INTERACTIONS BETWEEN SOLUBLE ENZYMES AND SUBCELLULAR STRUCTURE

Author: **Colin J. Masters**
School of Science
Griffith University
Brisbane, Australia

Referee: John E. Wilson
Biochemistry Department
Michigan State University
East Lansing, Michigan

I. INTRODUCTION

The interactions between enzymes and subcellular structure have been the topic of a great deal of scientific consideration in the past, and there is now a widespread realization of the biological significance of these interactions in the cellular microenvironment.[1-11] Much of the emphasis of previous research into enzyme-structure interactions, however, has been directed towards the more evident cases of such associations — those enzymes which are strongly bound, integral components of membranes or subcellular organelles — but there is now a discernible tendency towards a broadening of these considerations, and the inclusion of weaker, noncovalent and reversible interactions in topical research interests.

In recent years, for example, there has been considerable discussion and increasing acceptance of the possibility that the enzymes of the cytosol may interact with the cellular matrices or be organized into multienzymic complexes. The cytosol, of course, is known to be one of the most important metabolic compartments of the cell — containing as it does several major pathways and a large number of so-called soluble enzymes (Table 1). This latter terminology (i.e., the common reference to cytosolic proteins as "soluble" components) should not be taken as indicating that these compounds are freely dissolved within the cell, however. Rather it should be regarded as an operational definition referring more to the common application of centrifugal procedures in subcellular fractionation than to any established *in vivo* characteristic.

At the same time, it needs to be recognized that any unequivocal demonstration of an interaction between components of the cytosol and membranous structures by classical, centrifugal techniques is attended by considerable technical difficulties, which relate to the possible perturbations of cellular structure during homogenization, the common use of nonphysiological suspension media and the susceptibility of the cytosol to component redistribution during subcellular fractionation. Nevertheless, a number of scientists have directed their attention towards the cytoplasm in recent years,[7,10,12-17] and a growing list of reports in the literature using a variety of methodologies attest to the association of soluble enzymes with a variety of cellular components in different cell types.

In an historical sense, much of this current topicality of interactions between cytosolic components and cellular structure may be traced back to the seminal investigations of Green and co-workers in 1965. Considerable interest was aroused by these descriptions of membrane fractions which were capable of catalyzing the complete sequence of glycolysis with greater specific activities than those of the original homogenate, and their extrapolation to the general postulate that all metabolic sequences might be membrane

Table 1
MULTIENZYME SEQUENCES PREDOMINANTLY LOCALIZED IN THE "SOLUBLE" FRACTIONS OF CELLS

Amino acid activation and transfer to *t*RNA
Glutathione biosynthesis
Complete glycolytic sequence from glycogen to pyruvate; all enzymes involved in glyconeogenesis from *P*-enol pyruvate to glycogen
Pentose phosphate cycle
Biosynthesis of fatty acids
δ-Amino levulinate → coprophyrin III
Tryptophan catabolism
Catabolism of pyrimidines

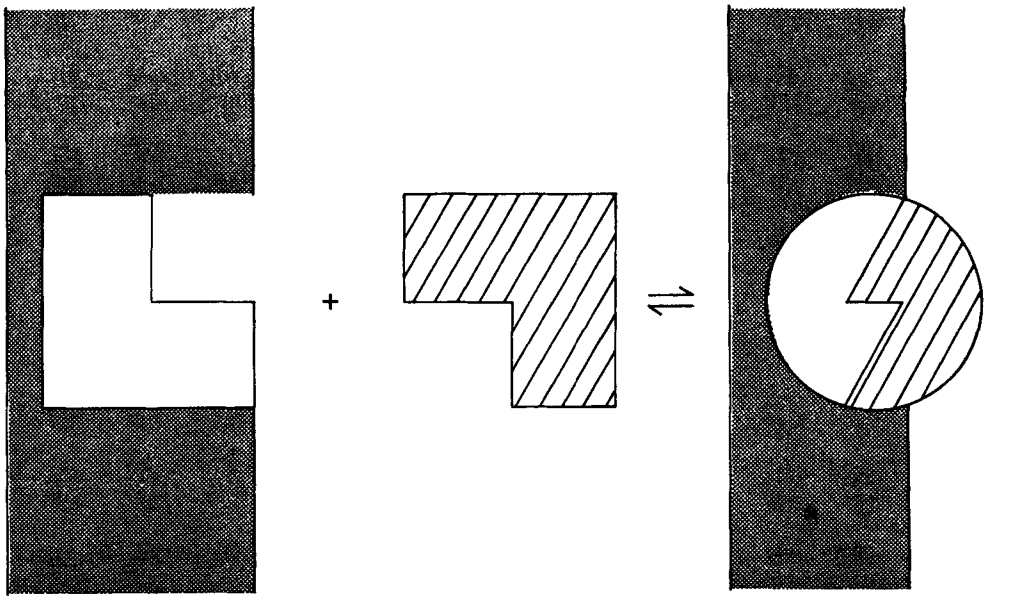


FIGURE 1. Diagrammatic illustration of interaction between cellular structure and soluble components. The principle features are the presence of specific binding domains on the particulate structures, the occurrence of reversible binding between the soluble and particulate components, and the possibility of attendant conformational alterations.

