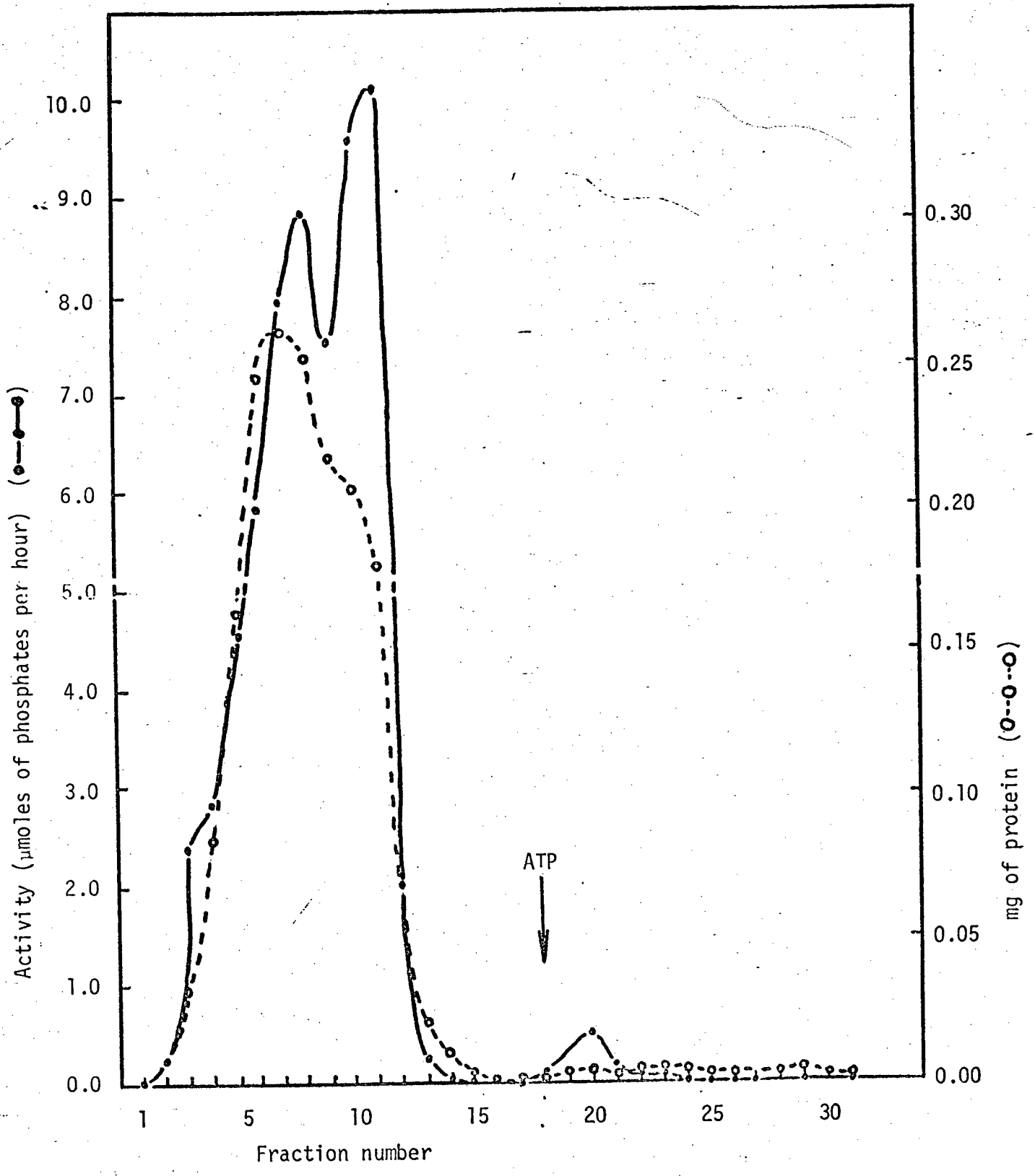

Fig. 13 Periodate oxidized ATP affinity chromatography
Na^+K^+-activated ATPase activity (●—●—●)
Protein measured by fluorescence method (O--O--O)

