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Association of Glycolytic Enzymes with Structural Proteins

by

Wayne William Bronstein

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DEDICATION

To my wife Debbie, whose inspiration, encouragement and patience is deeply appreciated.

To my parents, for their invaluable support throughout the course of this achievement.

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Abstract

The thesis contains data which supports the contention that glycolytic enzymes may interact with subcellular particulate structures. The structural proteins of skeletal muscle I band myofibrils may provide a base structure with which individual glycolytic enzymes may associate. Data presented in the literature review provide some evidence for enzyme interaction with structural muscle proteins. However, with the exception of aldolase, the studies did not indicate specific interactions between individual glycolytic enzymes and I band proteins. Previous attempts involved sedimentation of a mixture of enzymes with a purified structural protein preparation which provides a system for many types of protein-protein interactions. The strategy presented here involved covalent linkage of an actin or actin complex ligand to cyanogen bromide activated Sepharose 4B which served as an affinity matrix for enzymes passed through separate chromatographic columns. Enzyme activity was utilized as a marker in order to measure the degree of enzyme adsorption to the various actin columns. The ligands covalently coupled to Sepharose were comprised of purified F actin, G actin, an F-actin-tropomyosin complex cross-linked with glutaraldehyde and F actin which was also glutaraldehyde cross-linked. In an attempt to determine the degree of association of glycolytic enzymes

with the actin containing columns, individual enzymes were passed through each column at low ionic strength (0.015). A gradient of increasing ionic strength was then applied to the columns in order to release any bound enzyme. Fractions were collected and assayed. Certain glycolytic enzymes adsorbed to the various columns with different degrees of affinity. Triosephosphate isomerase, glucosephosphate isomerase, phosphoglycerate mutase, enolase and creatine phosphokinase did not adhere to the matrices. The enzymes which were found to adsorb in the columns were aldolase, glyceraldehydephosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase and yeast phosphoglycerate kinase. Experiments demonstrated that glutaraldehyde cross-linked F actin provided a better ligand for enzyme interaction than did the untreated F actin. The enzymes which adsorbed provided the following profile in order of decreasing affinity; F-actin-tropomyosin > F actin glutaraldehyde cross-linked > F actin > G actin. These data support the view that the contractile proteins may serve as a site or structure for the organization and possible close association of glycolytic enzymes in the I band muscle thin filament region. This type of interaction may play an important role in the energy production in muscle cells.

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LIST OF ABBREVIATIONS

HK	hexokinase (EC. 2.7.1.1)
PHI	phosphohexose isomerase (EC. 5.3.1.9)
PGI	phosphoglucoisomerase
PFK	phosphofructokinase (EC. 2.7.1.11)
ALD	aldolase (EC. 4.1.2.b)
TPI	triosephosphate isomerase (EC. 5.3.1.1)
GAPDH	glyceraldehydephosphate dehydrogenase (EC. 1.2.1.12)
PGK	phosphoglycerate kinase (EC. 2.7.2.3)
PGM	phosphoglycerate mutase (EC. 2.7.5.3)
E	enolase (EC. 4.3.1.11)
PK	pyruvate kinase (EC. 2.7.1.40)
LDH	lactate dehydrogenase (EC. 1.1.1.27)
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CK	creatine phosphokinase (EC. 2.7.3.2)
DHAP	dihydroxyacetone phosphate
K_m	Michaelis-Menten constant
V_{max}	maximum velocity
NAD	nicotinamide adenine dinucleotide
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
SDS	sodium dodecyl sulfate
F-A	F actin
G-A	G actin
T_m	tropomyosin
F-A-Tm	F-actin-tropomyosin
Ga, Gl	glutaraldehyde
F-A-Gl	F actin glutaraldehyde cross-linked
HEPES	N-2-Hydroxyethylpiperazine-N'2-ethanesulfonic acid
TRIS	TRIS (hydroxymethyl) aminomethane

Association of Glycolytic Enzymes with Structural Proteins

Introduction

The glycolytic enzymes have been traditionally viewed as residing in the cell cytoplasm as soluble unattached macromolecules. This concept was derived from the fact that these enzymes could be easily extracted in dilute aqueous solutions from disrupted cells. The question of compartmentation of these enzymes within a microenvironment has attracted a great deal of interest in recent years (1,2). There are several structural compartments in eukaryotic cells involved with the various phases of carbohydrate metabolism. The Krebs cycle enzymes are considered to be mitochondrial in location; enzymes of glycogen metabolism are complexed to glycogen particles (3), while the enzymes involved in the pentose phosphate shunt appear to be microsomal (4). Within these separate cytosolic compartments the possibility of a discrete organization of glycolytic enzymes exists. The cytoplasm <u>in vivo</u> is gel like and composed of a large number of organelles with multiple compartments separated and connected by a variety of different membranes and particulate structures such as microfilaments and microtubules. The fact that the cellular milieu consists of a highly concentrated protein solution with structural barriers suggests a possibility for definite organization

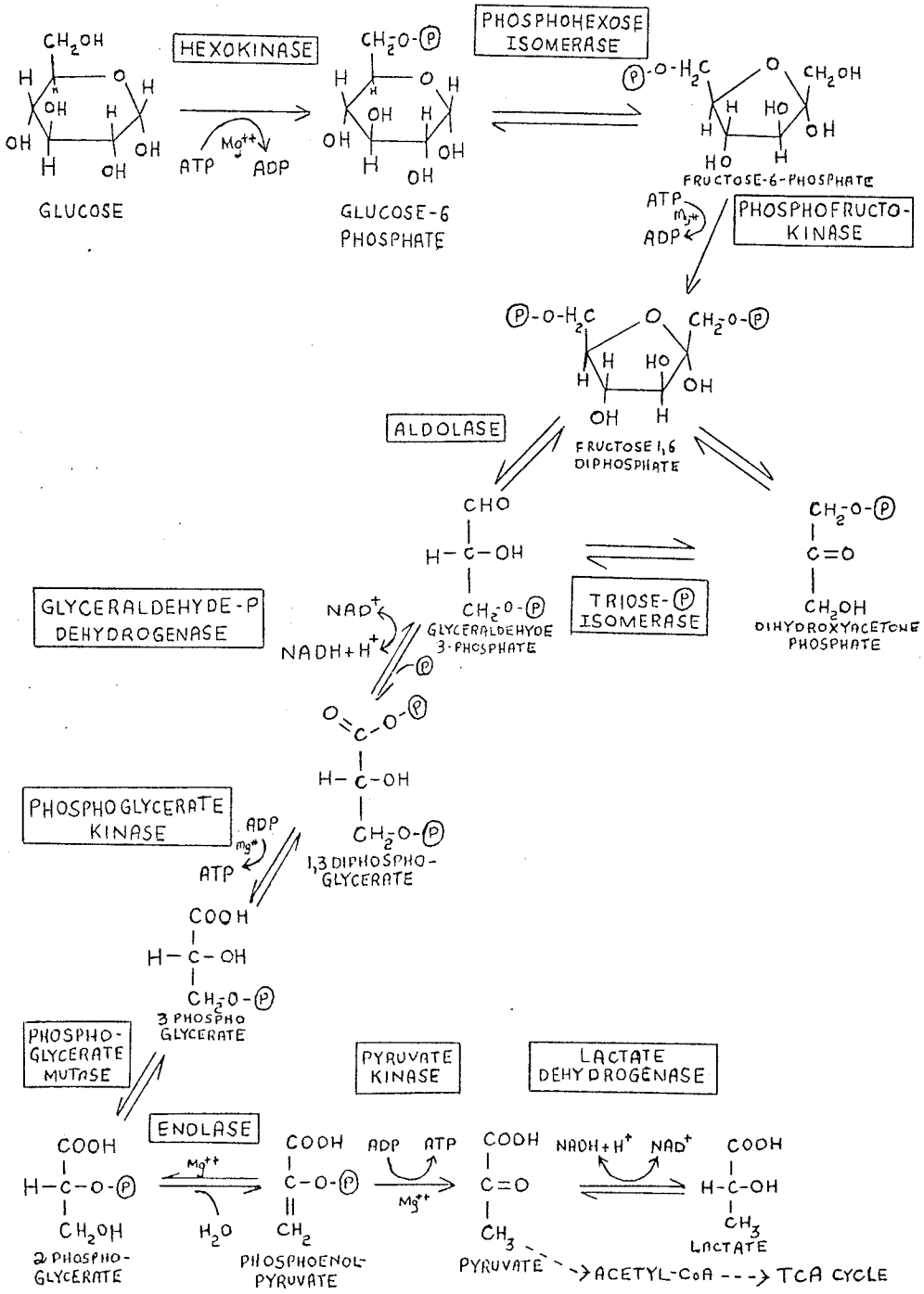
of the glycolytic enzymes rather than a disoriented random arrangement. It is important to note that the disruption of cellular structures and the dilution of the cytoplasm during tissue homogenization and fractionation produces a medium of lower protein concentration that can not be equated to the cellular environment in vivo. Evidence is accumulating that indicates glycolytic enzymes do associate with various subcellular components. In particular, certain glycolytic enzymes appear to reversibly interact with varying degrees of specificity to the skeletal muscle thin filament structural proteins of the I band; F actin, tropomyosin and troponin.

Metabolism of D-glucose for production of energy can be accomplished in two ways. Glycolysis (Embden-Meyerhof pathway) takes place in the absence or presence of oxygen and involves the breakdown of glucose into two pyruvate molecules. Under anaerobic conditions pyruvate is converted to lactate. Under aerobic conditions, pyruvate is oxidized in the mitochondria by way of the Krebs cycle. The glycolytic pathway involves a series of eleven reactions each catalyzed by a particular enzyme. A consideration of metabolic control reveals that certain key enzymes regulate the process. Notably, hexokinase, phosphofructokinase and pyruvate kinase activities can be influenced by interactions with metabolites which can activate or inhibit enzyme activity. The HK reaction is regulated by the levels

of glucose-6-phosphate, adenine nucleotides and Pi (5). PFK is influenced by ATP, ADP, AMP, fructose diphosphate, fructose-6-phosphate, citrate and phosphate (6) while PK is altered by phosphoenolpyuvate, fructose diphosphate, ATP, creatine phosphate and cations such as Mg^{2+}, K^{+} and NH_4^{+} (7). Even though all these factors are believed to regulate the rate of glucose metabolism, further control features are needed to account for the observed regulation of glucose catabolism (8). In view of the physiological reality of the cell's interior, the possibility of interaction of glycolytic enzymes with subcellular particles may provide alternate means of influencing enzyme catalysis and the regulation of the metabolic process as a whole. Adsorption of glycolytic enzymes to subcellular particles such as the thin filament proteins may increase the concentration of the enzymes at specific sites allowing for increased efficiency in glycolytic energy production.

Fig. 1. Diagrammatic representation of the glycolytic pathway.

GLYCOLYSIS


HEXOKINASE
ATP
ADP
Mg++
GLUCOSE
GLUCOSE-6 PHOSPHATE
PHOSPHOHEXOSE ISOMERASE
FRUCTOSE-6-PHOSPHATE
PHOSPHOFRUCTO-KINASE
FRUCTOSE 1,6 DIPHOSPHATE
ALDOLASE
GLYCERALDEHYDE-P DEHYDROGENASE
NAD+
NADH+H+
GLYCERALDEHYDE 3-PHOSPHATE
TRIOSE-P ISOMERASE
DIHYDROXYACETONE PHOSPHATE
PHOSPHOGLYCERATE KINASE
1,3 DIPHOSPHO-GLYCERATE
3 PHOSPHO GLYCERATE
PHOSPHO-GLYCERATE MUTASE
ENOLASE
2 PHOSPHO-GLYCERATE
PHOSPHOENOL-PYRUVATE
PYRUVATE KINASE
LACTATE DEHYDROGENASE
PYRUVATE
LACTATE
ACETYL-CoA
TCA CYCLE

Theory

The classical concept of glycolytic enzymes being homogeneously distributed throughout the cytoplasm requires reconsideration in view of recent reports. A large number of studies now support the realistic contention of glycolytic enzymes organizing by means of weakly interacting forces or perhaps in association with stable subcellular components. The purpose of this study is intended to illustrate the degree of glycolytic enzyme interaction with structural proteins of the skeletal muscle I band.

The majority of studies in this area involved ultracentrifugation of enzyme mixtures from muscle homogenates with purified actin and its associated contractile proteins (refer to pp 8, literature review). Duplication of the ultracentrifugation studies of Clarke and Masters (9) was initially attempted. F actin was suspended with a muscle myogen preparation (defined pp 10) and pelleted at forces greater than 100,000 x g. The data obtained from several experiments were not acceptable due to difficulty found in the quantitation of glycolytic enzymes in the supernatant and F actin pellet. A typical study is shown in Table 1. A total decrease in enzyme activity was consistently found in samples assayed after centrifugation. The results, however, did suggest

TABLE 1. EFFECT OF IONIC STRENGTH ON THE ADSORPTION OF GLYCOLYTIC ENZYMES TO F ACTIN

The mixtures contained 9.0 mg myogen and 2.0 mg F actin in a final volume of 2.5 ml of 0.005 M imidazole, 0.001 M $MgCl_2$, 2.0 mM β-mercaptoethanol, pH=6.8 which included 5.0 mM or 150 mM KCl.

SAMPLE	FRACTION	LDH	PK	ALD	GK	PHI	TPI
muscle	S	1.66	2.90	0.59	1.54	0.28	6.18
myogen	P	0.59	0.53	0.03	0.01	0.03	0.52
myogen	S	1.43	3.11	0.28	1.47	0.33	6.13
+ actin (5 mM KCl)	P	0.32	0.90	0.25	0.23	0.06	0.88
myogen	S	1.26	2.94	0.69	1.22	0.29	5.96
+ actin (150 mM KCl)	P	0.51	0.98	0.10	0.34	0.11	0.97

association of glycolytic enzymes with actin.

This scheme did not allow for differentiation of enzyme-enzyme interaction and enzyme-actin interaction. The techniques employed to identify the possible existence of enzyme interaction involved devising a biospecific affinity system between actin and individual glycolytic enzymes.

Model

The model described herein represents a means of illustrating specific interactions between glycolytic enzymes and thin filament structural proteins. F actin, G actin and F-actin-tropomyosin are each covalently immobilized to affinity support columns. Each of the ligands serves as a receptor to which individual glycolytic enzymes may associate. Enzymes are passed through the separate columns and the columns are washed with additional buffer in order to facilitate release of unbound enzymes. Enzymes that are bound to the column are then released by application of a continuous gradient of increasing ionic strength. The advantages of such a technique include: 1) quantitation of enzyme bound to each column, 2) investigation as to the nature of interaction, eg. electrostatic, hydrophobic, 3) indication of the relative binding affinities of each enzyme, 4) determination of the relative amounts of each enzyme in association with actin

and associated proteins, and, 5) study of enzyme-enzyme interactions as a consequence of binding of one enzyme to actin. Results of these studies may provide credence to the existence of a localized complex of glycolytic enzymes within the muscle I band *in vivo*.

LITERATURE REVIEW

A review of the literature supports the contention that glycolytic enzymes may interact with various types of subcellular components or interact with each other. Interaction has been observed in many cell types ranging from unicellular organisms to mammalian tissue cells.

Evidence for the Existence of a Glycolytic Complex or Particle

An active particle bound lactate dehydrogenase (LDH) sediments in homogenates of the ciliated protozoa Tetrahymena pyriformus (10). The LDH is retained by the particles even after detergent treatment suggesting that the enzyme is firmly bound in the cell and not an artifact of fractionation procedures. A later study established the localization of other glycolytic enzymes in this organism (11). At pH 6.8 most of the hexokinase (HK) activity occurred in the 100,000 x g supernatant fraction while at pH 7.9 most of the HK activity was bound to a mitochondrial fraction (ie. binding was dependent on pH).

A number of workers have searched for the existence of a "multienzyme glycolytic complex or particle". In the unicellular organism trypanasome, a stable microbody-like

organelle containing all the glycolytic enzymes in the sequence HK to phosphoglycerate kinase (PGK) and glycerol kinase {except for triosephosphate isomerase (TPI)} has been identified and termed the "glycosome" (12). The search for such a complex has been attempted in other cells . Recent data indicates that several glycolytic enzymes from E. coli K-12 can self aggregate (13,14). Application of this fraction to a gel chromatography column gave an elution pattern resembling an aggregate of high molecular weight. Electron microscopic pictures showed a specific aggregate. The fraction contained all of the glycolytic enzymes in equimolar proportions and the catalytic ability of the particle could be altered by a decrease in the protein concentration in the concentrated enzyme fraction.

It was also discovered that glycolytic enzymes present in many tissues are found in equimolar amounts. Pette et al were the first workers to show the activities of five enzymes-TPI, glyceraldehydephosphate dehydrogenase (GAPDH), PGK, phosphoglycerate mutase (PGM), and enolase (E) had a constant ratio in muscle types from different species (15,16,17). They postulated that these enzymes, referred to as the phosphotriose-glycerate (PTG) group or constant proportion group, were organized into a constant proportion multi-enzyme complex. Attempts to identify such a complex included application of the techniques used in gel filtration, DEAE ion exhange chromatography,

ultracentrifugation and kinetic analysis. Other data did not support the hypothesis of the existence of a constant proportion complex. No evidence was obtained that this group existed as a multi-enzyme complex in homogenates of chicken breast muscle (18). Deduve was unsuccessful in locating a glycolytic complex in liver cytoplasm (19). However, a specific aggregate of glycolytic enzymes in a skeletal muscle myogen preparation, a muscle extract to containing mostly glycolytic enzymes (at high u) , was shown to form a single slow moving peak during boundary electrophoresis, compatible with the behavior of a complex (20). In another study, utilizing the same technique, complexes were documented where several in vivo conditions were approximated ie. in vivo ionic strength, pH, protein concentration, myosin concentration and delta protein (a tropomyosin polymer) (21). It was also observed that several glycolytic enzymes in a myogen preparation co-sedimented (22). This association was aided by the presence of myosin.

Other data has indicated that glycolytic enzymes may associate or interact with each other. Foldi (23) examined the possibility of aldolase (ALD) binding to some other component in a concentrated rabbit muscle extract (myogen) using frontal analysis gel filtration. Myogen was applied at 20^{o} C to a Sephadex G-200 column equilibrated with buffer. It was found that the pure ALD eluted from the

column as expected on the basis of molecular weight, however ALD in the myogen preparation had an elution profile displaced towards a higher molecular weight indicating a complex formation with other macromolecules in the extract.

Sedimentation studies on a rat muscle cytoplasmic fraction provided further evidence of a multienzyme aggregate. A dialysed myogen preparation (24) approximating physiological conditions was centrifuged at high speeds along with control samples containing commercially purified enzymes (25). It was found that LDH, PK, ALD, and PFK sedimented at a much faster rate in the myogen than expected for the individual enzymes. It was also noted that this complex was very sensitive to factors such as pH, ionic strength, metabolites and the concentration of protein. Other tests for macromolecular association have been done by rapid kinetic analysis. An interaction was found between purified rabbit muscle ALD and GAPDH. Kinetic analysis indicated the reactions were more rapid than a random collision mechanism based on independent assay of each enzyme involved in the consecutive reactions catalyzed by this coupled enzyme sequence (26). It was also noted that ALD activity in myogen was augmented when GAPDH was added (27). These data suggest that enzymatic activities of adjacent metabolic sequence enzymes may be enhanced or

reduced by each other as a mechanism in which metabolic rates are regulated *in vivo*.