bound *in vivo* led to widespread discussion of the topic. In the intervening years, the original ideas have been modified and the relevant data extended by more detailed studies on individual glycolytic enzymes. Among the studies which have contributed most significantly to our present understanding of interaction phenomena are those involving the binding of hexokinase to mitochondria,[18,19,38] the association between lactate dehydrogenase and cellular structure,[20] and the binding of aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and phosphofructokinase to the particulate elements of many tissues[1,7,8,13] (Figure 1).

Viewing these interactions between soluble enzymes and subcellular structure from a biological point of view, one is immediately confronted with the possibility of metabolic advantage associated with such biphasic distribution of enzymes. Higher organisms characteristically display a compartmentation of cellular functions which is of fundamental importance to the processes of regulation and differentiation, for example, and a basic element of this compartmentation is often the provision of alternative means of catalyzing individual reactions. Hence, an understanding of the characteristics of these enzyme-structure assemblies would seem essential to any full and satisfying comprehension of the cellular processes.

In this review, attention is directed toward the current status of research efforts on the interactions between cytosolic enzymes and subcellular structure. The initial sections deal with some of the principle examples of cytosolic enzymes and their multiple forms of activity; then the available experimental data on interactions of the cytosolic components are considered, as well as the biological implications of these data. Comment is made on some of the procedural limitations in the past as well as some of the newer methodologies that are allowing the present rapid advancement in our understanding of subcellular localization. Because of space considerations, the major emphasis of these comments has been restricted to mammalian systems.

II. CYTOSOLIC ENZYMES AND ISOZYMES

As already seen, a number of important enzyme systems are identified as being localized in the cytosol. On the basis of studies involving the classical methods of subcellular fractionation, for example, the enzymes of glycolysis and the pentose phosphate pathway are commonly recognized as "soluble" enzymes, and many of the individual components of these and other cytosolic pathways have been intensively studied. It is beyond the scope of this review to examine the catalytic characteristics of each of these enzymes in detail, since the available data for some are sufficiently substantial to have justified separate reviews in their own right. Instead the major conclusions on the native interactions of such "soluble" enzyme systems have been discussed at some length in the later sections, and in regard to the more general properties of these enzymes readers have been referred to the many excellent reviews and other publications available.

There is one major feature of enzyme-structure interaction, however, that has not been generally included in previous reviews of this topic, yet is basic to any satisfactory treatment of these interactions — that is the characteristic of enzymes to occur in multiple forms of activity. In higher organisms, for example, most of the separate tissues display characteristic distributions of activity amongst the multiple enzyme forms, and these individual forms of an enzyme may interact quite differently with cellular structure and with metabolism. Also, cellular structure will vary between different tissues. Hence, it is of major importance to consider the isozyme status of any enzyme activity and the tissue distribution of the multiple forms before any attempt at definition of interactions are attempted.

The position with regard to the established heterogeneity of the major glycolytic enzymes is summarized in Table 2. With the exception of the phosphohexose isomerase, glyceraldehyde phosphate dehydrogenase, and phosphoglyceroisomerase, there is firm evidence that the individual glycolytic enzymes exist as a number of different protein forms. The most divergent tissues in terms of isozymic phenotype are those of liver and muscle which exhibit distinct isozymic profiles with at least seven of the glycolytic enzymes. Aldolase, for example, has been shown to exist in at least nine separate forms in most higher organisms. These forms are all tetramers and represent the different