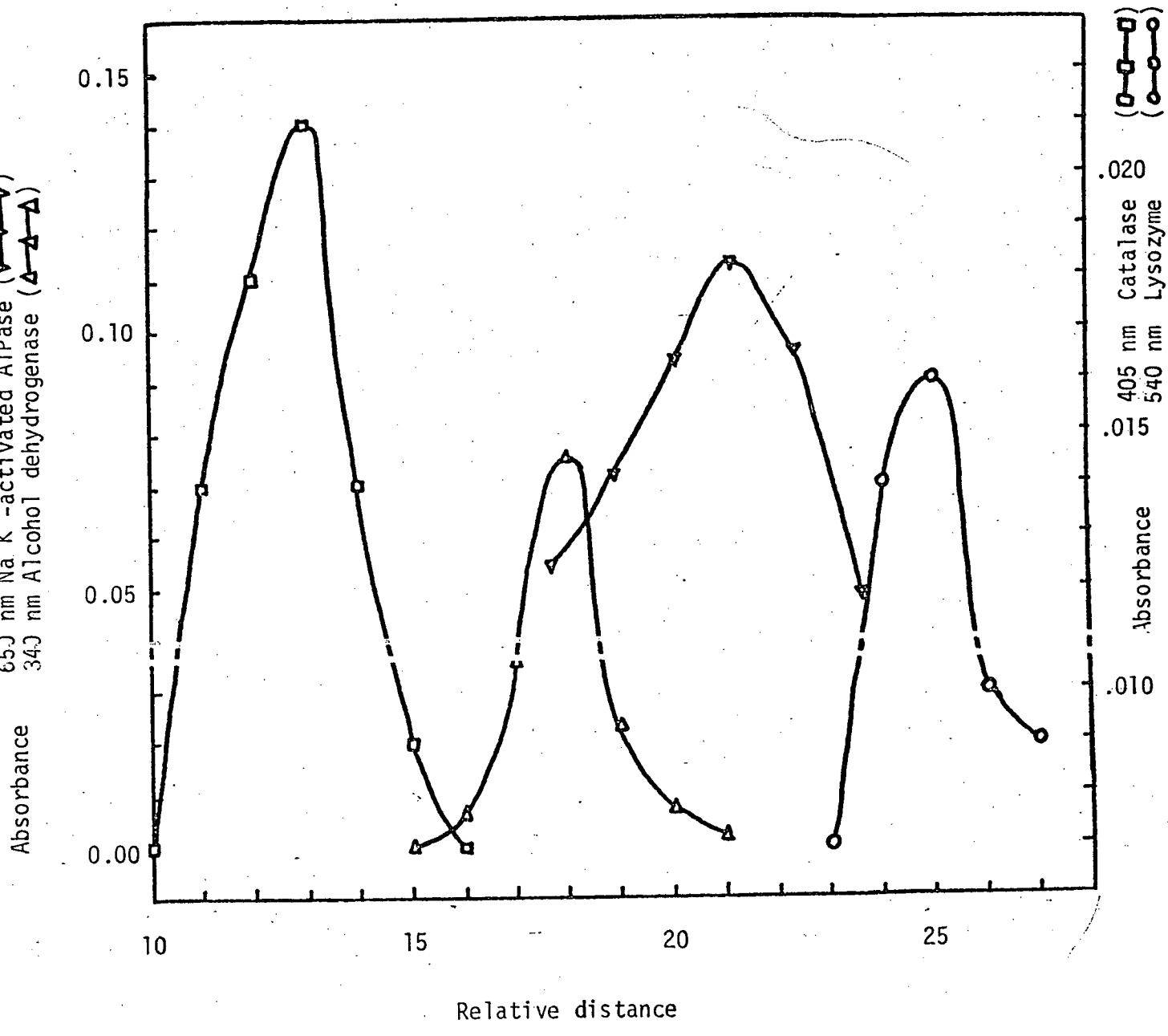

Fig. 14 Enzyme activity profile in sucrose gradient after centrifugation

Catalase (□—□—□)
Alcohol dehydrogenase (△—△—△)
Na^+K^+-activated ATPase (▽—▽—▽)
Lysozyme (○—○—○)