The existence of a multi-glycolytic enzyme complex has been postulated by many investigators. The proof for such a complex is lacking, which is probably attributable to the lack of well-researched examples of weak protein-protein interactions in the literature. Even though tightly bound multienzyme complexes are evident such as the pyruvate dehydrogenase complex and weaker interacting complexes have been found like the lactose synthetase and tryptophan synthetase complexes, weakly interacting associations been difficult to demonstrate, ie. glycolytic enzyme-membrane, enzyme-enzyme association.

There is however growing evidence that glycolytic enzymes may interact with a variety of subcellular components and particulate structures in different cell types.

Let us first examine recent work on the previously termed "soluble" (although not a glycolytic enzyme) enzyme creatine kinase (CK). The "M band" of muscle found in the middle of the A band region can be extracted with a dilute salt solution (u=0.05). A major component of this M band is creatine kinase (28,29,30). This type of evidence indicates that it is possible for an enzyme thought to be soluble to have a structural location. An important fraction of the total CK in the tissue is an integral part of the M line

while the rest is soluble or located between the inner and outer mitochondrial membrane. An antibody produced against chicken skeletal muscle CK bound specifically to the M line of the myofibrils (31). Furthermore, the incubation of monovalent F_{ab} fragments with myofibril bound CK solubilized most of the bound CK. The explanation given for the solubilization is that of a conformational change which occurs following CK interaction with F_{ab}. Thus CK no longer binds with the M line binding sites. These data support the view that CK is a physical part of the M line. Clearly it can be noted that there are forces which can bind an enzyme to a cytoplasmic structure in an intact cell even though in vitro low ionic strength (less than u=0.015) solubilizes the complex!

Mitochondria

The interaction of mammalian hexokinase with mitochondria has been documented. Since the observation by Crane and Sols (32) that HK can be found in a soluble and particulate bound form in many tissues, much work has been done involving the nature of the association with mitochondria and the physiological significance of the two forms of HK. A large fraction of the total HK activity is associated with mitochondria in chicken and rat skeletal muscle (33,34), intestinal tissue (34,35,36,37), heart

(38,39) and brain (5,40,41,42,43). Johnson (44) fractionated rat brain homogenates and found that eighty percent or more of the HK was particle bound while the majority of the other enzymes were found mainly in the supernatant fraction. This was an interesting observation since HK is known to be important in controlling glycolysis in brain (8,45). Certain metabolites and inorganic ions were able to cause solubilization of bound HK. These findings led to investigations of HK involvement in the regulation of glycolysis. Contradictory results have been reported. Kosow and Rose (47) examined ascites tumor cells under a variety of conditions but did not find enzyme redistribution. In contrast to their findings, physiological concentrations of glucose-6-phosphate or ATP specifically induced the release of ascites tumor bound HK which was first described by Rose and Warms (46). A number of factors affected the soluble-particulate distribution of HK activity. A high concentration of NaCl (0.3 mM) and ATP (1.6 mM) released ninety percent of the HK activity. Glucose-6-phosphate specifically eluted HK from ascites tumor mitochondria while inorganic phosphate or Mg^{2+} inhibited the elution. The capacity of mitochondria to bind HK was also evaluated. The amount of HK adsorbed varied with the treatment of the enzyme, eg. chymotrypsin caused loss of the capacity to rebind.

Sucrose extracts (0.3 M) of minced pig heart muscle

contained only ten to twenty percent of the total tissue HK activity (38). The particulate enzyme could be solubilized by glucose-6-phosphate and also with an increase in ionic strength and pH. A recent study by Font proved that a significant mitochondrial fraction of pig heart HK is firmly bound to the outer membrane (48). These data are in agreement with the data of Rose and Warms that there appears to be an equilibrium between soluble and mitochondrial bound heart HK.

Other workers have noted that a major portion of HK activity in brain homogenates is bound to mitochondria (49,50). Wilson observed that glucose-6-phosphate could elute bound HK from rat brain mitochondria and favored the view that HK is interconvertible between soluble and particulate forms (49). Approximately sixty percent of the HK activity is overt (51) ie. assayable activity. The other 40% has previously been described as latent. The latent form could be released by membrane disrupting techniques such as rapid freezing and thawing, treatments with detergents and sonification (52,53). ATP and glucose-6-phosphate solubilization was specific for HK as indicated by the higher specific activity of the solubilized enzyme. The specificity of glucose-6-phosphate in the release of bound HK (54,55) and solubilization by ATP has been clearly demonstrated (56). ATP affected solubilization to a greater extent than AMP. The ATP and

glucose-6-phosphate solubilization of HK was also altered in the presence of inorganic phosphate, divalent ions, an increase in temperature and chelating agents such as EDTA and citrate which also solubilized HK. The latent and assayable activities were found to be electrophoretically the same and identified as type 1 (52,57,58). Later Wilson determined the reason for latency of brain HK by fractionation of rat brain homogenates on linear sucrose density gradients (59). He discovered that the latent HK activity corresponded to enzyme trapped in synaptosomes during the homogenization process (59).

Craven and Basford found that the glucose-6-phosphate solubilized rat brain HK is part of a high molecular weight aggregate composed of membrane fragments with cholesterol, phosphatidylethanolamine and lipid composition similar to brain mitochondrial membranes (60). It was speculated that cholesterol or other membrane lipid components may play a role in the binding of brain HK to the mitochondrial membrane but does not affect catalytic activity (61). The addition of free fatty acids to mitochondrial bound HK had a solubilizing effect (62) which may occur during hypoxia as the level of free fatty acids increase (63).

Localization of HK on the membrane has been done by a variety of techniques. Inner and outer mitochondrial membranes were prepared and subjected to maximum HK binding conditions (64). Sixty three percent of the total HK

activity was associated with the outer membrane. At this point it was tentatively concluded that brain HK is localized on the outer membrane. More direct evidence involved fluorescent antibody immunohistochemical localization of HK in thin sections of cortex (65). The resulting fluorescent particles corresponded to the outer membrane mitochondrial fractions. Craven et al demonstrated that the outer membrane fractions had an increased specific activity of HK. In other studies HK binding sites were found on the outer membrane but not found on the inner membrane or on microsomal membranes (46,66,67). Kropp and Wilson verified this and suggested HK be used as a marker for the outer membrane (66).

The association of cellular HK to the outer surfaces of mitochondrial membranes in a form that can be specifically eluted with glucose-6-phosphate, adenine nucleotides and other intracellular metabolites led to investigations suggesting that changes in the soluble-particulate distribution may be of physiological significance. Evidence has been obtained that the soluble and mitochondrial HK forms have significantly different kinetic and regulatory properties. In contrast to these views Purich and Fromm evaluated the <u>in vitro</u> glucose-6-phosphate potential for solubilization of physiological levels of HK, substrates, products and

effectors (68). Their data suggested that there was no significant difference in the distribution of HK with a change in glucose-6-phosphate or Pi concentrations. They also stated that inorganic phosphate did not alter the location of the enzyme. These studies noted that HK partitioning may be of metabolic significance but a change in the glucose-6-phosphate concentration has little effect on the distribution under physiological conditions. Other workers considered the distribution of HK as a means of amplifying glucose-6-phosphate as a regulatory signal.

A measurement of the rate of glucose phosphorylation as compared to intermediate metabolite levels after addition of glucose to Krebs ascites cells revealed changes suggesting that feedback inhibition of HK by glucose-6-phosphate depended in part on the rate of bound HK release from mitochondria (39,69).

There is much evidence in support of significant metabolic and regulatory influence by glucose-6-phosphate. The importance of this metabolite acting as an allosteric effector of HK activity was first conceived by Crane and Sols (32,70). Wilson observed that the sedimentation properties of rat brain HK in sucrose density gradients in the presence of glucose-6-phosphate which induces solubilization of mitochondrially bound enzyme are different from those cenrifuged in the presence of Pi (71).

As the metabolic rate in the brain increases the amount of bound HK increases (72). Compared to the soluble form, the particulate enzyme is more catalytically active due to an increased affinity for ATP and a decreased susceptibility to inhibition by glucose-6-phosphate. These kinetic differences between soluble and mitochondrial HK have been reported in ascites tumor cells (73), bovine brain (58,74), frog skeletal muscle (75) and rat brain (72). A redistribution could influence the regulation of HK activity since binding of HK to mitochondria provides the tissue with a more active HK. The HK reaction may utilize mitochondrial ATP to phosphorylate glucose producing ADP which stimulates further ATP production by the Krebs cycle in mitochondria. This was the basis of an HK acceptor theory of insulin action in which insulin enhances binding of HK to mitochondria. The close association provides HK with access to mitochondrially generated ATP thus furthur stimulating glucose phosphorylation (36,76,77).

All of the above reports have provided information suggesting a distribution between the bound and soluble forms of HK. This has been supported by physiological evidence. Hexokinase has been shown to play a regulatory role in glycolysis in brain (8,45). It was noted that brain cells utilize ATP for glucose metabolism as well as at the membrane sodium/potassium active transport pump. Competitive challenge for this ATP could be damaging to the

cell (50). An increased glycolytic rate in brain is accompanied by decreased ATP and glucose-6-phosphate levels while there is an increase in inorganic phosphate (8). It was found that insulin-induced hypoglycemic chicks or galactose fed chicks, of which have decreased brain energy fluxes, caused the soluble HK (normally 50% soluble: 50% bound) to move to a mitochondrial location (25% soluble: 75% bound). These data indicated that partitioning of HK between the soluble and bound form varies with the brain's energy status. In order to show reversibility of this condition the ischemic chicks were intraperitoneally injected with glucose which increased the ATP, glycolytic intermediate levels and the soluble-particulate HK distribution (50%-50%) to control values (50). This in vivo redistribution between the soluble and particulate HK results in the more active bound form predominating in the cell under conditions of energy stress. The HK particulate form may also undergo reversible desorption when induced by glucose-6-phosphate and ATP as noted by others (38,46,49,78). HK control via this equilibrium has been established as the Ki (for glucose-6-phosphate) for the soluble form (6 uM) is several fold less than for the particulate form (30 uM) (79). This mechanism serves as a fine "tuning" control over the activity of the very important first step in the glycolytic pathway.

Another study included a look at the multiple

molecular forms of HK associated with soluble and several particulate fractions of various tissues of normal and streptozotocin diabetic rats (51). Most of the tissues in the rat contain three molecular forms of HK designated as HK 1, 11 and 111. In addition to these three, liver has a fourth type designated type 1V or liver glucokinase. The main form bound to hepatic mitochondria is type 11 (76,36). In all tissues studied, the only isozymes found associated with mitochondria were types 1 and 11. It was found that the amount of type 11 found in the mitochondrial, sarcoplasmic vesicle rich, and soluble fractions of heart, diaphragm and gastrocnemius muscle from diabetic rats was less than the amount of this type in the same fractions as compared to controls. However, type 11 increased in the soluble fraction of diabetic small intestine. Diabetes had little effect on type 1 HK content in any fractions of brain or kidney. There was an increase in type 111 HK in the soluble fractions of cardiac and skeletal muscles. It appears that tissues most dependent on insulin have predominately type 11 while the brain where the majority of HK is in the bound state and is not insulin dependent has type 1 isozyme. Type 1 is generally bound to mitochondria while type 11 requires insulin to mediate the binding process (33). The role of insulin in binding has been examined. Borrebaek and Spydevold performed <u>in vivo</u> and <u>in vitro</u> binding studies on rat epidymal fat pads and found

increased bound HK (30%) in tissues incubated with insulin (80,81). Intraperitoneal injections of insulin in rats increased the level of HK bound to skeletal muscle mitochondria (77). Other workers showed that an anti-insulin antibody caused a release of bound HK from mitochondria of lactating mammary gland (82). These data provide evidence for the HK acceptor theory of insulin action and indicate that association of HK with mitochondria is not only related to metabolite status ie. ATP and glucose-6-phosphate, but is also related to the hormonal status of the animal (33).

Other glycolytic enzymes have been observed compartmented in cells.

Erythrocyte Membrane

In red blood cells it also been shown that glycolytic enzymes may exist in association with the erythrocyte membrane. Green et al isolated hemoglobin free membranes from red cells hemolyzed in low ionic strength buffer and found that twenty percent of the total glycolytic activity was associated with the membrane (83). The glycolytic complex bound to the membrane and could also be eluted and reassembled by altering the pH. TPI appeared to be tightly bound. Extensively washed red cells still retained some

enzyme activity, ie. ALD, glucose-6-phosphate dehydrogenase (84). Fractions were obtained that could catalyze the complete glycolytic pathway. This apparent binding of glycolytic enzymes to the membrane provided a model system for studying how an enzyme may associate with a biological membrane. Shin and Carraway were among the first workers to find that GAPDH is one of the major protein components associated with the red cell membrane (85). Under hypotonic conditions, sixty to eighty percent of GAPDH remained associated. In the presence of 1 mM ATP, the amount of associated GAPDH was reduced to five from twenty percent. This release could also be effected by treatment of the membrane with a high ionic strength salt solution. GAPDH has now been identified as an integral component of the red cell membrane (85,86) in vivo. The band 6 polypeptide has been established as the monomeric form of GAPDH. It's location is adjacent to the inner surface band 3 peptide as evidence demonstrates it preferentially binds to this site. Band 3 is the predominant polypeptide species spanning the red cell membrane.

McDaniel investigated GAPDH binding to human erythrocyte ghosts (87). A large proportion of the enzyme bound and could be eluted by 0.15 M NaCl at pH 7.5. It was also noted that a freely reversible interaction could occur between enzyme and membrane (81). Specificity in the binding was indicated since GAPDH from human red cells and

rabbit muscle bound similarily to human red cell membranes while yeast GAPDH did not bind nearly as well. Kant and Steck (88) attempted to establish the physiological significance of this association. The binding of GAPDH to membranes decreased with an increase in ionic strength and pH which suggested an electrostatic attraction.

Investigation into the release of membrane bound GAPDH revealed that repeated washings of bound GAPDH with 10 mM Hepes pH 7.2 did not affect, association demonstrating the stability of GAPDH attachment (89). The effects of pH, ionic strength and NAD(H) on the association were also studied (89,90). The influence of electrolytes showed that an ionic strength of up to 0.04 did not release bound enzyme but an ionic strength of between 0.05-0.1 did cause detachment. NADH (200 uM) dissociates all the GAPDH from the membrane while addition of NAD^+ stabilizes the enzyme membrane complex (90). An extension of the previous study has revealed that GAPDH (band 6), associated with band 3, can be solubilized from ghosts with Triton X-100 (91,92,93). The sole site of GAPDH binding <u>in vitro</u> is the inner cytoplasmic surface of the erythrocyte (91,92,94). It's function is believed to facilitate anion transport across the membrane (95). GAPDH associated with band 3 glycoprotein has also been implicated in the transport of phosphate into the erythrocyte (95,96) and the Hela cell (96).

Recent work has shown an ALD binding site on the red cell membrane (97,98) specifically at the inner cytoplasmic surface with band 3 (93,99). Hemoglobin-free ghosts retained fifteen percent of the total cellular ALD. Elution of the endogenous ALD with 1mM fructose diphosphate, high ionic strength (150 mM KCl) and Triton X-100 to membranes in sucrose density gradients has been demonstrated. Utilizing another isolation procedure, up to eighty percent of the cellular ALD could be retained on the human red cell membranes (100). Reassociation of pure erythrocyte ALD to sealed and unsealed ghosts confirmed the exclusive binding site of ALD to be the cytoplasmic surface of band 3. It was also shown that ALD catalytic activity could be decreased in the bound form when attached to either whole membranes, solubilized band 3, or proteolytic fragments from the cytoplasmic surface of band 3. Reduced ALD activity in the bound state *in vivo* may yet have undefined physiological significance.

Phosphoglycerate kinase is also associated with the human erythocyte membrane. Schrier first noted that PGK is embedded in the membrane (101). He proposed that PGK converted 1,3 diphosphoglycerate produced by GAPDH into 3-phosphoglyceric acid plus ATP as an energy supply for the (Na^+-K^+)-ATPase within the membrane. Parker and Hoffman reported a functional interaction between PGK and the ATPase (102). During the transport of ions the energy

requirements of the ATPase are intimately linked to the PGK reaction in the membrane. A recent study showed tight binding of PGK to the red cell membrane which could be eluted with 0.15M NaCl (103). NAD^+ (1 mM) and ADP (0.2 mM) favored binding while NADH and ATP did not. However,a definite coupling of the PGK to the (Na^+-K^+) ATPase was not indicated.

Compartmentation has been recognized, in general, as a structural feature of cell systems in metabolic regulation. Within cells this is achieved within organelles or attachment to membranous structures (104), eg. mitochondria, endoplasmic reticulum. In the absence of membrane imposed organelle compartmentation, organization appears to exist by means of isolated stable enzyme complexes or associations with a basic structure. There may be a microenvironment of weakly interacting proteins or associations with structural elements in a cell that may serve as a convenient and efficient means of regulating metabolism (105,106). A number of advantages of such an organization to the cell include: 1) specific environments to enhance specific reactions (eg. hydrophobic), (107), 2) decreased competition for a substrate in a certain locale (108), 3) decreased diffusion or transit time (109,110), 4) alteration of enzyme catalysis due to conformational change upon association, and 5) allosteric control mechanism

(111).

It appears that in vivo most enzymes function bound in a structured state (ie. membrane bound, complexed to a protein).

Brain

In brain, glycolytic enzymes appear to be assembled with microsomal or membranous particulate components. Aldolase bound to a particulate fraction of rat brain homogenates (112,113,114). The degree of adsorption and desorption of ALD was found to be influenced by ionic strength, pH, and the level of functionally dependent metabolites such as fructose diphosphate and Pi. The binding of ALD isozymes to particulate matter was such that A4 (muscle type) bound firmly while C4 (brain type) weakly adsorbed. The binding characteristics of hybrid forms ie. A2C2, A1C3, demonstrated that addition of an A subunit into the ALD molecule greatly enhanced the affinity of the enzyme for the structural components of the cell. The effect of an increase in ionic strength and pH on the elution of bound aldolase suggests an electrostatic interaction between the enzyme and the structural components. Fractionation studies showed that the microsomal fraction contained the bound form of ALD (mostly

A type) (114). In fact, two-thirds of the total ALD activity was associated with the particulate matter.

Measurement of the activity under Vmax conditions for soluble ALD indicated that the particulate ALD has less activity than the soluble enzyme. Therefore, these data suggest that binding may exert control over the ALD reaction (112,114).

Exposure of the bound enzyme to phospholipase or ribonuclease treatment removed a significant part of the membrane but had little effect on the association of ALD A4 to the microsomal membranes (115). Aldolase adsorption resulted in a reduction in the catalytic activity as compared to the soluble form. This property could provide an *in vivo* control mechanism dependent on the metabolite levels such as fructose diphosphate and Pi.

The subcellular distribution of pyruvate kinase (PK) in the cerebral cortex of rat brain was studied. It appeared in microsomal, mitochondrial and synaptosomal membrane fractions (116). The metabolic importance of PK includes a regulatory role in glycolysis, and it is thought to be involved in ionic transport and lipid biosynthesis. Data indicate that brain PK is 30%-40% particulate while in nerve endings it is 85% particulate. Pyruvate kinase interaction with membrane fractions of rat brain homogenates was shown to be electrostatic (117). The

solubilization of PK occurred with high ionic strength and pH. It was later observed that several glycolytic enzymes had between forty to ninety percent of their activities associated with particulate structures of lysed nerve endings (118). The enzymes were HK, ALD, phosphohexose isomerase (PHI), PFK, GAPDH, PK and LDH. Eighty percent or more of this associated activity could be released with salt treatment (ie. increased u), which indicated an electostatic association. Subfractionation showed that HK and fumarase (a mitochondrial marker) had the highest specific activities in one fraction which is consistent with HK associating with mitochondria in brain.