Table 2
MULTIPLE FORMS OF GLYCOLYTIC ENZYMES

Enzyme	Multiple forms	Major localization	Ref.
Hexokinase*	A	Brain	102
(E.C.2.7.1.1)	B	Muscle	
	C	Liver	
	D	Liver	
	E	Sperm	
Phosphoglucomutase	A	Wide distribution	103
(E.C.2.7.5.1)	B	Wide distribution	
	C	Fibroblasts	
Phosphofructokinase*	A_4	Muscle, heart	104
(E.C.2.7.1.11)	B_4 + AB hybrids	Live, red cells	
	C_4 + AC hybrids	Brain	
Aldolase*	A_4	Muscle, heart	23
(E.C.4.1.2.13)	B_4 + AB hybrids	Liver, kidney	
	C_4 + AC hybrids	Brain	
Fructose bis phosphate	A_4	Muscle	125
(E.C.3.1.3.11)	B_4	Liver	
Phosphoglycerate kinase	A	Wide distribution	105
(E.C.2.7.2.3)	B	Sperm	
Enolase	A_2 + AB hybrids	Muscle, heart	106
(E.C.4.2.1.11)	B_2	Liver	
	C_2 + BC hybrids	Brain	
Pyruvate kinase*	A_4	Muscle, heart	22
(E.C.2.7.1.40)	B_4 + BC hybrids	Liver, kidney	
	C_4 + AC hybrids	Wide distribution	
Lactate dehydrogenase*	A_4 + AB hybrids	Wide distribution	22
(E.C.1.1.1.27)	B_4	Heart	
	C_4 + BC hybrids	Sperm	

* These enzymes are known to bind in significant proportion to enzyme structure with consequent changes in their activity. Metabolites are also known to influence the degree of adsorption.

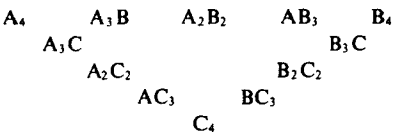
possibilities of hybridization between three types of gene product (Table 3). Not only do these multiple forms display different kinetic characteristics, but there is also firm evidence of differential binding properties between the individual multiple forms.[21-23]

In retrospect, it is perhaps not surprising that the binding characteristics of different multiple forms of an enzyme should be distinctive. If we accept that the interactions of these enzymes are significant *in vivo* (and there now seems little doubt on this score), then it is not difficult to envision the possibility that evolutionary pressures might well bring about divergences in the binding domains of the separate multiple forms of an enzyme, in a similar fashion to the divergence which is widely accepted for the carrier portion of proteins.

It is also clear, however, that the possibility of different binding characteristics between individual enzymes has not been thoroughly investigated in the case of all the individual soluble enzymes, and that the principle needs to be widely established that in investigations of interactions between soluble enzymes and structure, it is important to specify both the nature of the structural component and the isozymic status of the catalytic component.

A number of other factors may also be involved in determining the *in vivo* significance of the binding characteristics of a particular enzyme. There are several difficulties in translating from the binding properties of the purified enzyme *in vitro* to the realities and complexities of the cellular microenvironment, and many of these are referred to in the

Table 3
SUBUNIT STRUCTURES OF MAMMALIAN ALDOLASES



subsequent sections of the review. It might be appropriate at this point, however, to establish that not only is it important to specify the natural heterogeneity of an enzyme as a prerequisite to studies of its interactions, but it is also often necessary to take account of the possibility of artifactual heterogeneity. It has recently been shown, for example, that both fructose bisphosphatase and fructose-1,6-bisphosphate aldolase may be isolated as proteolytically modified form from tissue extracts in which lysosomal proteases had been released into the cytoplasm.[24-26] It appears that early results on these enzymes which were thought to be representative of the native catalysts were in fact grossly modified, and hence the specter of a similar artifactual modification of the binding properties must be considered in relation to other soluble enzymes.

One further aspect of major importance in considerations of the role of cytosolic enzymes in interactions is the influence of adsorption on the catalytic properties of the enzymes. It is becoming increasingly clear that binding to particulate material may significantly modify the kinetic parameters of an enzyme. This being so, the role of the enzyme in metabolism may also be influenced, and the available data appear to support this possibility. Readers will find a number of examples in the subsequent sections, but to cite one case at this point, the data of Karadshen and Uyeda might be mentioned[27] (Figure 2). These workers have shown that there is a specific interaction between phosphofructokinase and the inner surface of the erythrocyte membrane. Associated with this binding is a change in the allosteric properties of the enzyme. Unlike free phosphofructokinase, the membrane-bound enzyme is not inhibited by ATP or 2,3-diphosphoglycerate, and its fructose-6-phosphate saturation curve reverts to a nonsigmoidal shape. Apparently the interaction has reduced or "frozen" the conformational flexibility of the enzyme. Results such as these obviously imply that the classical regulatory role of this key allosteric enzyme may be markedly influenced by adsorption to cellular structure, and that the biological function of other enzymes may also be quite different to that assumed on the basis of monophasic kinetics. Hence, a major new element is introduced to control considerations in cellular metabolism.