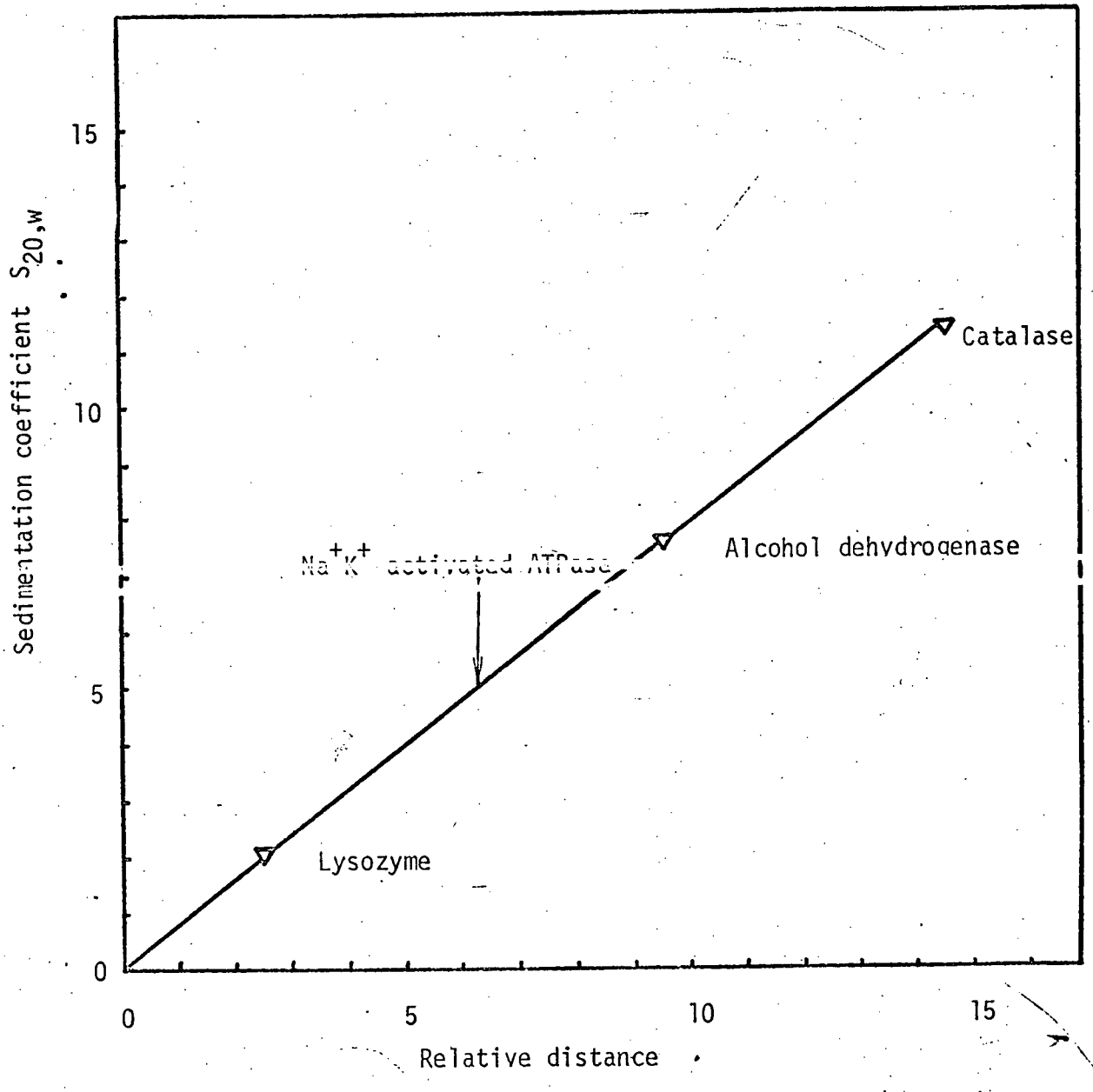

Fig. 15 Velocity sedimentation in an isokinetic sucrose gradient

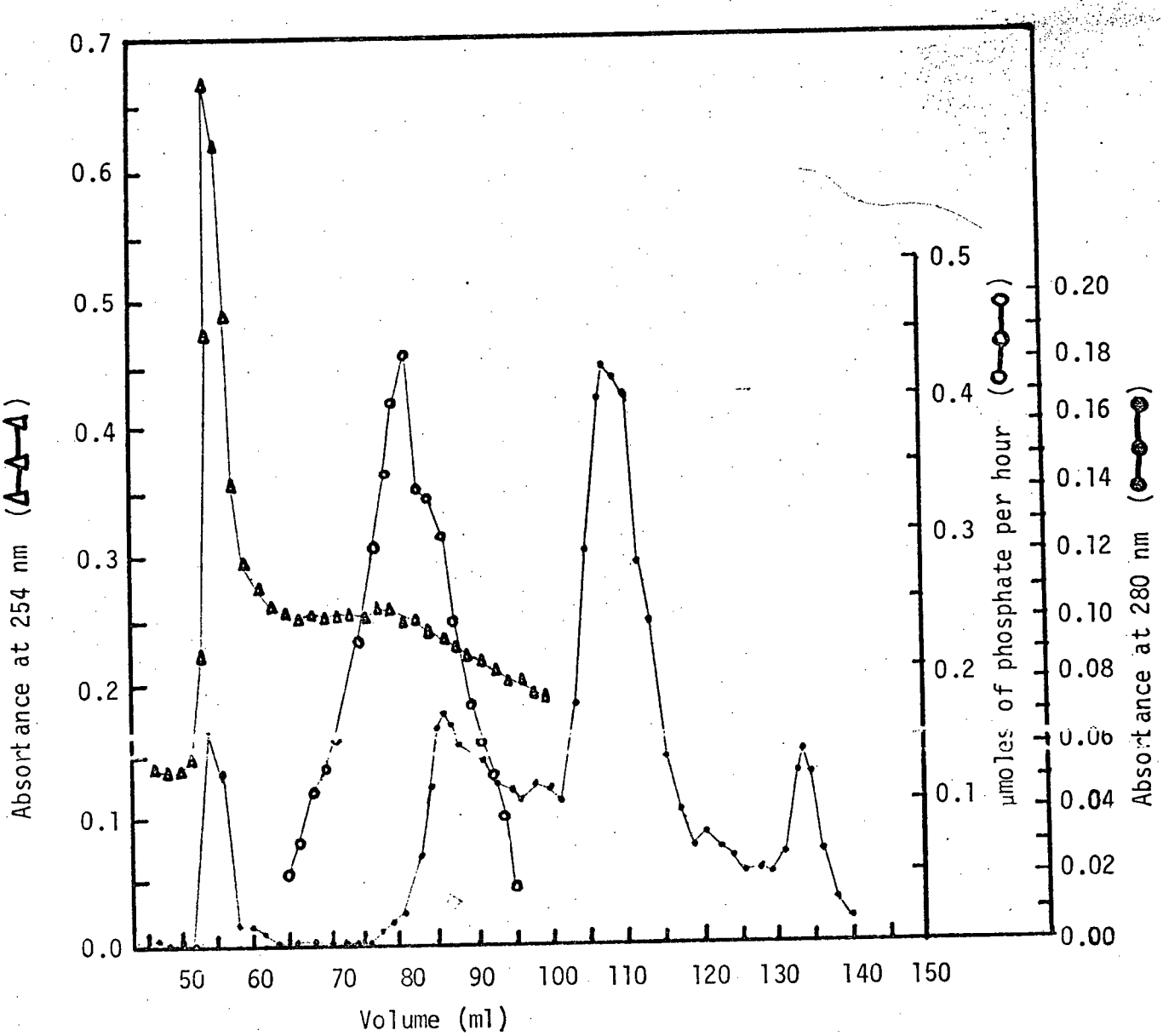

Fig. 16 Sepharose 6B gel filtration of Na^+K^+-activated ATPase & standard proteins

Blue Dextran 2000 (Δ—Δ—Δ)
Na^+K^+-activated ATPase (O—O—O)
Standard proteins (⊛—⊛—⊛)