In squid retinal membrane fractions, certain enzymes of glycolysis were also retained, ie. PGK, PK, GAPDH, ALD, HK and glucose-6-phosphate dehydrogenase (119).

Liver

In liver homogenates, sixty to seventy percent of the total ALD is sedimentable (120). The association was sensitive to changes in pH, salt concentration, and metabolite concentrations. Ultrastructural studies demonstrated ALD associated with the endoplasmic reticulum.

Skeletal Muscle

Association of glycolytic enzymes in muscle systems has been extensively examined.

Histochemical staining patterns of "soluble" glycolytic enzymes in skeletal muscle has been documented. Lactate dehydrogenase has been studied in frog (121) and rabbit skeletal muscle (121,122,123), and in locust flight muscle (124,125). The stained areas appeared in a striated distribution aligned with the I bands (thin filaments) of the myofibrils. GAPDH gave exactly the same visual pattern in the rabbit muscle and locust flight muscle (123,124). Measurements of LDH activity in fractions of homogenized chicken breast muscle unexpectedly showed that a considerable amount of LDH was associated with particles rather than in the soluble phase following centrifugation (126). Increasing amounts of attached LDH were released into the soluble fraction as the pH and ionic strength of the homogenizing buffer was increased. LDH appearance in the particulate fraction is likely due to an interaction with F actin (24,127). A shift towards enzyme solubility also occurred as the tissue concentration in the centrifuged suspension was decreased. Particulate LDH could be eluted by an increase in ionic strength, pH, and by metabolites. NADH (1 mM, and NAD^+ to a lesser extent) could solubilize bound LDH , but at a high protein concentration a significant portion of LDH remained bound. The bound form of LDH also differed kinetically from the soluble form

(128). LDH 5 bound to the particulate structures is not inhibited by pyruvate. In contrast, the soluble form is strongly inhibited. Thus association protects the enzyme from pyruvate inhibition. Also, the inhibition of bound LDH-5 by NAD^+ was eliminated. The K_m (pyruvate) for the soluble enzyme varied as a function of enzyme concentration, but the K_m for the bound form remained constant. Another important finding was that LDH-1 did not bind to particulate structures of skeletal muscle or heart homogenates indicating specificity of binding for type LDH-5. The behavior of LDH in homogenates of trout skeletal muscle was similar to the enzyme in chicken muscle (129).

Mammalian LDH is a tetrameric enzyme composed of two different subunits (M-muscle type) and (H-heart type) yielding five isozymic forms. These isozymes differ in physical, catalytic and immunological properties. LDH-3, (M2,H2) bound to particulate matter and was solubilized with an increase in ionic strength, pH and metabolite levels (130). LDH-1 (H4) did not bind. The concentration of insoluble sedimentable protein also increased the degree of binding of LDH-3 and LDH-5. A report on the kinetic properties of LDH-1, 3 and 5 as a function of temperature revealed that the binding of LDH-3 and LDH-5 decreased the Vmax at cellular temperature (127). Each of the isozymic forms had an increased K_m (pyruvate) in the bound over the soluble form. The K_m also increased as the temperature was

raised from 4-40° C. The K_m for LDH-3 and LDH-5 increased upon binding to the particulate fraction at all temperatures recorded. The data show that the kinetics of chicken LDH isozymes are complex and dependent on the temperature and the state of the enzyme.

The association of LDH isozymes with subcellular fractions in rat kidney and testis was also studied (131). LDH-5 was the only isozyme selectively attached to microsomes while a mitochondrial fraction of testis was found to be rich in LDH-1, 2 and 3. The action of different ionic strength solutions and detergents on the association demonstrated that a large portion of the LDH was firmly bound, even at intracellular ionic strength (u=0.15).

The results with regard to pH and salt elution indicate that ionic interactions are important in the binding of LDH to subcellular particulate fractions. The amount of binding is also influenced by the protein concentration and the NADH level. The distribution between LDH in bound and soluble form is greatly affected by isozymes but the physiological importance of this is unknown at the present time.

Fractionation of chicken breast muscle homogenates in water caused eighty-six percent of the total GAPDH to associate with a particulate fraction (132). Solubilization of GAPDH was enhanced by increasing the ionic strength, or pH (at a low ionic strength 50% became solubilized at pH

7.5), and partial desorption could be effected by presence of glyceraldehyde 3-phosphate or 2,3 diphosphoglycerate. The solubilized GAPDH could also rebind to the particulate fractions. The particulate enzyme had a lower Vmax (7.0 vs 29 uM NAD^+) and K_m (0.07 vs 0.2 mM glyceraldehyde 3-phosphate) than for the soluble form. Binding of glyceraldehyde 3-phosphate to GAPDH has been reported to cause a conformational change in the enzyme (133). It is quite possible that the release of bound enzyme is a result of the alteration in enzyme conformation. ATP (1 mM) did not cause solubilization of GAPDH as has been found for GAPDH binding to muscle actin (134).

Quantitation of the association of phosphofructokinase (PFK) with cellular particles has been found to be difficult due to the enzyme's aggegation and dissaggegation tendencies. There have been however, a few reports on specific location of PFK in cells. Margreth et al (135) observed that twenty-five percent of frog muscle PFK associated with a sarcoplasmic reticulum microsomal fraction. Hofer and Pette (136) found that twenty-five percent of rabbit muscle PFK is not extracted with a sucrose buffer, but a 0.1 M phosphate buffer caused extraction of all the PFK present in the tissue. Craven and Basford suggested that in brain, PFK adsorbed to mitochondria if the ADP concentration was high (20 mM)

(137). They suggested that these conditions would enhance PFK binding to mitochondria at a site of ATP generation which would increase access to ATP to facilitate glycolysis. PFK was particle bound in fractions from guinea pig and sheep heart centrifuged at 24,000xg after 1-2 hours after ischemia (138). The particulate enzyme was inactive but could be activated and solubilized with addition of a solution containing $MgSO_4$, ATP, Tris-Cl, and B-mercaptoethanol at pH 8.0 (6).

The histochemical staining patterns of other glycolytic enzymes using a gel film technique has shown that PFK, ALD, PHI and TPI appear specifically along the I band region of skeletal muscle (123,139). An exception was HK which was located at mitochondrial sites. In order to verify that ALD was localized in the myofibril, Arnold et al (139) extracted ALD from minced rabbit psoas muscle to show the absence of I band staining. Addition of ALD to this same preparation resulted in the reappearance of the stained pattern at the identical intracellular location. It was postulated that localization of these soluble enzymes at particular regions of the I bands is dependent on an attraction or affinity between enzyme and structural protein. Immunofluorescent localization of glycolytic enzyme proteins with rabbit striated skeletal muscle and heart muscle has been demonstrated (140). The enzymes ALD, GAPDH, PFK, LDH and E were located within the isotropic

zones. These data provide strong evidence for a specific *in vivo* association of glycolytic enzymes to actin filaments.

Other approaches utilized in order to study these apparent interactions include ionic strength fractional studies (20,24,141), investigations with press juices (20,142), electrophoretic analysis of protein-protein interactions (21), binding studies with various components (134,143) and affinity chromatography techniques (thesis presentation).

Amberson et al used "press juices" of heart and skeletal muscle to test if glycolytic enzymes were totally soluble. They found that a significant fraction of the total LDH and ALD activity was attached to the muscle matrix (20,142). Pette and co-workers (24,134) investigated the reversible association of ALD and GAPDH in striated muscle. In rat or rabbit muscle homogenates, only sixty to seventy percent of the total ALD activity could be extracted with low ionic strength buffer solutions. The complete extraction of ALD occurred only after a buffer of ionic strength 0.2 was used. It was noted that ALD release from the tissue was dependent on the ionic strength of the extraction solution. The fractional extraction of GAPDH and PFK from muscle tissue was also dependent on this factor as an increase in u was necessary to achieve complete solubilization. In order to identify the muscle component(s) responsible for the association, purified

muscle structural proteins were prepared. A sample of F actin, myosin, actomyosin and stroma protein were mixed with purified ALD and centrifuged at high speed. F actin completely bound the ALD. Bound enzyme was defined as the particulate F actin associated activity determined by desorption (150 mM KCl) or calculation of the difference in enzyme activity assayed prior to centrifugation minus the activity present in the supernatant fraction after centrifugation. Myosin also bound the ALD but to a very small extent as compared to F actin.

The F-actin-enzyme complex could be dissociated in the presence of 150 mM KCl suspended in the myogen extract. Other glycolytic enzymes which sedimented with actin (100,000 x g) were GAPDH, PGK, LDH and PK. Creatine kinase which constitutes part of the M line in muscle did not bind.

Other experiments with muscle tissue provided evidence that glycolytic enzymes would associate with subcellular entities. Research continued in this area and led to the study of ALD and TPI binding to F actin (134). In the case of ALD, the presence of the metabolites fructose diphosphate, ATP, ADP and Pi in the suspension with actin prior to centrifugation led to decreased adsorption. TPI binding to actin was decreased in the presence of glyceraldehyde-3-phosphate. Kinetic studies of ALD

performed under conditions of total binding (ie. low u) resulted in a modification of the catalytic properties of the enzyme in the bound form. The Vmax (for fructose diphosphate) of the bound enzyme was doubled, while the K_m (fructose diphosphate) was increased by two fold. The binding of ALD, GAPDH, LDH and other glycolytic enzymes to F actin was dependent on ionic strength (an increase suppressed the interaction, an increase in pH decreased binding), as well as the presence of ions Mg^{2+} and Ca^{2+}. At pH 6.5 the enzymes were more firmly bound than at pH 7.5 (24,126). Contrary to these findings, the enzymes PGM and E did not bind to F actin while LDH and PGK bound to a lesser degree. This was important as differences were found to exist in the binding of various glycolytic enzymes to actin (24)-(to be discussed later). Centrifugation studies also led to complex formation between a G actin sample and ALD.

Clarke and Masters studied the association of glycolytic enzymes present in a "myogen" preparation of sheep semitendinosus muscle with the structural proteins of the thin filament, F actin, tropomyosin and troponin (9). The degree of enzyme adsorption to the filaments at high centrifugal forces (100,000 x g for 1 hour) was determined by measuring the difference in activity between the uncentrifuged control sample and the supernatant obtained after centrifugation . The enzymes ALD, PK, LDH, GAPDH, PHI and PFK pelleted with the filaments under the conditions

employed while PGK, PGM, E, TPI and HK displayed weak adsorption if any. Interestingly, association also occurred under conditions of physiological ionic strength (u=0.15) if the protein concentration was high. Furthermore, an actin-tropomyosin-troponin ligand increased the degree of binding of the bound enzymes. The presence of Ca^{2+} and fructose diphosphate slightly increased the adsorption of ALD, PK and LDH while there was a decrease in the constant proportion enzyme group. PFK binding to the complex was also demonstrated with a press juice of minced muscle. Creatine kinase did not absorb to F actin nor to F-actin-tropomyosin-troponin.

Recently, Clarke and Masters reported on the formation of aggregates between ALD and actin containing filaments (144). Paracrystalline structures of ALD bound to F actin were viewed under carbon grid electron micrographs. The structures consisted of filaments in an ordered parallel arrangement with regular cross banding at 36 nanometers (nm). Aldolase bound to F-actin-tropomyosin-troponin provided a more extensive and clearer picture. These visual interactions have provided more information as to the nature of ALD interaction with the structural I band proteins. Aldolase appeared to have multiple sites of attachment to the protein complex and also suggested that troponin may be an important component involved in the interaction. Further electron micrograph studies showed

that in the absence of troponin, minor aggegates of ALD attached to the F actin. In the case of the fully reconstituted F-actin-tropomyosin-troponin filament, a well formed lattice was evident (145). The formation of the lattice required a minimum troponin inhibitory (I) and troponin tropomyosin binding subunit structure in association with F actin and tropomyosin. The cross-links appearing between parallel filaments were regarded as being ALD, as no cross-linking appeared in a preparation in the absence of ALD. The filaments were cross-linked at regular intervals of 38.7 nm which coincides with the periodicity of troponin (38.5 nm) calculated from X-ray diffraction patterns. The cross-links and orderly structure appear to be due to ALD contact with troponin. It has not yet been fully established which troponin subunit binds ALD with the greatest affinity.

The kinetic characteristics of ALD were shown to be altered upon binding to actin containing filaments (146). The K_m (fructose diphosphate) and Vmax of ALD bound to pure F actin were similar to the values obtained by Arnold and Pette (134). An ALD-F-actin-tropomyosin association produced slight increases in both the K_m and Vmax (both unaffected by Ca^{2+}). In contrast, ALD binding to F-actin-tropomyosin-troponin caused a hundred fold increase in K_m as compared to the K_m for the soluble ALD, while the

Vmax increased by a factor of four . Another interesting finding was that the presence of Ca^{2+} increased K_m by only twenty fold and Vmax 2.5 times. These values are quite significant in view of the I band composition of skeletal muscle (ie. complexed contractile proteins). Another portion of this study compared the ALD catalytic capacity as a function of fructose diphosphate concentration. At low fructose diphosphate concentration, an increase in the amount of enzyme adsorbed resulted in a decrease in the catalytic potential of the enzyme. At a higher fructose diphosphate concentration (130 um), enzyme adsorption resulted in a greater catalytic activity than would be expected of the soluble enzyme form at an equivalent fructose diphosphate concentration. This type of regulation would be desirable to a cell during rapid glycolysis when the fructose diphosphate concentration is raised (45). Lowering of the K_m with a small decrease in the Vmax in the presence of Ca^{2+} may be conceived as a means of facilitating the attainment of an increased catalytic capacity after initiation of contraction. The biological significance of this data remains to be fully elucidated.

The influence of electrical stimulation on the adsorption of the glycolytic enzymes in muscle under physiological conditions has been examined. These experiments have shown that the soluble-particulate distribution of certain glycolytic enzymes such as ALD, PFK

and GAPDH is affected by the muscle state (147). In sheep muscle, six to ten fold changes in the adsorption of the important control enzyme PFK has been observed on stimulation. These data suggest a functional correlation between enzyme adsorption and the physiological and metabolic status of the muscle. Starlinger also found a connection between enzyme binding and the muscle state (148). Lower ALD catalytic activities were found in low ionic strength extractions of tetanically stimulated muscle than in extracts of resting muscle. This non-extractable ALD was present in a subcellular particulate fraction which could be released by treatment with higher ionic strength solutions. These results correspond to Arnold and Pette's fractional extraction studies of ALD from muscle (24).

Materials

The enzymes, co-factors, substrates, ATP, B-mercaptoethanol and Trizma base and cyanogen bromide activated Sepharose 4B were purchased from Sigma Chemical Co., St. Louis Mo. All inorganic chemicals used were of reagent grade or better. Acrylamide, bisacrylamide, N,N,N′,N′-tetramethylethylene diamine, ammonium peroxydisulfate and Coomasie blue R-250 were from Eastman Kodak (Rochester, New York). Sodium dodecyl sulfate was from Bio Rad Laboratories (Richmond, California). The protein molecular weight markers obtained from Mann Research Lab. Inc. New York, New York, were kindly provided by Dr. E.T. Pritchard.

Methods

Preparation of Actin

The actin utilized in the studies was isolated and purified from rabbit back and hind leg skeletal muscles as described by Spudich and Watt (149). Acetone dried muscle powder was prepared in the cold room (0-4° C) through a series of washes and cheese cloth filtrations of freshly minced muscle (150). Actin was isolated from the acetone powder by a series of three polymerization - depolymerization steps. The initial extraction with 0.1 mM ATP, pH 7.6 was performed for 30 min. The residue was then poured over Whatman #1 filter paper in a Buchner funnel and gently filtered by suction. The remaining residue was re-extracted with ATP for 30 min and filtered once again. The combined filtrates were clarified by ultracentrifugation of the crude actin at 100,000 x g for 30 min at 4° C in an International Preparative Ultracentifuge Model B-60 in an A-321 type rotor (International Equipment Co., Needham Hts., Mass.). The pellet was discarded. Salt was added to the supernatant to a final concentration of 0.05 M KCl, 0.1 mM $MgCl_2$, and 0.05 M tris-nitrate pH 7.6 and the actin was allowed to polymerize (6-12 hrs). The resultant viscous material was

centrifuged at 100,000 x g for 2.5 hr at 4° C. The supernatant was discarded and the gel-like pellet carefully rinsed with ice cold distilled water. This crude F actin (filamentous actin) was depolymerized by the addition of 0.1 mM ATP and centrifuged for one hour to remove impurities. The polymerization-depolymerization procedure was repeated until a purified F actin was obtained as seen in Figure 2. G actin (globular form) was obtained by depolymerization of the final F actin sample by gentle homogenization in a Ten Broeck ground glass homogenizer followed by a final ultracentrifugation step for 2.5 hr to ensure removal of any denatured protein. The G actin was then extensively dialysed against 1.0 mM tris-nitrate pH 7.6 and passed through a Sephadex G-150 column. Purity of the actin during the purification scheme was monitored by sodium dodecyl sulfate gel electrophoresis on 7.5% acrylamide gels (151). A tris-glycine buffer (tris 12g/l, glycine 58g/l, 0.2% SDS) was used in both chambers of a Bio-Rad Model 150-A Gel Electrophoresis Cell. Electrophoresis was performed at 20° C with a constant current of 1.5 ma per gel with a Buchler power source. The gels were stained with Coomassie Brilliant Blue R-250 for 1 hour. The gels were subsequently destained in a solution of 45% methanol and 7% acetic acid for 4 hrs then transferred to 5% methanol and 7% acetic acid. The purified F actin and G actin were judged to be greater than 90% pure as examined

on the SDS gels. The molecular weights of the proteins were calculated from Rf values obtained by co-electrophoresis of samples with the marker proteins bovine serum albumin MW 67,000, myoglobin MW 17,800 and ovalbumin MW 43,000. Muscle tropomyosin was received as a gift from Dr. L. B. Smillie, Department of Biochemistry, MRC Muscle Research Group, University of Alberta, Edmonton, Canada. SDS gel electrophoresis revealed tropomyosin to be a highly purified protein. Cross-linking of F actin and an F-actin-tropomyosin complex was accomplished with fresh glutaraldehyde (Polysciences Inc.)(152,153). The cross-linking of F-actin-tropomyosin was performed with 20 mM glutaraldehyde in 5 mM Hepes, pH 7.0, at 20^{o}C. Ten mg of F actin and 2 mg of tropomyosin in 10 ml of buffer were utilized in each preparation. After 30 min the reaction was quenched with the addition of 0.2 M $NaHSO_3$ in 0.1 M phosphate buffer pH 7.0. The sample was then extensively dialyzed at 4^{o} C against 10 mM sodium phosphate pH 7.0 buffer. A portion of the cross-linked product was dialyzed against the same buffer at room temperature which also included 1% SDS and 1% B-mercaptoethanol. Electrophoresis was performed using SDS acrylamide gels to determine the extent of molecular cross-linking.

Affinity Column Preparation

Cyanogen bromide activated Sepharose 4B (Sigma Chem. Co., St.Louis Mo.) was washed, swollen and activated on glass filter paper in 1 mM HCl for 15-20 min. Ten mg of F actin was added to 5 ml of the activated gel in 10 ml of coupling buffer which consisted of 5 mM triethanolamine-HCl, 50 mM KCl, 2.5 mM $MgCl_2$ at pH 8.5 (154,155).

G actin (10 mg) was coupled to the Sepharose beads utilizing the same procedure except that the buffer composition was 5 mM triethanolamine-HCl, pH 8.5. Salts were eliminated in order to prevent the possibility of polymerization of G actin to the F form (155,156).