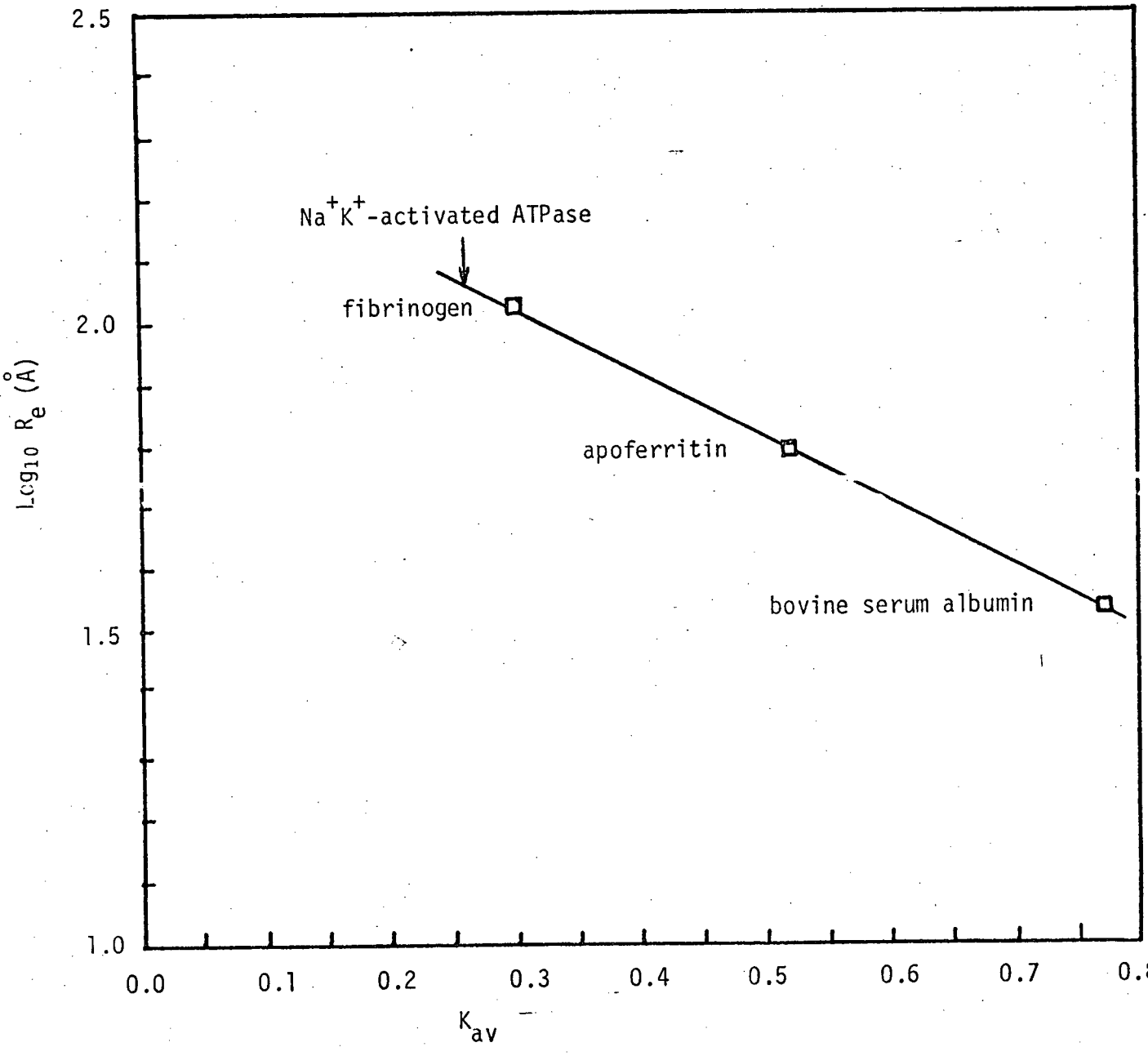

Fig. 17 Stokes' radius as a function of partition coefficient

DISCUSSION

(1) Extraction of Na^+K^+-activated ATPase

From Table VI, it is obvious that out of the four detergents tested, Lubrol WX is best suited for the extraction of Na^+K^+-activated ATPase from the rectal gland of dog fish. Although trimethyldodecylammonium bromide was not useful for this work, it has been found to be promising in the extraction of acetylcholinesterase from the electroplax of Electric Eels and Torpedoes (92), and could be a potentially useful detergent for other membrane bound proteins. Lubrol WX was therefore chosen for this work. It has been observed that a detergent which could be used for extraction at low concentration could become inhibitory at higher concentrations (93). Thus, it is desirable to investigate extraction as a function of Lubrol WX concentration. Fig. 5 shows that extraction of both Na^+K^+-activated ATPase and total protein increases for increasing Lubrol WX concentration, but the increase in total protein is less drastic when Lubrol WX concentration is higher than 0.5% (w/v), such that the specific activity increases moderately with Lubrol WX concentration. It appears that high Lubrol WX concentration can be used to advantage, but the threshold of its solubility at 5°C is approximately 2% (w/v). Hence 1.5% (w/v) was chosen for routine extraction.