The F-actin-tropomyosin complex (5:1 w/w) in buffer (coupling buffer described for F actin) adjusted to pH 8.5 was added to an equivalent amount of CNBr-activated Sepharose 4B in the coupling triethanolamine salt containing buffer, pH 8.5, utilized in the linkage of F actin (157). The F actin cross-linked with glutaraldehyde was also coupled to Sepharose.

Control columns were prepared in the absence of the proteins (F actin, G actin, or F-actin-tropomyosin) in the identical manner as described above.

The Sepharose-protein mixtures were gently swirled in the cold room overnight (16 hr) in order to facilitate chemical coupling. An excess amount of 1M ethanolamine (pH 8.8-9.0) was added to each conjugate to ensure blockage of

any remaining activated groups. Each of the mixtures was poured into 2.5 cm x 1.2 cm support columns filled with coupling buffer (4° C) and extensively washed alternately with 2 M KCl and then 5 mM triethanolamine-HCl pH 7.5. Near total binding of all forms of actin was confirmed by absorbance readings at 280 nm of the above effluents. SDS gel electrophoresis of samples taken of the solution on top of the settled Sepharose beads also confirmed the linkage between protein and matrix because no proteins were detected on the gels (Figure 3). All of the chromatographic procedures were carried out at 4° C.

The muscle glycolytic enzymes were obtained in salt suspensions or in crystallized form (other enzyme types noted): aldolase and lactate dehydrogenase Sigma Type 1X were in crystallized form; glucosephosphate isomerase triosephosphate isomerase, phosphoglycerate mutase, enolase, glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase (yeast), pyruvate kinase, creatine phosphokinase and hexokinase (yeast) were obtained in ammonium sulfate suspensions.

The glycolytic enzymes were desalted by passage through a Sephadex G-25 (coarse) column previously equilibrated with a low ionic strength buffer consisting of 20 mM tris-acetate , 1 mM $MgCl_2$, 5 mM B-mercaptoethanol pH

6.5. The peak enzyme fractions were pooled. The quantity of protein present in the fractions was also measured. The amount of each enzyme to be applied to the actin columnns was established by trial and error. When established, that amount of enzyme was passed through the control, F actin, glutaraldehyde treated F actin, G actin, and F-actin-tropomyosin glutaraldehyde linked columns. Many of the enzymes applied to the actin-Sepharose matrices were found to adsorb. An initial 10 ml wash of 20 mM tris-acetate low ionic strength buffer pH 6.5 was collected. Elution of bound enzymes was performed by application of a linear gradient with 20 mls of 20 mM tris acetate buffer and 20 mls of 20 mM tris-acetate buffer with the inclusion of 150 mM KCl. A final 10 ml eluant solution of 20 mM tris-acetate, 500 mM KCl, 1 mM $MgCl_2$, 5 mM B-mercaptoethanol pH 6.5 was applied to the columns in order to ensure release of the total amount of bound enzyme. One ml fractions were collected during each chromatographic run and assayed for activity. Conductivity meter readings were taken on a Radiometer Conductivity Meter of each fraction and converted to ionic strength values.

Enzyme activities were assayed utilizing pyridine nucleotide coupled assay procedures and monitored at 340 nm in a temperature controlled multi-cell Gilford recording

spectrophotometer. Activities of the various enzymes were determined using the following assay procedures: hexokinase (EC 2.7.1.40) (158), glucosephosphate isomerase (EC 5.3.1.9) (159), aldolase (EC 4.1.7.b) (112), glyceraldehydephosphate dehydrogenase (EC 1.2.1.12) (160), triosephosphate isomerase (EC 5.3.1.1) (33), phosphoglycerate kinase (EC 2.7.2.3) (161), phosphoglycerate mutase (EC 2.7.5.3) (161), enolase (EC 4.3.1.11) (161), pyruvate kinase (EC 2.7.1.40) (162), lactate dehydrogenase (EC 1.1.1.27) (163) and creatine phosphokinase (EC 2.7.3.2) (164). The enzyme activities were expressed as micromoles of substrate catalyzed to product per minute. The activity of each fraction was assayed under conditions of saturating level of substrate.

Protein was determined by the colorimetric method of Lowry et al (165) or spectrophotometrically by the method of Kalb and Bernlohr (166).

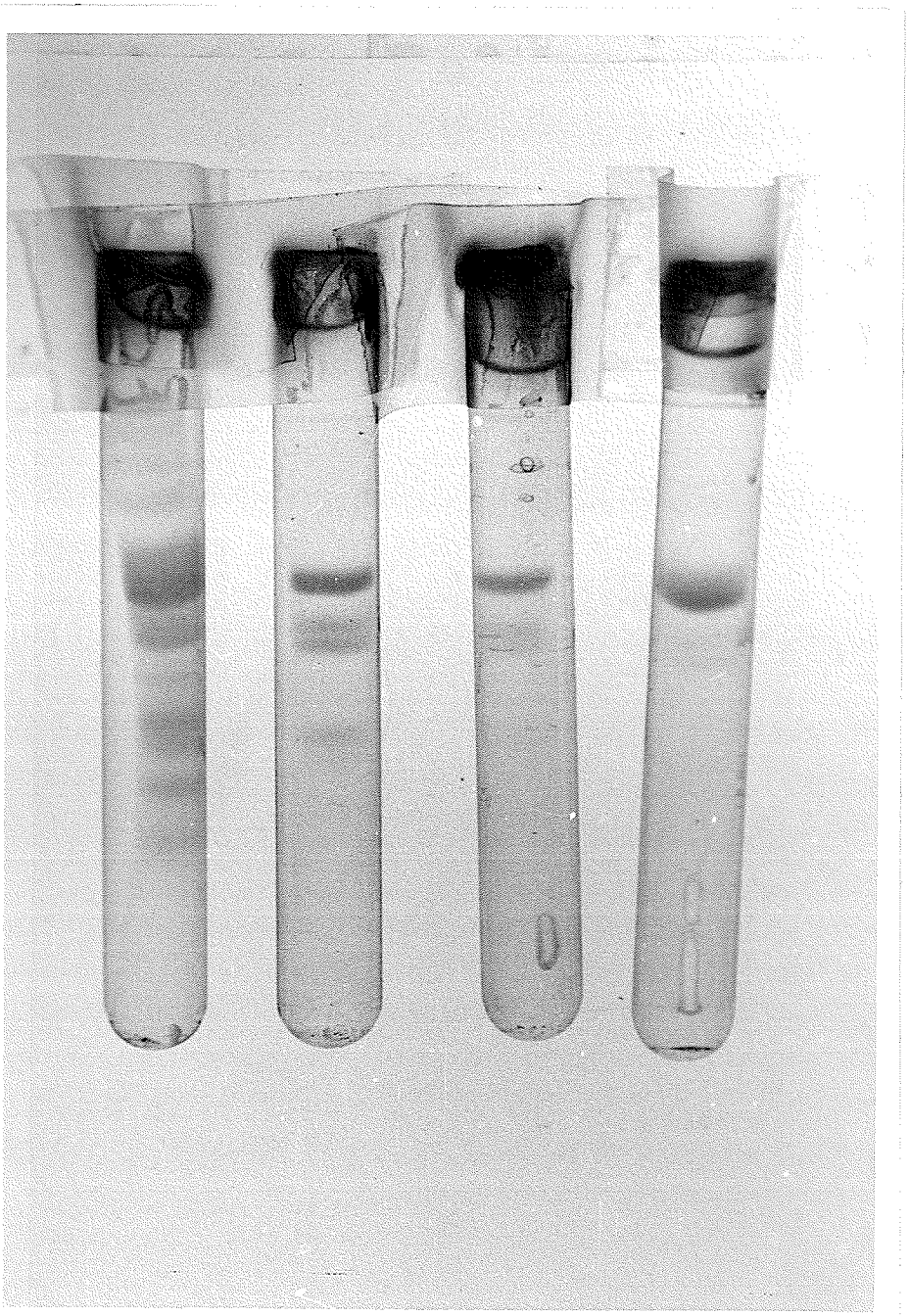
Results

The SDS electrophoretic gels show the degree of actin purification during the isolation procedure. The purification sequence shown in Figure 2 presents evidence of a nearly homogeneous protein preparation. Both F and G actin samples appeared to be greater than 90% pure as judged from the gels. The actin band corresponded to a molecular weight of 45,000 ± 2400 (n=5) calculated from its relative mobility which compares favorably with reported literature values (Figure 4) (167,168,169). The tropomyosin utilized in this study was estimated to have a molecular weight of 39,000 (Figure 3) [Literature values are 36,000 MW (170,171)]. F actin filaments were also prepared for identification by electron microscopy. Microfilaments were present which visually corresponded to photographs of actin appearing in a recent paper on actin fixation with osmium tetroxide (172) (Figures 5 and 6). The data in Figure 7 represents the results of SDS gel electrophoresis of F actin and the glutaraldehyde cross-linked forms of actin. Glutaraldehyde treatment of F actin creates intermolecular cross-links between actin subunits. The first gel indicates that SDS denatures F actin to the monomeric form (ie. single band). The second gel represents purified tropomyosin. The third gel shows that glutaraldehyde cross-linked F-actin-tropomyosin even after SDS treatment

results in a polymeric protein form too large to penetrate the gel. This aggregate is the stained portion at the top of the gel. The bands co-migrating with actin and tropomyosin revealed that a number of tropomyosin and actin subunits were not adequately linked. The fourth gel shows that F actin treated with glutaraldehyde also resulted in a high molecular weight complexed protein located at the front of the gel. These purified protein preparations were utilized in a study to determine if individual glycolytic enzymes would interact with either F or G actin. The experimental design involved immobilizing actin by covalent linkage to cyanogen bromide activated Sepharose 4B followed by passing enzymes through the matrix.

The data represented in Figures 8,9,10 and 11 shows that aldolase, glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase and lactate dehydrogenase adsorbed to the F and G actin columns when applied in a low ionic strength buffer. Elution from the columns required an increase in eluant ionic strength. Enzymes were present in a low ionic strength buffer (u=0.014) adjusted to pH 6.5 prior to application to the columns. In initial studies a pH 7.3 buffer system was utilized which appeared to result in weaker enzyme adsorption in the actin linked columns. For example, in one study at pH 7.3 bound aldolase was released within fractions #16-18 (u=0.022-0.03) while at pH

Fig. 2. SDS polyacrylamide gel electrophoretic patterns of F actin at various stages of purification. [A]. crude F actin after extraction from an acetone powder of muscle. [B]. The preparation shown in gel A was depolymerized to G actin and repolymerized to F actin. A sample was then subjected to SDS electrophoresis. [C]. Actin after a second depolymerization-repolymerization step. [D]. Actin after a third depolymerization-repolymerization step. 60-80 ug of protein was applied to each gel. Refer to methods for details of actin purification.



A B C D

Fig. 2a. The photograph shows the pellet obtained after centrifugation of F actin at 100,000 x g for 2.5 hr.

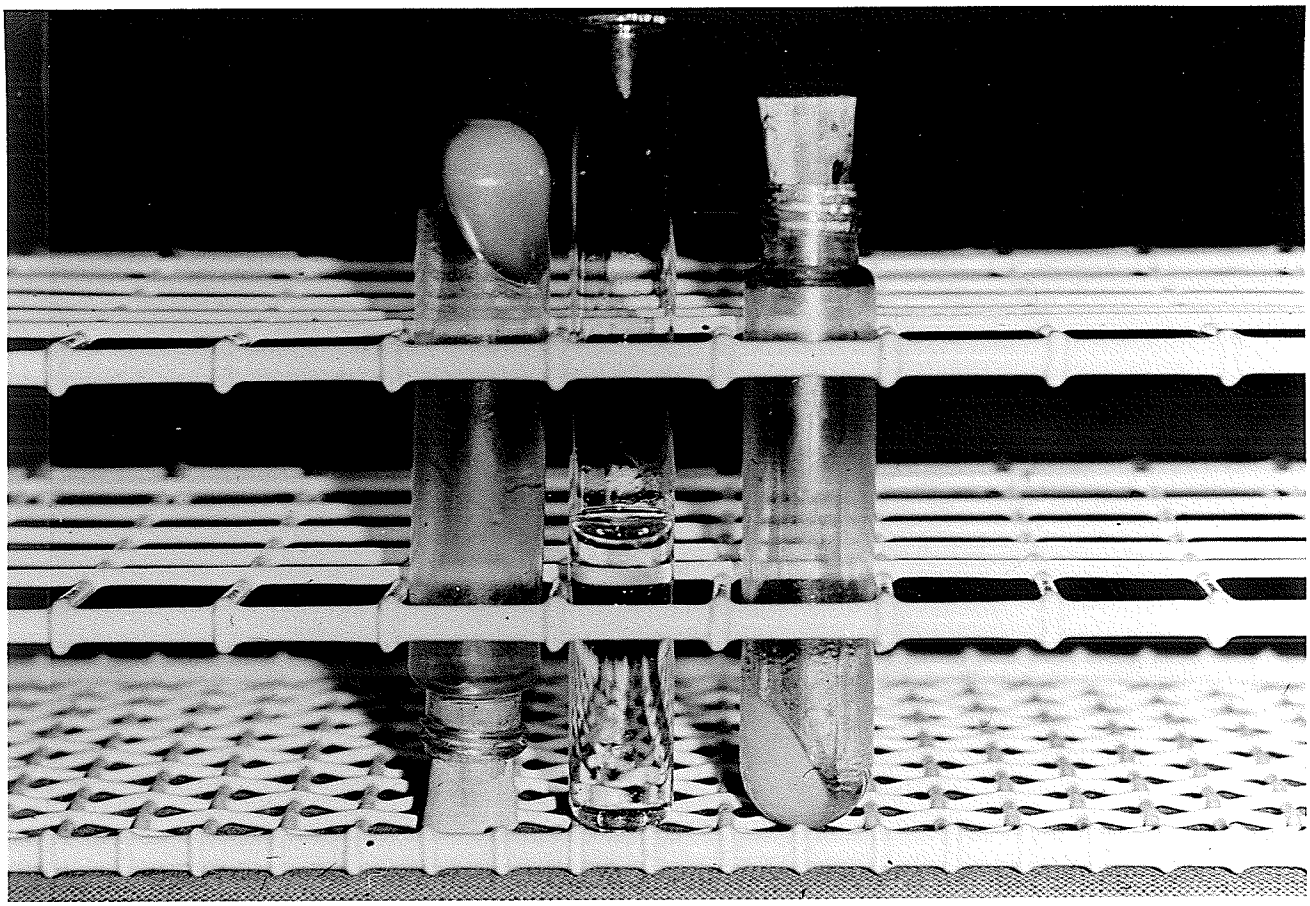
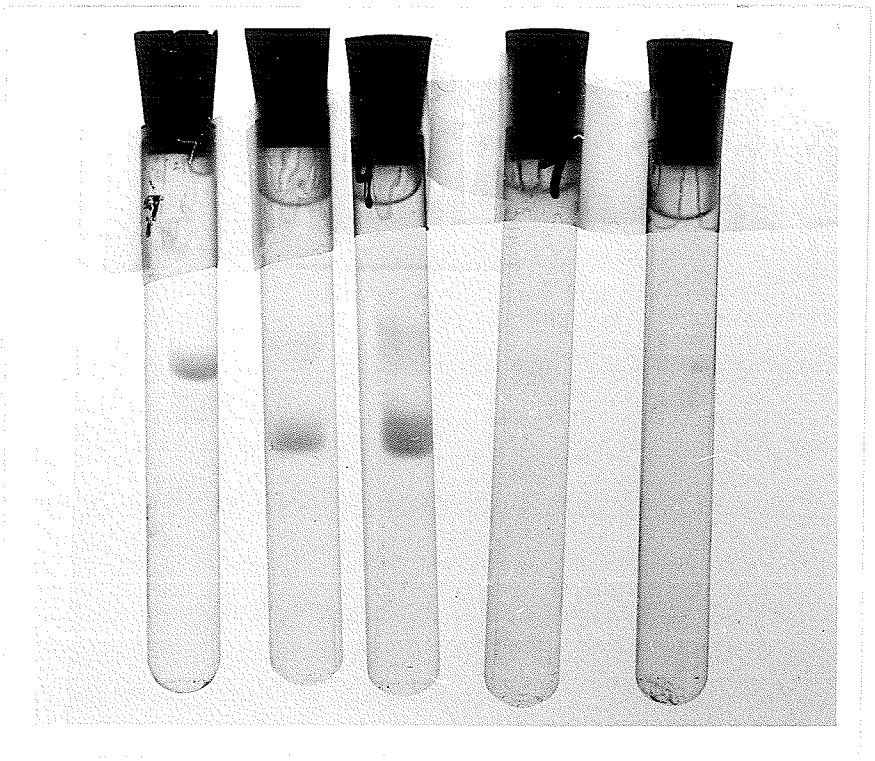
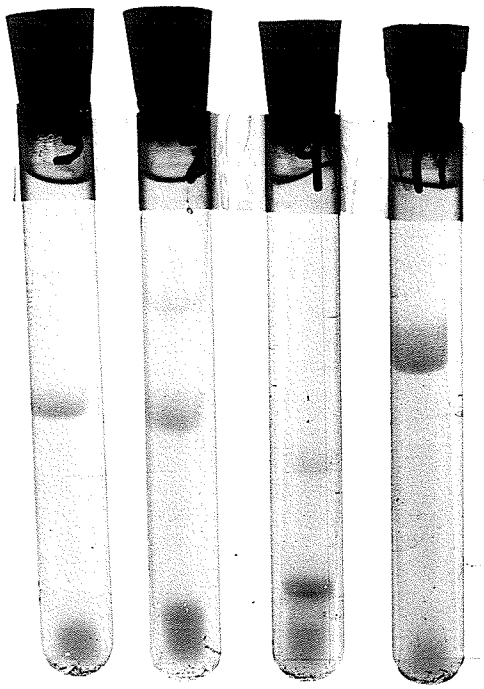


Fig. 3. SDS electrophoretic patterns on 7.5% acryamide gels of; [A]. F actin, [B]. and [C]. tropomyosin, [D]. and [E]. Aliquot (50 ul) of solution obtained from the top of settled CNBr activated Sepharose 4B actin coupled beads. Approximately 20 ug of protein was applied to gels A and B and 40 ug protein to gel C. Gels D and E represent an experiment in which F actin was cross-linked to CNBr activated Sepharose 4B. Had there not been any cross-linking of actin with the Sepharose beads, protein would have appeared in gels D and E.



A B C D E

Fig. 4. SDS polyacrylamide gel electrophoresis of purified actin and the molecular weight markers; myoglobin 17,800 daltons, ovalbumin 43,000 daltons and bovine serum albumin 67,000 daltons. A. F actin, B. ovalbumin, C. myoglobin D. bovine serum albumin



A B C D

Fig. 5. Electron micrograph of actin filaments treated with glutaraldehyde and osmium tetroxide followed by dehydration in ethanol. Thin sections were stained with 1% uranyl acetate and lead citrate. Grids were examined on a Hitachi HU-12 transmission electron microscope. (x 75,000)

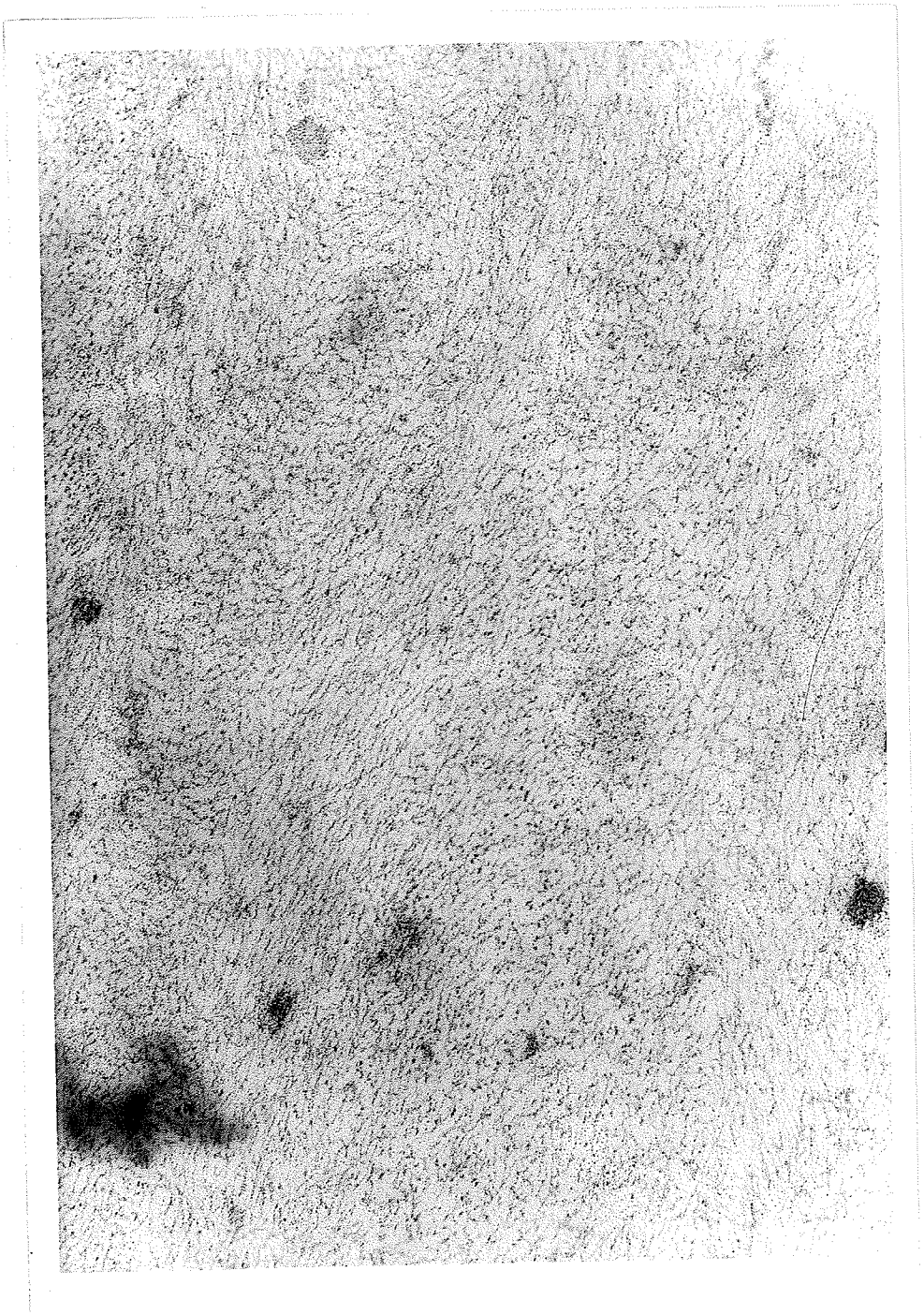


Fig. 6. Electron micrograph of actin filaments (x 137,000). Refer to figure legend 5 for details

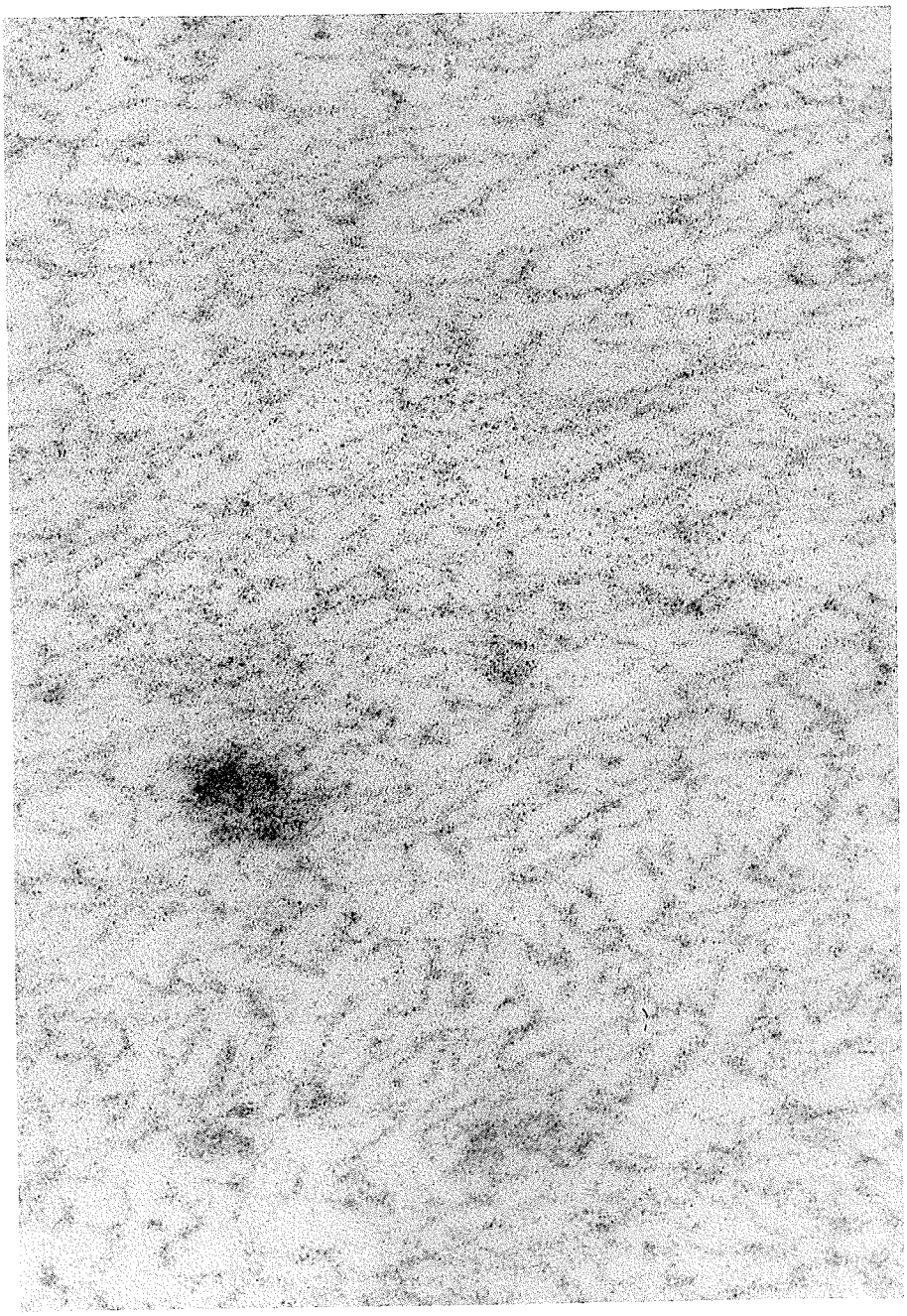
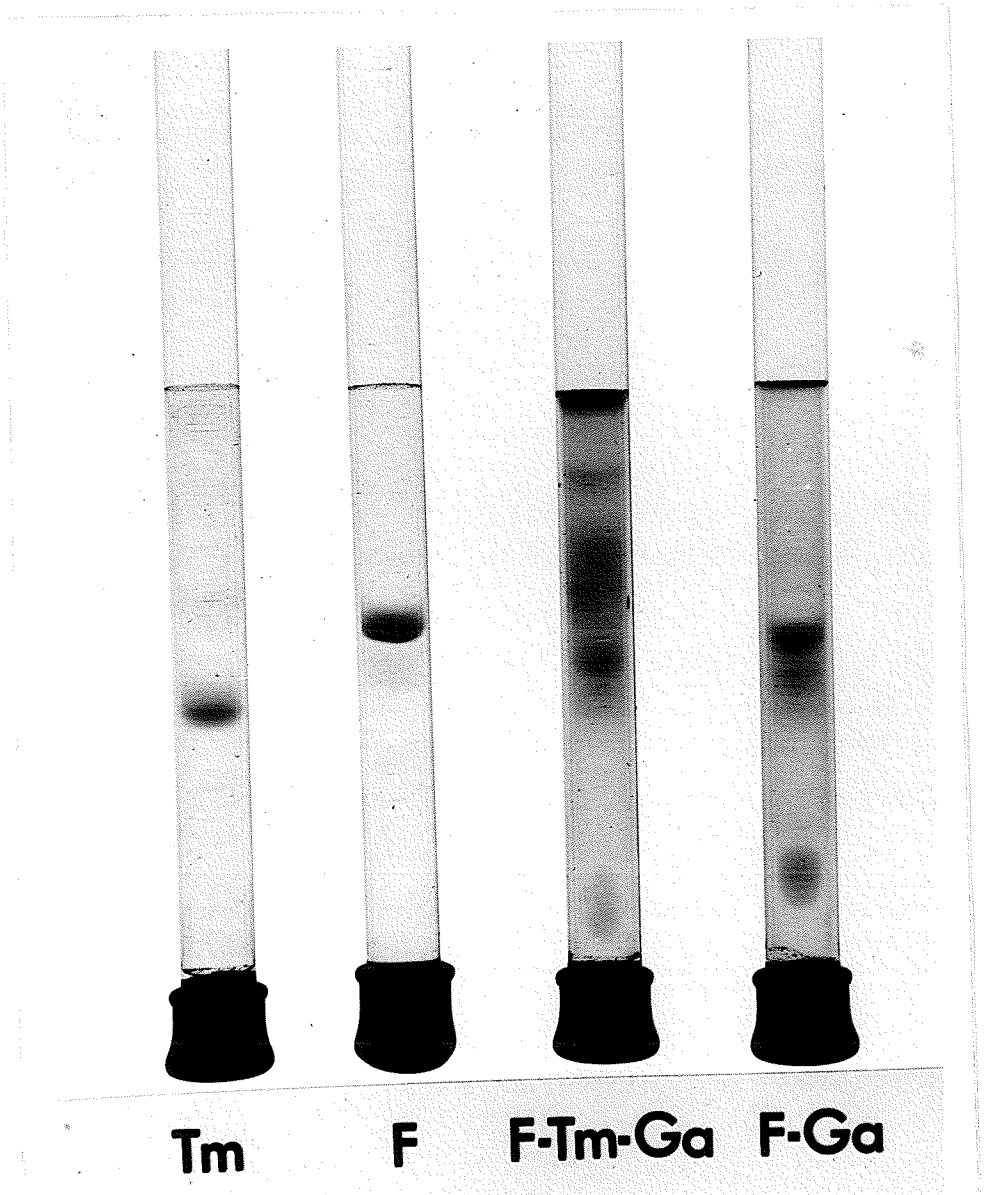


Fig. 7. SDS electrophoretic patterns of tropomyosin (Tm), F actin (F), F-actin-tropomyosin cross-linked with glutaraldehyde (F-Tm-Ga) and F actin glutaraldehyde cross-linked (F-Ga). The protein samples were dialysed for 2 hr. at 20^{o} C against 20 mM sodium phosphate buffer, 1% SDS and 1% B-mercaptoethanol pH=7.0. 30-50 ug of protein was applied to each gel.


Tm
F
F-Tm-Ga
F-Ga

6.5 the peak enzyme activity was measured between fractions #20-23 (u=0.042-0.051). Similarily, glyceraldehydephosphate dehydrogenase eluted at u=0.02-0.03 at pH 7.3 and u=0.035-0.085 at pH 6.5. Therefore, the following studies employed pH 6.5 buffer solutions.

Initial control studies indicated non-specific binding of enzymes to Sepharose. It was deemed necessary to compare results of control column non-specific enzyme binding to specific actin enzyme adsorption. In each case, the bulk of enzyme activity emerged in the first few low ionic strength fractions. However, sometimes enzyme activity was detected in the gradient as shown in control column profiles Figures 8-11. Aldolase demonstrated the greatest affinity for the control column. Due to this adsorption (usually minor amounts) to the control columns the amount of enzyme applied to each column was always several fold above that required to saturate binding to the control column. A few of the enzymes ie. creatine phosphokinase, enolase, glucosephosphate isomerase, phosphohexose isomerase, hexokinase and phosphoglycerate mutase did not adhere to the control columns (Figure 12).

The elution of the enzymes bound to both F and G actin columns occurred as the gradient shifted towards conditions of increasing ionic strength. In Figure 8 ,the elution of bound aldolase from F actin was found in the range of

0.04-0.1 u. G actin bound aldolase was released at slightly lower values of 0.03 - 0.075 u. The affinity of glyceraldehydephosphate dehydrogenase (Figure 9), and lactate dehydrogenase (Figure 11) was also greater for F actin that for G actin. Phosphoglycerate kinase (Figure 10) adsorbed to the F and G actin with the same relative affinities. Several enzymes did not adhere in the columns but were detected in the void volume after being applied to the column. These included phosphoglucose isomerase, triosephosphate isomerase, phosphoglycerate mutase, enolase (Figure 12), hexokinase and pyruvate kinase (Figure 13). Perhaps slight association of pyruvate kinase to F and G actin was observed as indicated in Figure 13. Interaction of phosphofructokinase (PFK) with actin was also attempted. PFK activity could not be detected due to its instability in the absence of ammonium sulfate.

Tropomyosin was incorporated into the F actin filaments by covalent linkage with glutaraldehyde (Figure 7). The results of passing glycolytic enzymes through an F-actin-tropomyosin column is shown in Figures 8 through 13. It was interesting to note that the same four enzymes found to associate with F and G actin were also observed to associate with the F-actin-tropomyosin complex. The enzymes aldolase, glyceraldehydephosphate dehydrogenase, lactate dehydrogenase and phosphoglycerate kinase demonstrated a greater affinity for the tropomyosin containing matrix.

There is also evidence that pyruvate kinase (Figure 14) adsorbed in the F-actin-tropomyosin columns as an elevated ionic strength was necessary for elution. An increase in ionic strength of between 0.02 - 0.04 u was required to elute these enzymes from columns containing tropomyosin, as compared to the F and G actin columns. The enzymes were desorbed from this column in the range of 0.05 - 0.12 u. The desorption of bound glyceraldehydephosphate dehydrogenase from F-actin-tropomyosin occurred at ionic strength values of 0.1 - 0.15 u which is considered physiological.

The possibility of a cross-linked F actin being a better ligand for enzyme interaction as a result of modification of the protein with glutaraldehyde prior to coupling to Sepharose was also investigated. Evidence obtained from the SDS gels indicated the intermolecular cross-linking of the F actin subunits (Figure 7). The elution profiles of glycolytic enzymes from columns in which the F actin was cross-linked with glutaraldehyde are shown in Figures 15 through 18 . The data indicates that the enzymes aldolase (Figure 15), glyceraldehydephosphate dehydrogenase (Figure 16), phosphoglycerate kinase (Figure 17), pyruvate kinase (Figure 14) and lactate dehydrogenase (Figure 18) had a greater affinity for F actin treated with glutaraldehyde than for the untreated F actin.

The enzymes which did not adhere to the supports

listed in Figures 12 and 19 were also passed through the glutaraldehyde cross-linked F actin matrix. The same enzymes did not adhere to this column ie. triosephosphate isomerase, creatine phosphokinase, phosphoglucoisomerase, enolase and hexokinase.

Triosephosphate isomerase has been reported to associate with F actin using techniques described in Table 1 (9). Since it did not bind to actin, another experiment was performed in which triosephosphate isomerase was passed over an F-actin-tropomyosin column which had previously been equilibrated with 500 ug of aldolase. Aldolase was applied to the column at low ionic strength after which 50 ug of triosephosphate isomerase were applied and then the usual column development followed. The results of Figure 19 suggest the presence of aldolase is necessary to enable triosephosphate isomerase to associate with the F-actin-tropomyosin matrix. This enzyme which did not adhere to any of the other columns did adsorb to a slight extent in the presence of aldolase bound to F-actin-tropomyosin suggesting an enzyme-enzyme interaction rather than an enzyme-actin interaction.

The amount of protein applied to the F and G actin columns was as follows: aldolase 500 ug, glyceraldehydephosphate dehydrogenase 500 ug, lactate dehydrogenase 300 ug and phosphoglycerate kinase 300ug.

Protein applied to F-actin-tropomyosin and F actin glutaraldehyde treated columns was: aldolase 800 ug, glyceraldehydephosphate dehydrogenase 1 mg, lactate dehydrogenase 1 mg, phosphoglycerate kinase 300 ug and triosephosphate isomerase 50 ug. The amount of protein appearing in the fractions corresponding to enzyme activity peaks from the F-actin-tropomyosin column was 500 ug for aldolase, 300 ug for glyceraldehydephosphate dehydrogenase, 250 ug of lactate dehydrogenase and 100 ug of phosphoglycerate kinase. Recovery of all enzymes applied to the columns was always greater than 80% (except phosphofructokinase which was often 10-25%).

Saturation of the binding capacity of the columns was not attempted, except for the control column.

Judging from the data it is evident that at these concentrations aldolase, lactate dehydrogenase and glyceraldehydephosphate dehydrogenase saturated both F and G actin columns. However, the capacity for enzyme binding by the F-actin-tropomyosin column was not exceeded by the amounts used in these experiments. To illustrate the capacity of the columns, the application of lactate dehydrogenase to various columns will be considered. In Figure 11, 300 ug protein of lactate dehydrogenase was applied while approximately 1 mg was used in the study referred to in Figure 18. One hundred and ten of a possible 300 ug protein eluted between fractions #15-23 from the F

actin column (Figure 11) while the same portion of the gradient in Figure 18 for identical pooled fractions contained 150 ug of a possible 1000 ug of protein. In comparison, the F-actin-tropomyosin and F actin-glutaraldehyde cross-linked columns retained all of the applied enzyme. The evidence indicates that these two columns possessed a much greater capacity for lactate dehydrogenase than either the F or G actin supports. Similarily, the retention of glyceraldehyde phosphate dehydrogenase, aldolase and pyruvate kinase was greater in the glutaraldehyde treated F actin columns than on the F actin columns not treated with glutaraldehyde.

Fig. 8. Profile for the elution of aldolase from F actin, G actin and F-actin-tropomyosin cross-linked to Sepharose 4B and control columns. Application of enzyme to the columns is described in the methods section. The column was developed with 40 ml of a linear gradient of increasing ionic strength. One ml fractions were collected and each was assayed for enzyme activity and ionic strength. Preparation of the columns is described in the text.