The impure enzyme is highly unstable once detergent-extracted, but glycerol is known to stabilize it (7). The effects of the presence of 5, 10,and 20% (v/v) glycerol during extraction were shown in Fig. 6, 7, and 8. The presence of glycerol does not significantly change the extraction. In order to find the optimum concentration of glycerol for stabilization, the activity of the enzyme extracted in different concentrations of glycerol was followed with time and the results were shown in Fig. 9. It can be seen that without glycerol for stabilization, the activity falls to less than half the original value upon storage at 4°C for two days. The enzyme is more stable in 5% (v/v) glycerol, and even more so in 10% (v/v) glycerol, but there is no detectable improvement in stability from 10% to 20%. Hence 10% (v/v) glycerol was routinely used for stabilization.

(2) Affinity chromatography

Concanavalin A and wheat germ agglutinin are haemagglutinating agents known to have a strong affinity for D-glucoside or D-mannoside (94) and N-acetylglucosamine (90) respectively. Concanavalin A has been applied successfully for the purification of Rhodopsin (61), and it is also known to bind acetylcholinesterase strongly (95). Since Na^+K^+-activated ATPase is known to be a glycoprotein (7,11), it was hoped that the carbohydrate portion might contain either D-glucose, D-mannose or N-acetylglucosamine at the nonreducing end and be accessible to

Concanavalin A or wheat germ agglutinin.

As shown in Fig. 11 and 12, neither of these ligands was able to bind Na^+K^+-activated ATPase strongly enough. The activity and protein profile of the Concanavalin A column in Fig. 11 are essentially the same as that of the control in Fig. 10. No activity could be detected upon elution with α-D-methylmannoside. The initial peak at fraction 5 was probably due to large aggregates of proteins eluting in the void volume. The activity profile of the wheat germ agglutinin column in Fig. 12 suggests that there might have been some weak binding. A small peak of activity appeared in fraction 21 to 26 after elution of N-acetylglucosamine started, but it only accounts for approximately 4% of the total input activity.

As shown in Fig. 13, the periodate oxidized ATP ligand did not have a strong affinity for Na^+K^+-activated ATPase. No significant activity could be detected upon elution with ATP. The failure of binding is not due to hydrolysis of the ligand. Inorganic phosphate was found to be absent in fraction 18 to 31, and a phosphate analysis of the gel recovered after the experiment showed that it contained essentially the same concentration of intact ligands as before. These results suggest that an intact ribose ring may be necessary for proper binding to occur.

The total amount of ligand was approximately 100 fold in excess of the total amount of Na^+K^+-activated ATPase used, on a one to one binding basis for Concanavalin A and wheat germ agglutinin. For periodate oxidized ATP it was more than 1,000 fold in excess. In all three cases the recovery of total protein was quantitative (>95%). The recovery of

total activity, including the control, was poor (60% or less). It appears that appreciable amount of Na^+K^+-activated ATPase was denatured in the column.

(3) Estimation of molecular weight and subunit composition

The plot of the sedimentation coefficients of the standard proteins versus their relative distances of migration in Fig. 15 is linear, and the line passes through the origin. These are in agreement with the theory. Fig. 17 shows that the logarithm of the Stokes' radius has a linear relationship with the partition coefficient. It appears that Na^+K^+-activated ATPase is a relatively asymmetric protein. As comparison, β-galactosidase, a protein with an unusually high molecular weight of 515,300 and an effective Stokes' radius of 71 Å (44), consistently eluted after Na^+K^+-activated ATPase in Sepharose 6B gel filtration (data not shown).

The molecular weight of 240,000 for Na^+K^+-activated ATPase estimated from the combined result of velocity sedimentation and gel filtration is that of the catalytically active subunit assembly because the enzyme was detected in these methods by its own activity. Sodium dodecyl sulfate gel electrophoresis measured the molecular weights of the separated subunits. The molecular weight of 250,000 obtained for Na^+K^+-activated ATPase from the rectal gland of dog fish and electroplax of electric eels is based on the assumption of an $\alpha_2\beta$ subunit structure (11,16,48), as

described in part I of the introduction. The agreement between the molecular weight obtained from this work and those mentioned above suggests that $\alpha_2\beta$ is the correct subunit structure of the intact enzyme in solution.

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