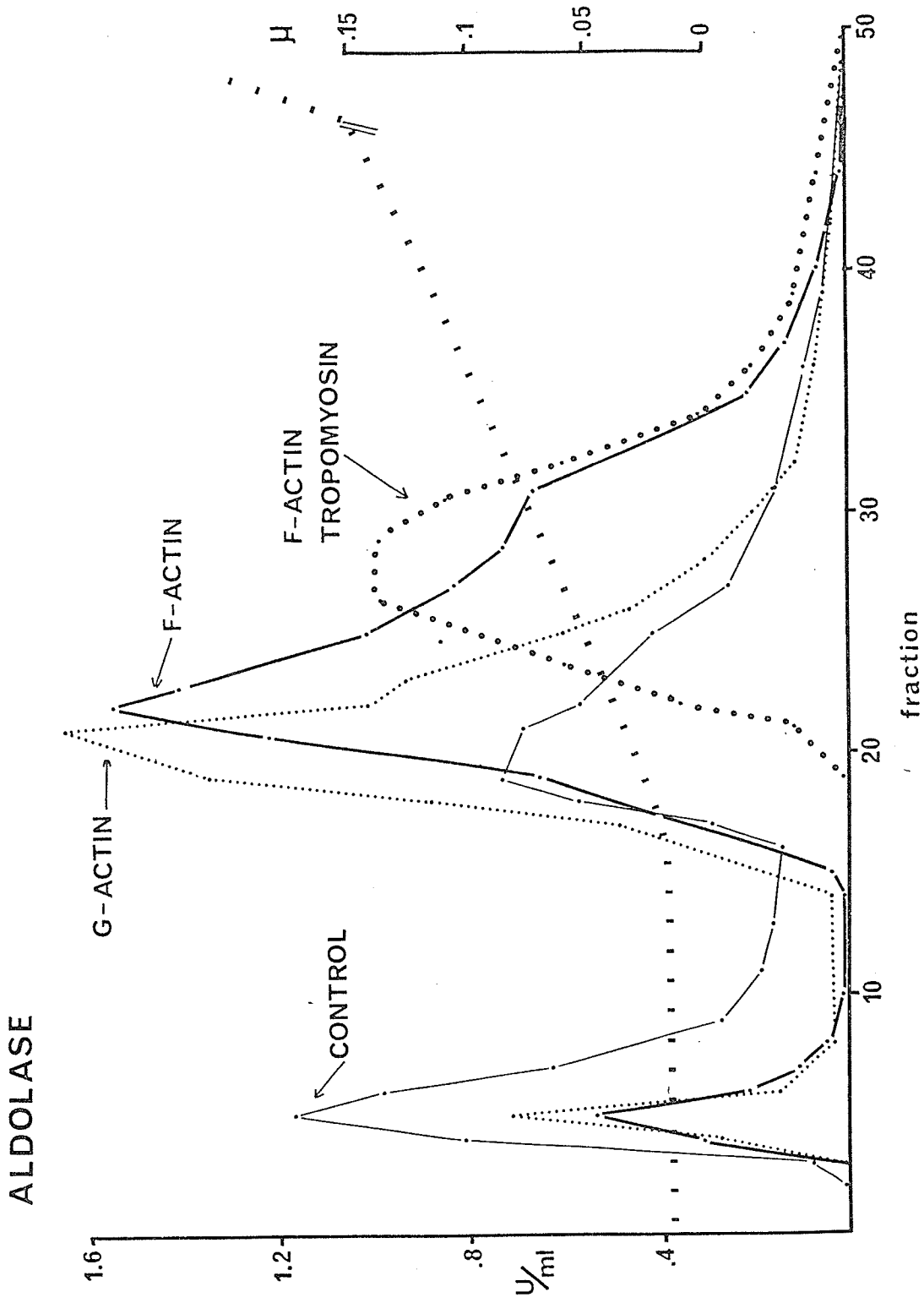

ALDOLASE
G-ACTIN
F-ACTIN
F-ACTIN
TROPOMYOSIN
CONTROL
μ
.15
.1
.05
0
1.6
1.2
.8
.4
U/ml
10
20
30
40
50
fraction

Fig. 9. Profile for the elution of glyceraldehydephosphate dehydrogenase from control, F actin, G actin and F-actin-tropomyosin columns. (For details see legend to Fig. 8.)

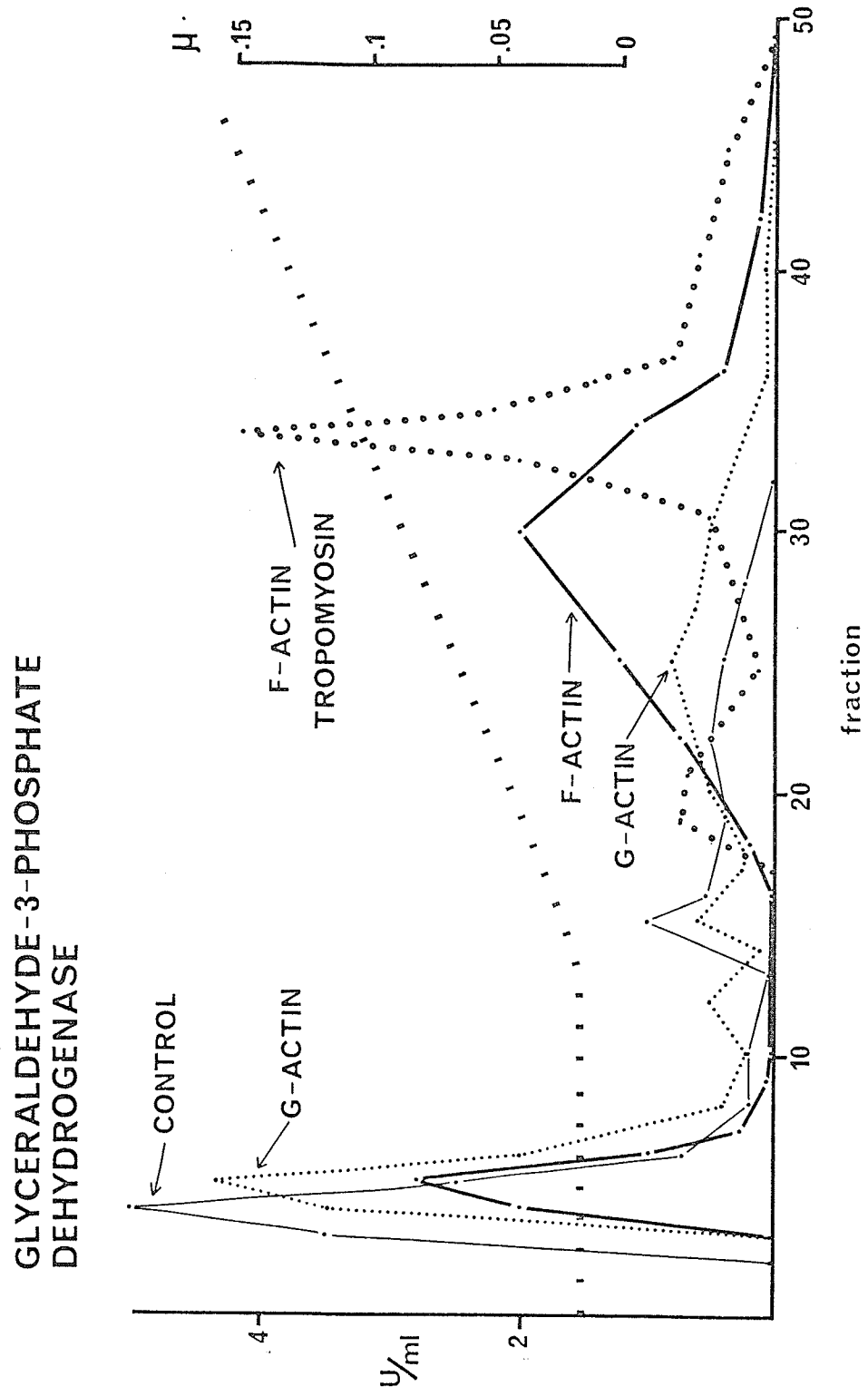

GLYCERALDEHYDE-3-PHOSPHATE
DEHYDROGENASE
CONTROL
G-ACTIN
F-ACTIN
TROPOMYOSIN
F-ACTIN
G-ACTIN
μ
.15
.1
.05
0
U/ml
4
2
10
20
30
40
50
fraction

Fig. 10. Profile for the elution of phosphoglycerate kinase from control, F actin, G actin and F-actin-tropomyosin columns. (For details see legend to Fig. 8.)

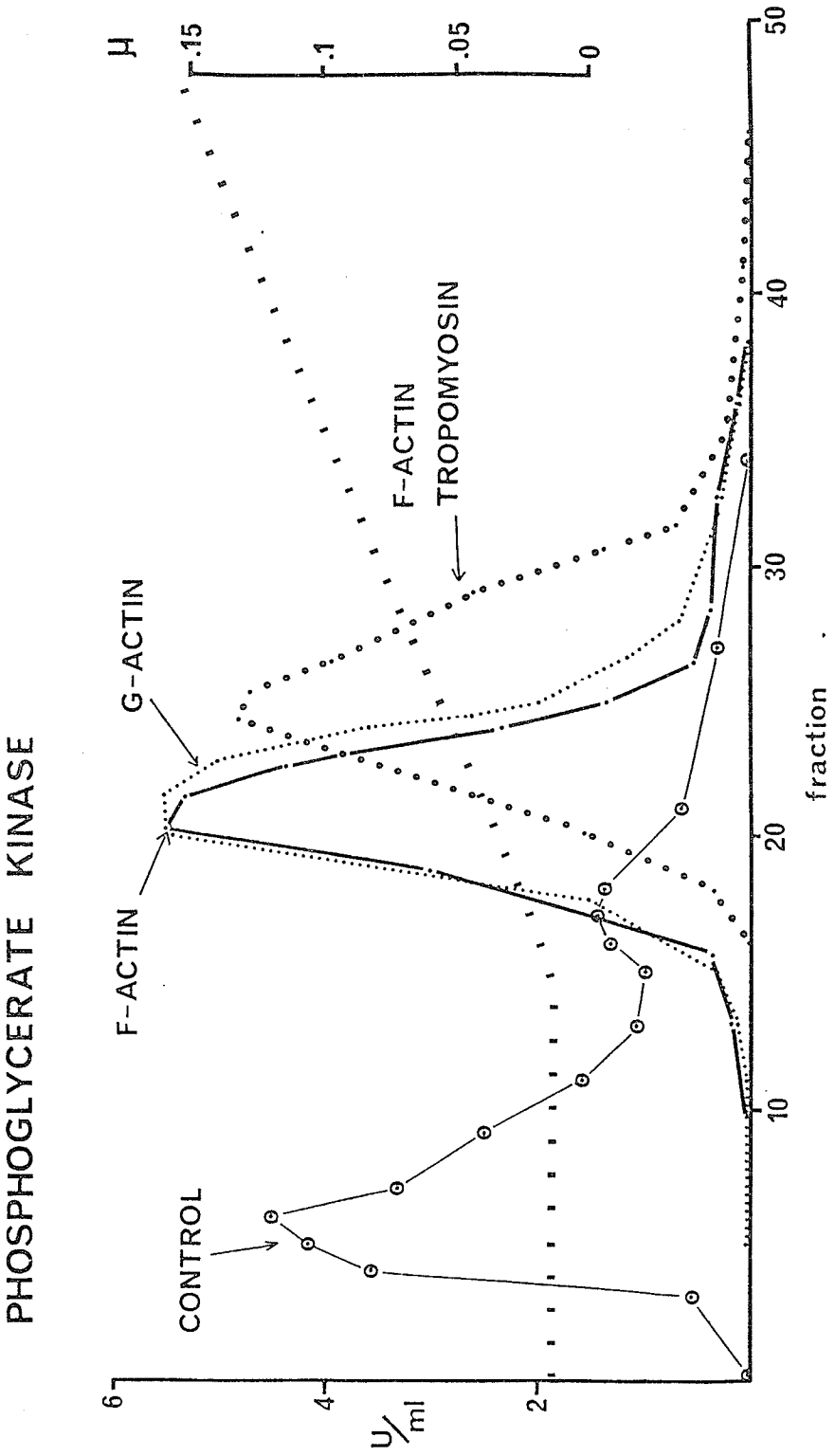

PHOSPHOGLYCERATE KINASE
μ
.15
.1
.05
0
G-ACTIN
F-ACTIN
TROPOMYOSIN
F-ACTIN
CONTROL
6
4
2
U/ml
10
20
30
40
50
fraction

Fig. 11. Profile for the elution of lactate dehydrogenase from control, F actin, G actin and F-actin-tropomyosin columns. (For details see legend to Fig. 8.)

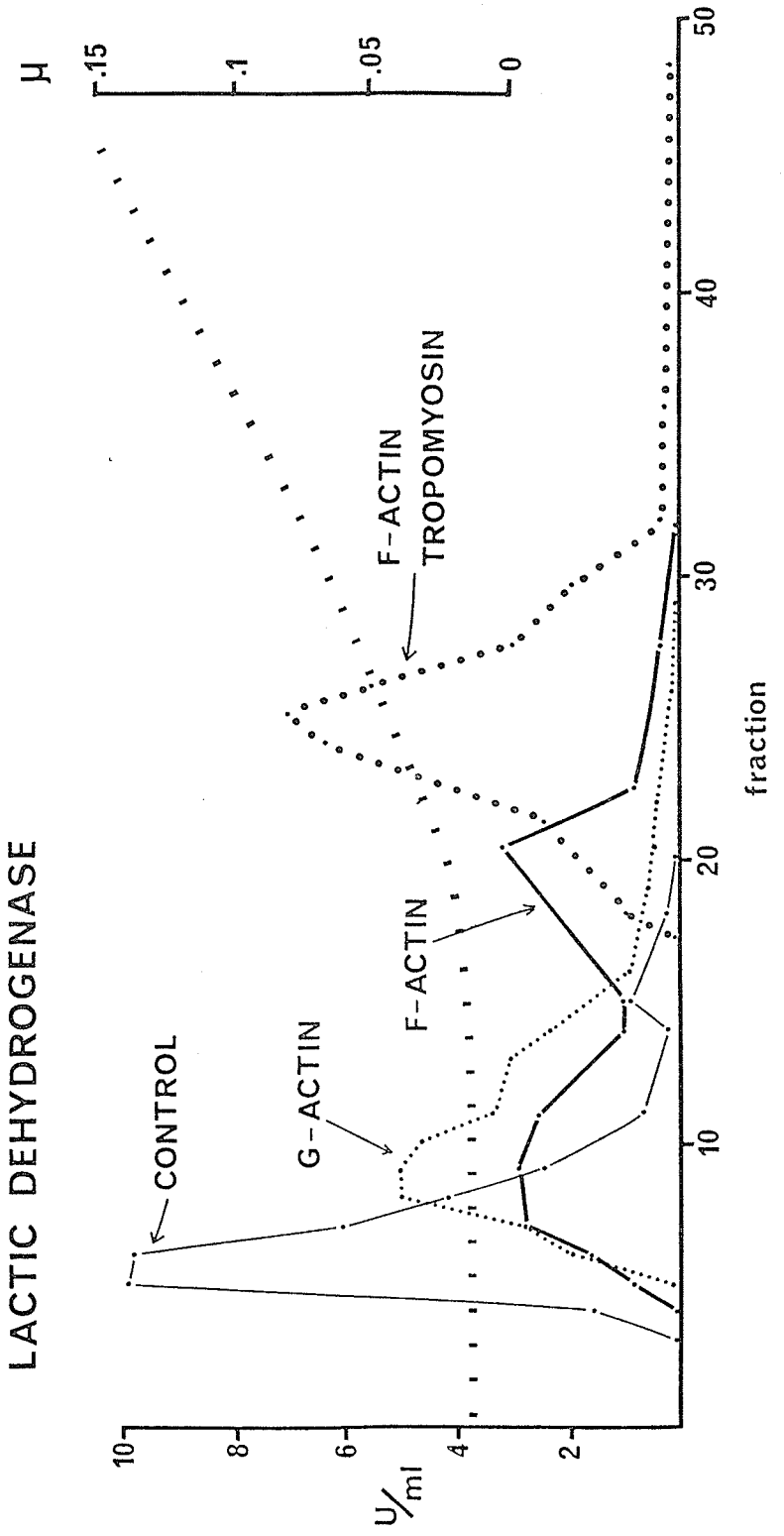

LACTIC DEHYDROGENASE
μ
.15
.1
.05
0
CONTROL
G-ACTIN
F-ACTIN
F-ACTIN
TROPOMYOSIN
10
8
6
4
2
U/ml
10
20
30
40
50
fraction

Fig. 12. Profile for the elution of creatine phosphokinase, enolase and glucosephosphate isomerase from control, F actin, G actin and F-actin-tropomyosin columns. (For details see legend to Fig. 8.)

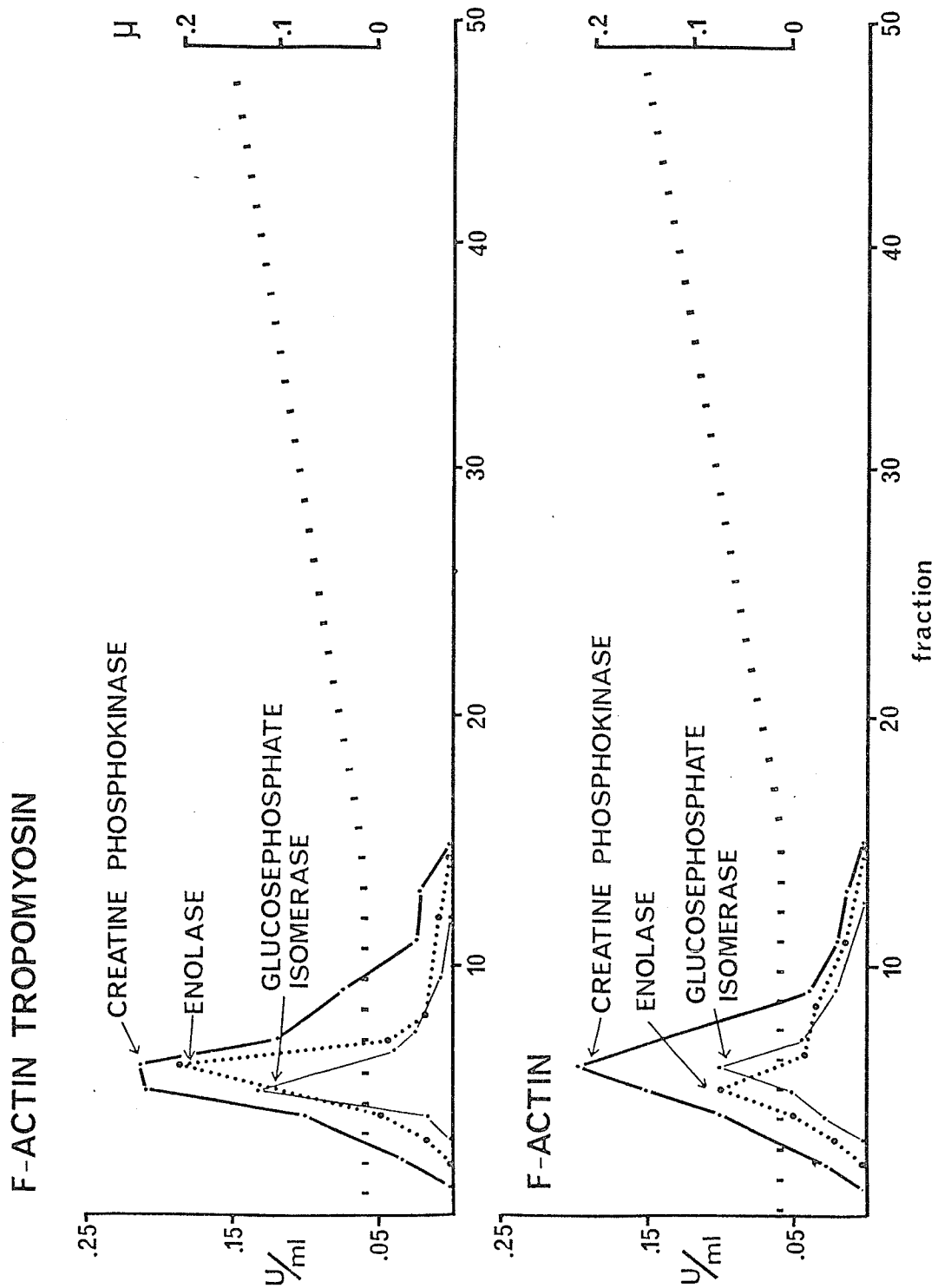

F-ACTIN TROPOMYOSIN
CREATINE PHOSPHOKINASE
ENOLASE
GLUCOSEPHOSPHATE ISOMERASE
μ
.2
.1
0
.25
.15
.05
U/ml
10
20
30
40
50
F-ACTIN
CREATINE PHOSPHOKINASE
ENOLASE
GLUCOSEPHOSPHATE ISOMERASE
.2
.1
0
.25
.15
.05
U/ml
10
20
30
40
50
fraction

Fig. 13. Profile for the elution of pyruvate kinase from control, G actin, F actin and F-actin-tropomyosin columns. (For details see legend to Fig. 8.)

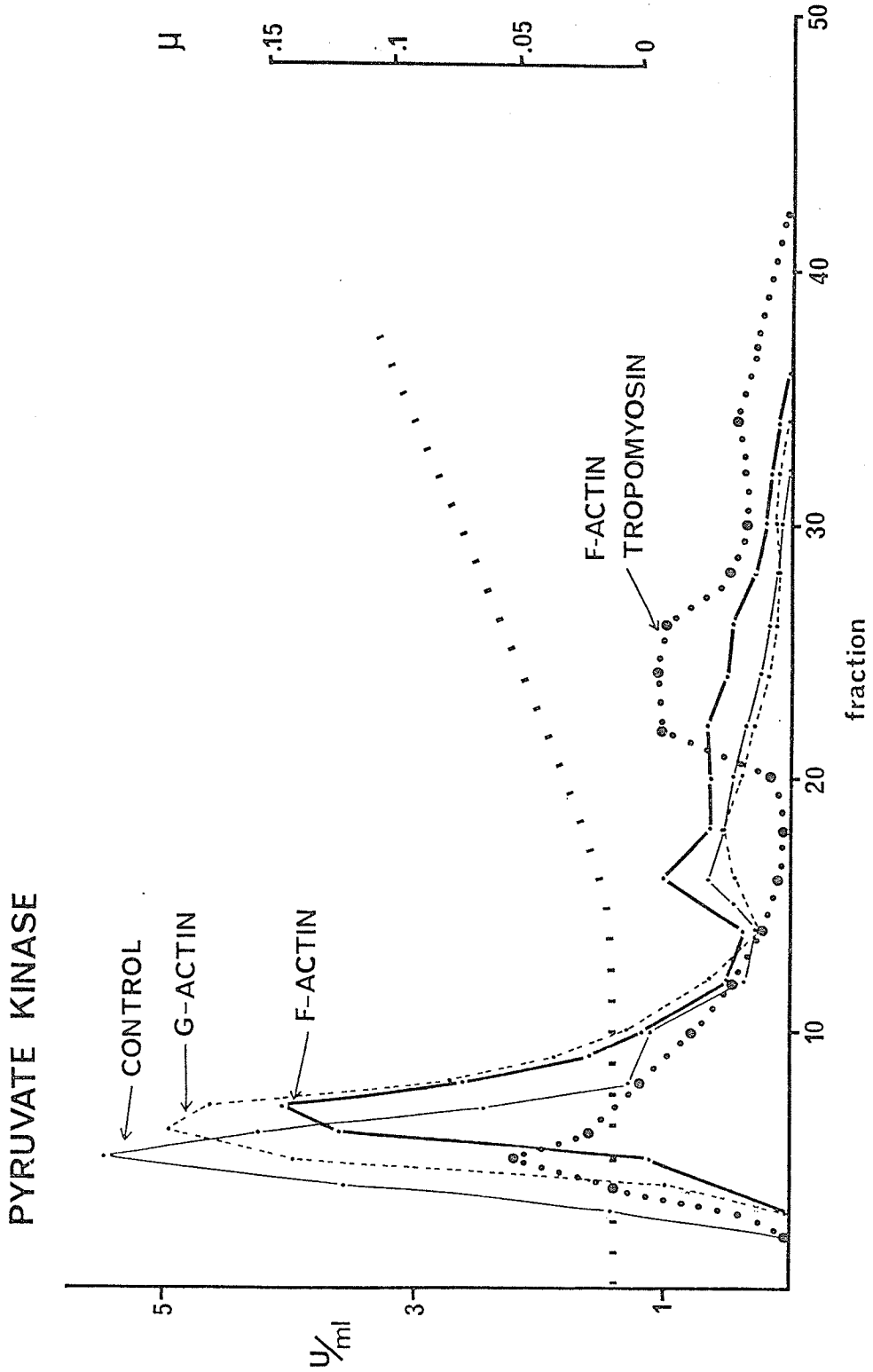

PYRUVATE KINASE
CONTROL
G-ACTIN
F-ACTIN
F-ACTIN
TROPOMYOSIN
μ
.15
.1
.05
0
U/ml
5
3
1
10
20
30
40
50
fraction

Fig. 14. Profile for the elution of pyruvate kinase from F actin, glutaraldehyde cross-linked F actin and F-actin-tropomyosin cross-linked with glutaraldehyde columns.
(For details see legend to Fig. 8.)

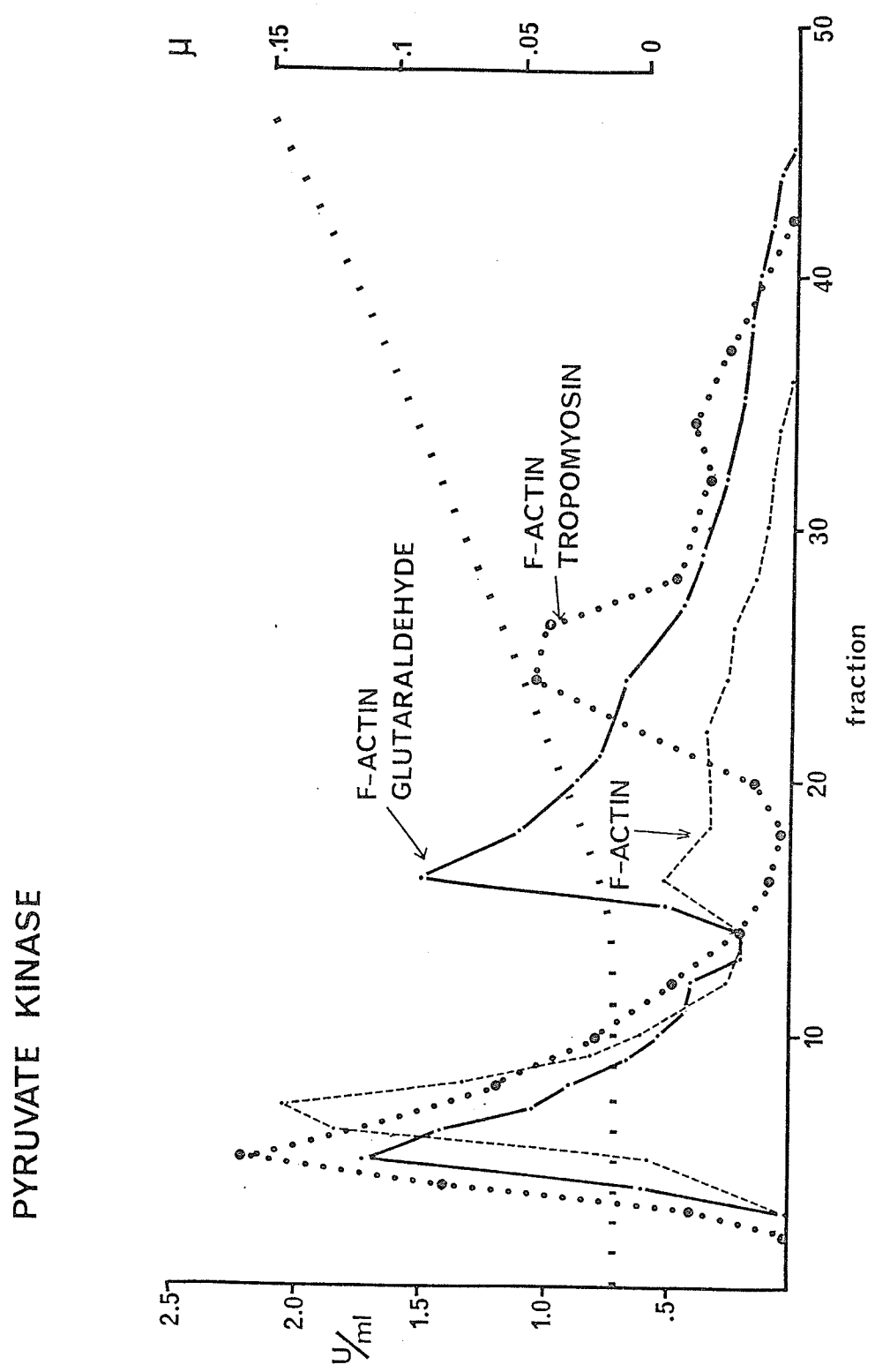

PYRUVATE KINASE
μ
.15
.1
.05
0
2.5
2.0
1.5
1.0
.5
U/ml
F-ACTIN
GLUTARALDEHYDE
F-ACTIN
TROPOMYOSIN
F-ACTIN
10
20
30
40
50
fraction

Fig. 15. Profile for the elution of aldolase from F actin, glutaraldehyde cross-linked F actin and glutaraldehyde cross-linked F-actin-tropomyosin columns.
(For details see legend to Fig. 8)

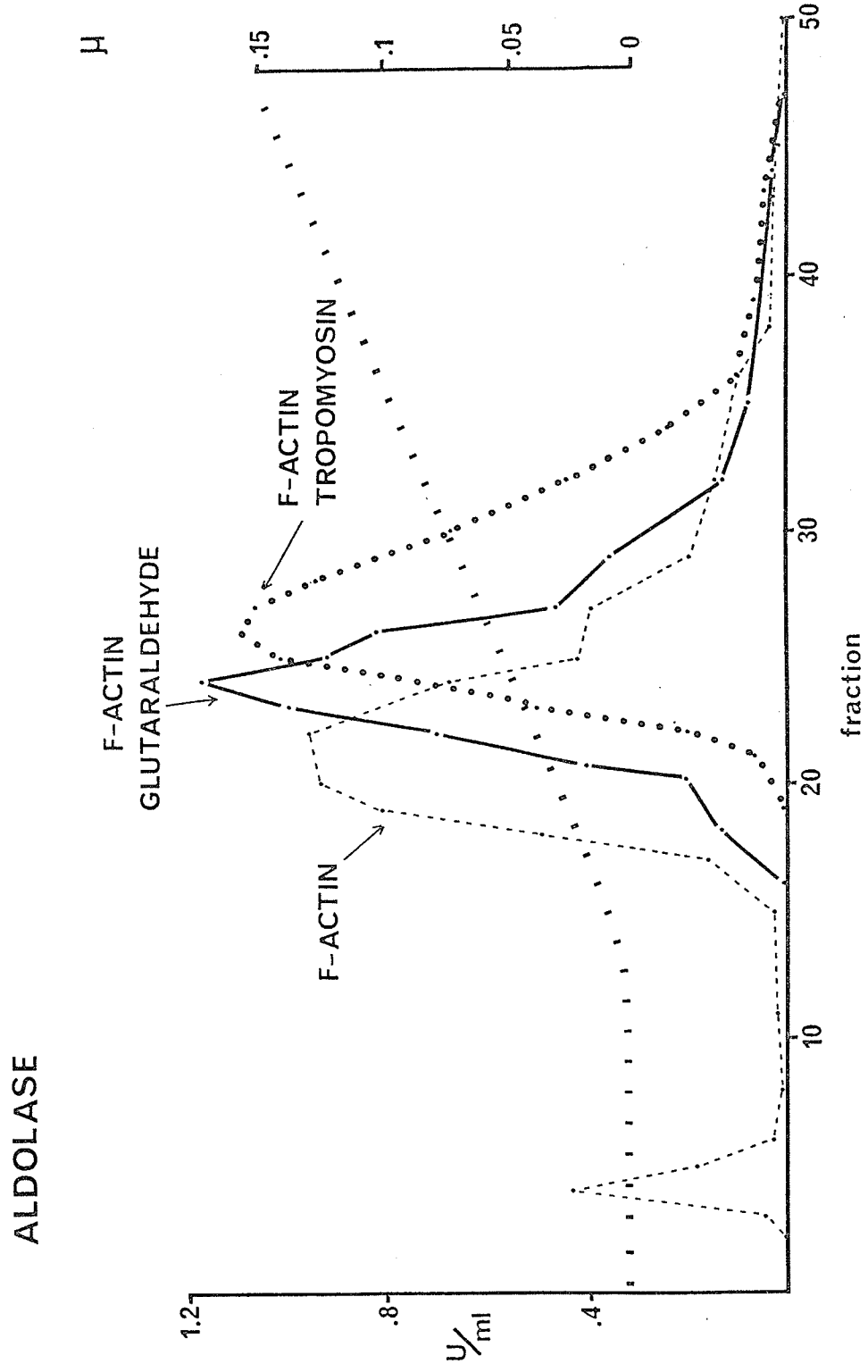

ALDOLASE
μ
.15
.1
.05
0
F-ACTIN
TROPOMYOSIN
F-ACTIN
GLUTARALDEHYDE
F-ACTIN
1.2
.8
.4
U/ml
10
20
30
40
50
fraction

Fig. 16. Profile for the elution of glyceraldehydephosphate dehydrogenase from F actin, glutaraldehyde cross-linked F actin and F-actin-tropomyosin glutaraldehyde cross-linked columns. (For details see legend to Fig. 8)

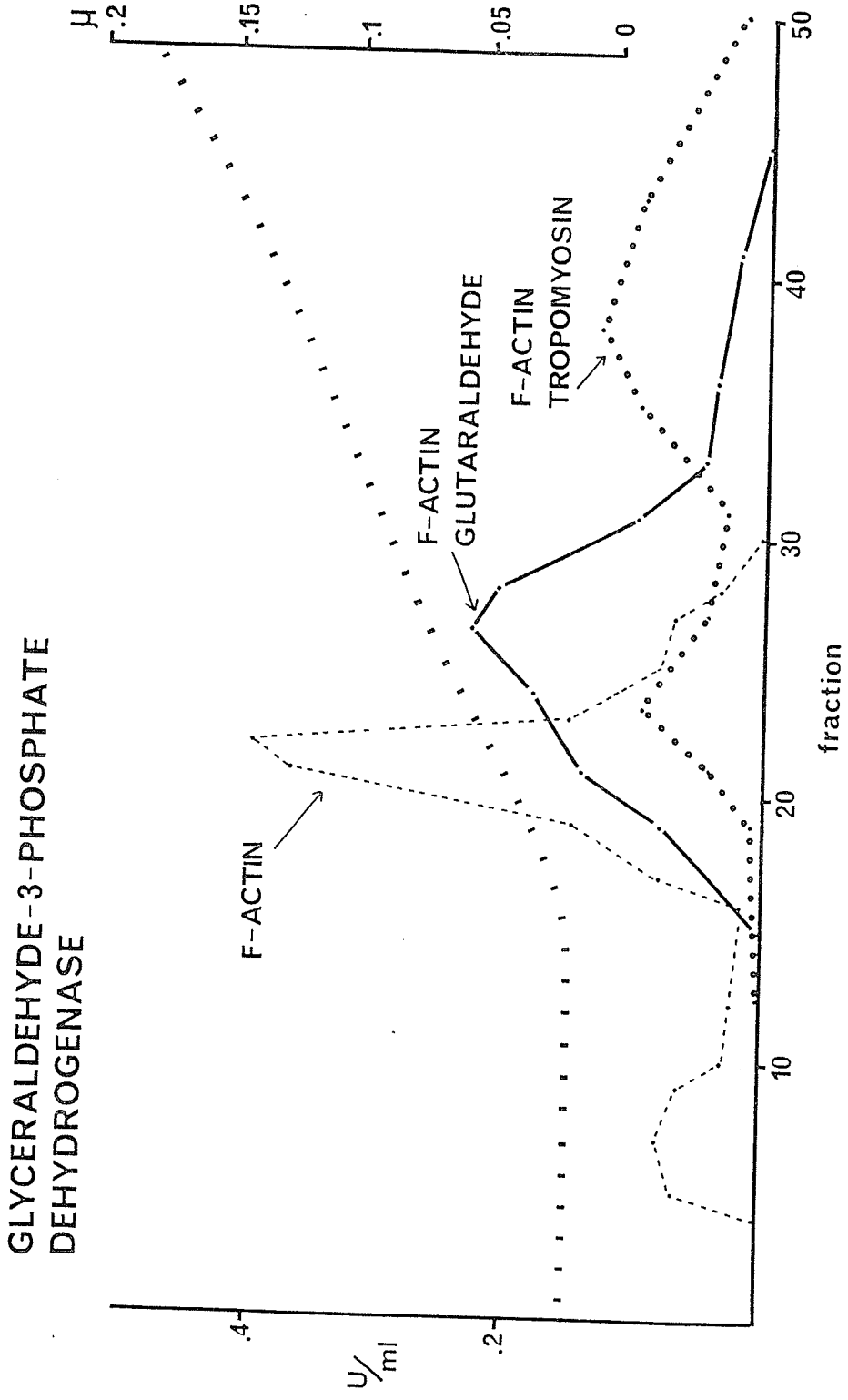

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE
F-ACTIN
F-ACTIN GLUTARALDEHYDE
F-ACTIN TROPOMYOSIN
U/ml
μ
.4
.2
.2
.15
.1
.05
0
10
20
30
40
50
fraction

Fig. 17. Profile for the elution of phosphoglycerate kinase from F actin, glutaraldehyde cross-linked F actin and F-actin-tropomyosin glutaraldehyde cross-linked columns.
(For details see legend to Fig. 8.)

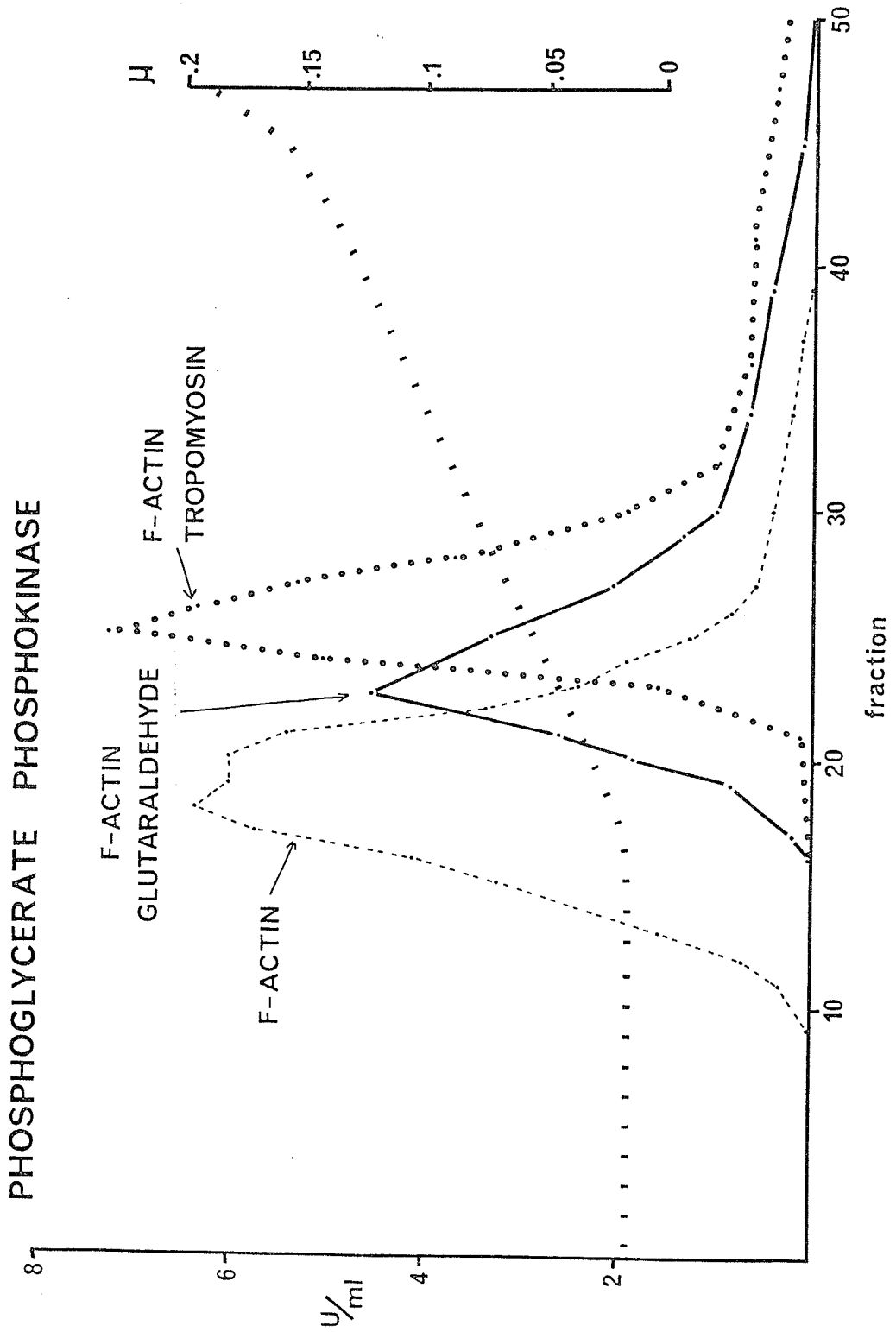

PHOSPHOGLYCERATE PHOSPHOKINASE
F-ACTIN
GLUTARALDEHYDE
F-ACTIN
TROPOMYOSIN
F-ACTIN
μ
.2
.15
.1
.05
0
8
6
4
2
U/ml
10
20
30
40
50
fraction

Fig. 18. Profile for the elution of lactate dehydrogenase from F actin, glutaraldehyde cross-linked F actin and F-actin-tropomyosin glutaraldehyde cross-linked columns. (For details see legend to Fig. 8.)

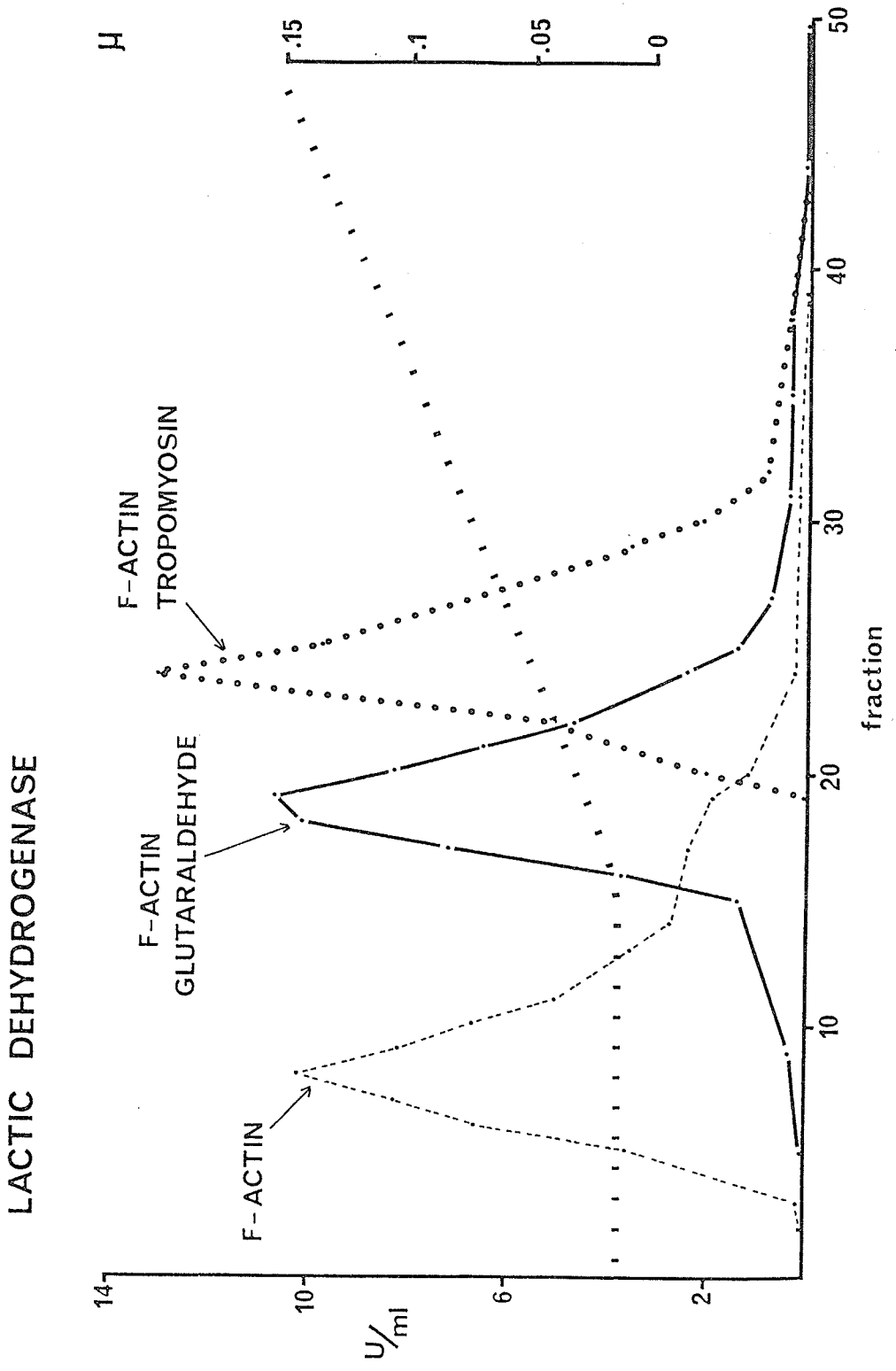
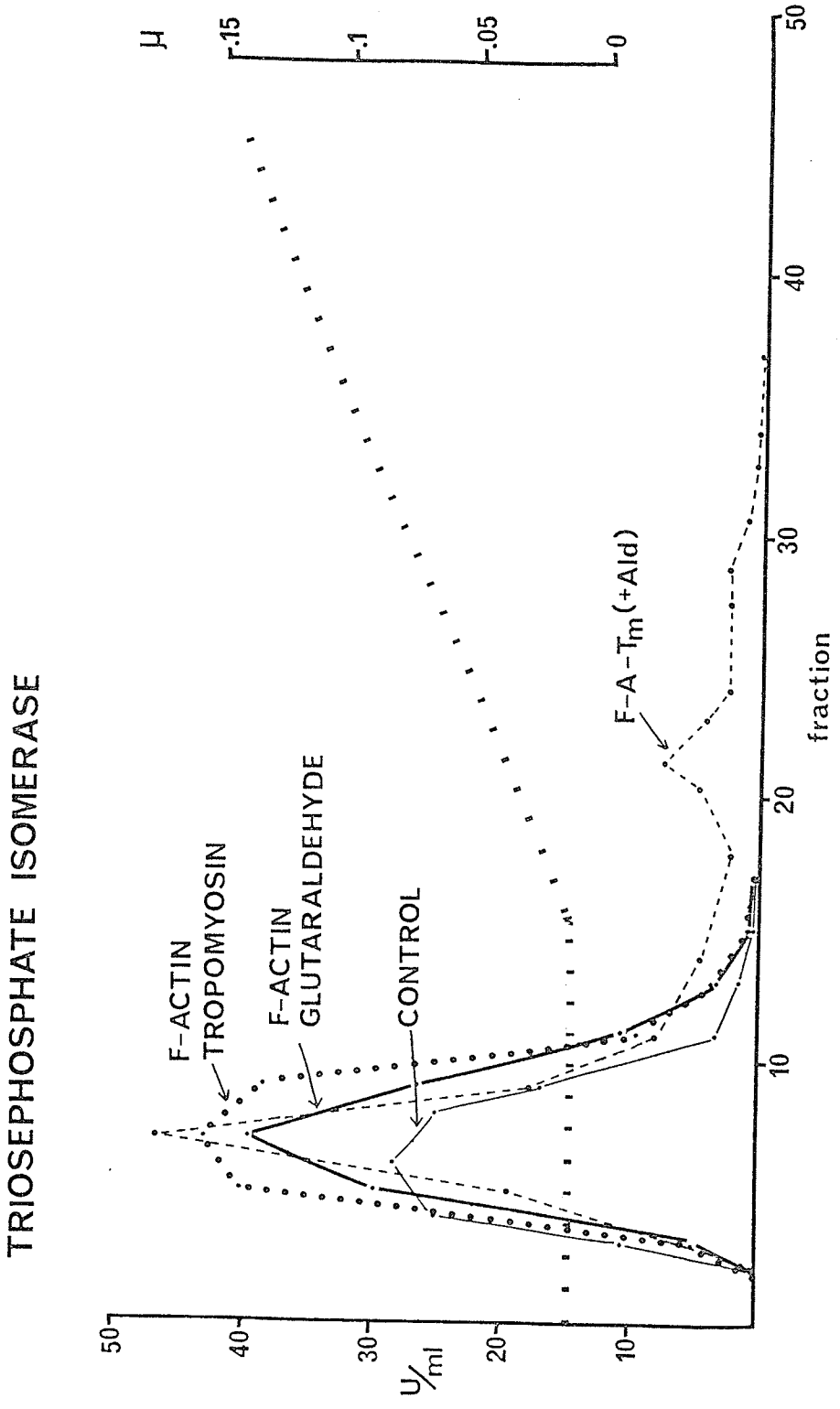

LACTIC DEHYDROGENASE
F-ACTIN
F-ACTIN
GLUTARALDEHYDE
F-ACTIN
TROPOMYOSIN
μ
.15
.1
.05
0
14
10
6
2
U/ml
10
20
30
40
50
fraction

Fig. 19. Profile for the elution of triosephosphate isomerase from control, F actin glutaraldehyde cross-linked , F-actin-tropomyosin glutaraldehyde cross-linked and an F-actin-tropomyosin column containing bound aldolase (+Ald). (For details see legend to Fig. 8.)


TRIOSEPHOSPHATE ISOMERASE
F-ACTIN TROPOMYOSIN
F-ACTIN GLUTARALDEHYDE
CONTROL
F-A-Tm (+Ald)
μ
.15
.1
.05
0
U/ml
50
40
30
20
10
10
20
30
40
50
fraction

Discussion

The results indicate that certain glycolytic enzymes interact with the various forms of matrix bound actin. Hexokinase, which is known to have a specific association with mitochondria, did not bind (33). Creatine kinase, which is a component of the M line in muscle (30), eluted immediately in the void volume as expected. The other enzymes which displayed no affinity for the F actin, G actin, and F-actin-tropomyosin matrices were triosephosphate isomerase, phosphoglycerate mutase, enolase and phosphohexose isomerase.

An interesting finding was that triosephosphate isomerase did not interact with the immobilized actin (Figure 19). In contrast, previous studies have shown this enzyme to pellet with actin along with other enzymes present in a muscle extract (9). One explanation is that actin structure may be altered during the cross-linking treatment with glutaraldehyde and/or the coupling to Sepharose. Another reasonable explanation is that triosephosphate isomerase requires other components present in the extract in order to sediment. The data presented in Figure 19 suggests that aldolase has a role in the binding of triosephosphate isomerase to F-actin-tropomyosin. The implication is that triosephosphate isomerase interaction with actin may not be direct, that is, it interacts with

aldolase or an aldolase-actin complex.

Evidence for the existence of a glycolytic multi-enzyme complex was not found. These data suggest that F-actin-tropomyosin is not an adequate structure required as a nucleus for complex formation since the column containing this ligand did not bind all of the enzymes. The data obtained in this study would suggest that if a glycolytic enzyme complex exists then the enzymes which did not adsorb to actin ie. glucosephosphate isomerase, phosphoglycerate mutase, enolase and triosephosphate isomerase must be interacting with other enzymes which bind to actin. Five enzymes were found to bind directly to F-actin-tropomyosin. The slight affinity of triosephosphate isomerase to an aldolase bound F-actin-tropomyosin ligand (Figure 19) has furthur significance if one considers interaction of the postulated constant proportion group (16). Three of the five constant proportion group enzymes did bind to actin, ie. glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase and triosephosphate isomerase, which suggests interaction or co-ordination of these three enzymes which may in turn interact with phosphoglycerate mutase or enolase (the other two enzymes of the group) which did not bind to actin. These latter associations remain to be determined.

The effect of glutaraldehyde treatment on actin structure was considered in view of the findings that the

preparatory steps performed between the addition of F actin to the CNBr activated Sepharose 4B and the application of enzymes to the column lead to partial alteration or denaturation of the native structure of F actin. Another possible explanation for the increased affinity is that glutaraldehyde fixation links amino groups in actin leading to a net increase in anionic charge. The changing ionic strength over a period of time may also affect F actin structure such that it becomes more like G actin than F actin. The glutaraldehyde treatment appears to maintain or stabilize much of the structure required for glycolytic enzyme binding. Direct comparison between untreated and glutaraldehyde treated enzyme affinity profiles, presented in Figures 14 to 18, provides evidence suggesting the presence of tropomyosin may be required for best interaction of glycolytic enzymes with this filament structure since all of the interacting enzymes displayed highest affinity for the tropomyosin containing columns.

The data presented here are in agreement with the results of other workers in that certain glycolytic enzymes associate with muscle structural proteins. Evidence for the interactions include the histochemical localization of glycolytic enzymes within the I band where the major structural protein is actin (123,139), the immunofluorescent localization of various enzymes at this same site (140), the sedimentation of glycolytic enzymes

with actin under physiological conditions (9), and the electron micrographs showing aldolase linkage to actin and associated proteins (144,145). With the exception of the latter study, the specific protein-protein interactions responsible for increasing the concentration of glycolytic enzymes in the I band regions have not been established. The data obtained in this study indicate that actin itself is responsible for adsorbing as many as six of the enzymes.

Previous investigations, with the exception of those on aldolase (9,24), utilized ultracentrifugation of tissue extracts containing a variety of proteins including glycolytic enzymes with purified actin. That is, whether specific enzymes were associating with actin or with other proteins that were associating with actin has not been determined. Specific interactions of six individual glycolytic enzymes with different forms of immobilized actin has been established in this study.

The elution profiles of the enzymes provides a survey of the relative affinities of the enzymes for the different forms of actin. Enzyme association with G actin has not been previously noted [except complex formation between aldolase and G actin (143,173)]. Interaction of glycolytic enzymes with G actin may be of some significance in cell types other than muscle since G actin has been shown to be present in brain and other non-muscle cells (174,175).

The enzymes observed to bind to the G and F actin

columns adsorbed with a greater affinity to the filamentous form. It appears that these enzymes have the strongest affinity for the F-actin-tropomyosin ligand. Significance of this phenomenon would be that tropomyosin plays a role in enhancing interaction. This protein must also be considered as a potential binding site of enzymes within the I band as has been found in ultracentrifugal studies of mixtures of aldolase with tropomyosin (176). This remains to be determined in the column affinity system.

The possibility of glutaraldehyde fixation of the actin subunits with tropomyosin creating this effect was ruled out with the following observations. A direct comparison of the enzyme elution profiles from the glutaraldehyde treated F actin column and the F-actin-tropomyosin cross-linked column definitely suggests a role for tropomyosin in the binding of aldolase, glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase, lactate dehydrogenase and pyruvate kinase. All of these enzymes had a greater affinity for the tropomyosin containing columns. This finding is likely the reason why Clarke and Masters (9) observed increased amounts of glycolytic enzymes sedimenting with F-actin-tropomyosin as compared to F actin. The finding that aldolase was necessary for the binding of triosephosphate isomerase to F-actin-tropomyosin suggests that enzyme-enzyme interactions may exist and be important in providing a

locus for possible arrangement of glycolysis *in vivo*.

Summary

1. Certain glycolytic enzymes interact with F and G actin.
2. Enzymes that reversibly bind to actin are aldolase , glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase and lactate dehydrogenase.
3. Enzyme interaction with F actin appears to be stronger than G actin.
4. F actin treated with glutaraldehyde has an increased affinity for these enzymes when compared to untreated F actin.
5. The greatest affinity was observed with F-actin-tropomyosin as the ligand.
6. Pyruvate kinase has only slight affinity for F and G actin but the inclusion of tropomyosin enhanced it's binding.
7. Triosephosphate isomerase was adsorbed in the column if aldolase had previously been bound to F-actin-tropomyosin suggesting an enzyme-enzyme interaction.
8. The association of the enzymes with the various actin ligands appears to be ionic.

Table 2

IONIC STRENGTH AT WHICH GLYCOLYTIC ENZYMES ELUTED FROM COLUMNS CONTAINING ACTIN

This table is a brief summary of portions of the data shown in Figures 8-19

	G Actin	F Actin	F-Actin-Gl	F-Actin-Tm	F-Actin-Tm-ALD
HK	Δ	Δ	Δ	Δ	
PHI	Δ	Δ	Δ	Δ	Δ
TPI	Δ	Δ	Δ	Δ	0.04-0.05
ALD	0.03-0.04	0.05-0.06	0.06-0.07	0.06-0.09	
GAPDH	0.05-0.06	0.06-0.07	0.07-0.08	0.12-0.13	
PGK	0.03-0.04	0.03-0.04	0.05-0.06	0.06-0.07	
PGM	Δ	Δ	Δ	Δ	
E	Δ	Δ	Δ	Δ	
PK	Δ	Δ	0.03-0.04	0.05-0.06	
LDH	0.017-0.025	0.02-0.03	0.03-0.04	0.04-0.05	
CK	Δ	Δ	Δ	Δ	

Δ: denotes no binding

Other Possible Experiments

Application of this model system utilizing affinity chromatography serves as a excellent method for studying specific glycolytic enzyme interaction with structural proteins. Other parameters that influence the association should be investigated. Troponin subunits may be linked to F-actin-tropomyosin and coupled to an affinity support in order to determine its role in enzyme binding. Furthur studies may be performed to show the effect of pH, temperature, substrates and various ions on the apparent association. No attempt was made to saturate the enzyme binding capacity of each column. This may be done in an attempt to stoichiometrically analyze enzyme-actin filament association. Myosin may be used to standardize the columns. It also may be possible to show enzyme-enzyme-actin interactions. Enzymes may be bound to actin, eg. glyceraldehydephosphate dehydrogenase and aldolase, followed by passage of enzymes that did not bind in the above study. This model could be used as a future working hypothesis in an attempt to reconstitute an F-actin-tropomyosin-troponin glycolytic enzyme complex. Since not all of the enzymes found in the I band actually interacted directly with actin , it is suggested that some enzymes may be interacting with enzymes associated with the actin filament. This model

may also be utilized with materials from other tissues. Actin has been isolated from non-muscle tissues such as brain. The problem with centrifugation studies is that the amount of sample required to study the interactions makes it difficult to quantitate. Immobilizing a small quantity of brain actin to an affinity support may provide an effective means of detecting glycolytic enzyme association. The possibility of enzyme interaction with non-muscle actin is intriguing. Actin has been found in many tissues. This structure may serve as an important component involved in the organization of glycolysis leading to a more efficient means of producing cellular energy.

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