A word of caution is necessary in regard to the exciting possibilities of this rapidly developing area, however. Very careful experiments are necessary to determine whether the alteration of the kinetic characteristics of an enzyme or binding to particulate structures occurs under conditions which are characteristic of the cellular microenvironment. The distinction between an effect which has physiological credibility and one that is merely artefactual is often subtle, and has been clearly achieved only in few instances to this time.

III. INTERACTIONS OF CYTOSOLIC COMPONENTS

A. Assemblies of Soluble Enzymes

Hopefully, the preceding outline of some of the complexities involved in the interpretation of the interaction of multiple enzymatic components within the cell and their microlocalization furnishes a context in which the available data on the interactions

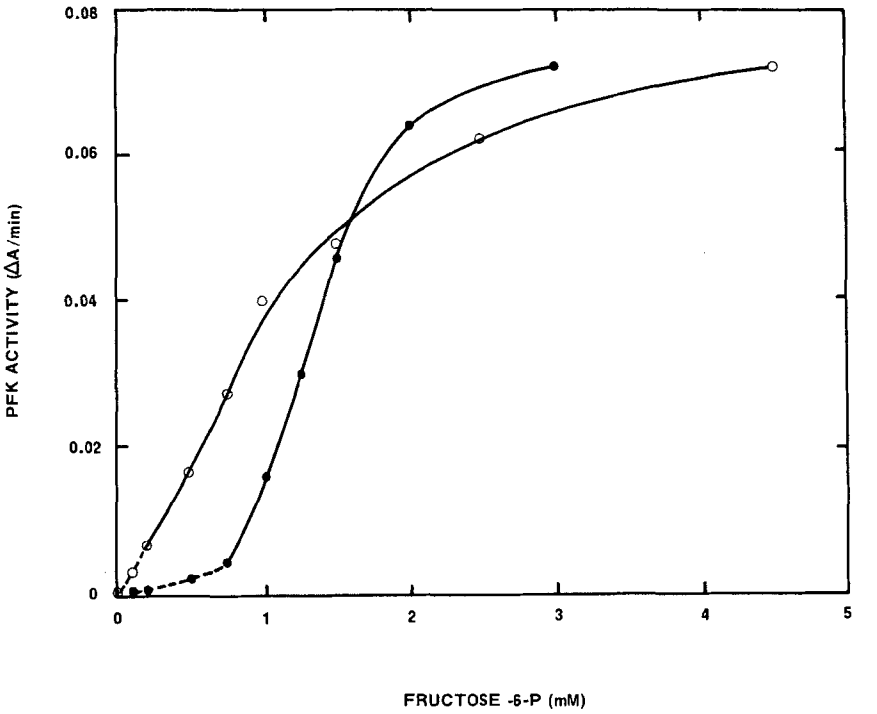


FIGURE 2. Fructose-6-phosphate saturation curves for membrane-bound phosphofructokinase (○) and the free soluble enzyme (●). (After Karadsheh, N. S. and Uyeda, K., *J. Biol Chem.*, 252, 7418, 1977.)

of some major individual enzyme systems can be considered appropriately. But before moving to a discussion of individual enzymes, it is relevant to mention the additional possibility of macromolecular associations between cytosol components leading to the formation of a "soluble" metabolic complex. There is, of course, a considerable current scientific emphasis on multienzyme aggregates,[25,28] but while some of these macromolecular assemblages (e.g., the pyruvate dehydrogenase complex and fatty acid synthetase) appear to be relatively stable and well substantiated, other possibilities exist which are more subject to contention. As one instructive example, the possible existence of a complex of the glycolytic enzymes may be considered.

A long history of controversy has attached to attempts to define the reality of the "glycosome". As previously described, more than a decade ago Green and his coworkers[30] reported the disruption of erythrocytes and yeast cells and the derivation of fractions which were capable of catalyzing the complete sequence of glycolytic reactions with greatly increased specific activity over that of the whole homogenates. These results led these workers to assume that the entire glycolytic sequence was membrane bound, and indeed to favor the general postulate that all major metabolic sequences might be membrane bound *in vivo*. Many other scientists have found Green's concept attractive and have sought substantiating proof in the intervening years. In particular, mention may be made at the conceptual level of the growth of emphasis on a limitation of solvent capacity in the cell,[14] and at the practical level, the ingenious press juice experiments of Amberson et al.[31]

More recently, substantial support for the concept of a glycolytic complex has arisen from a variety of sources. In *Escherichia coli,* evidence has been obtained for a multienzyme complex with glycolytic activity[32] (Figure 3), and the localization of nine glycolytic enzymes in a microbody-like organelle has been established in trypanosomes.[33]

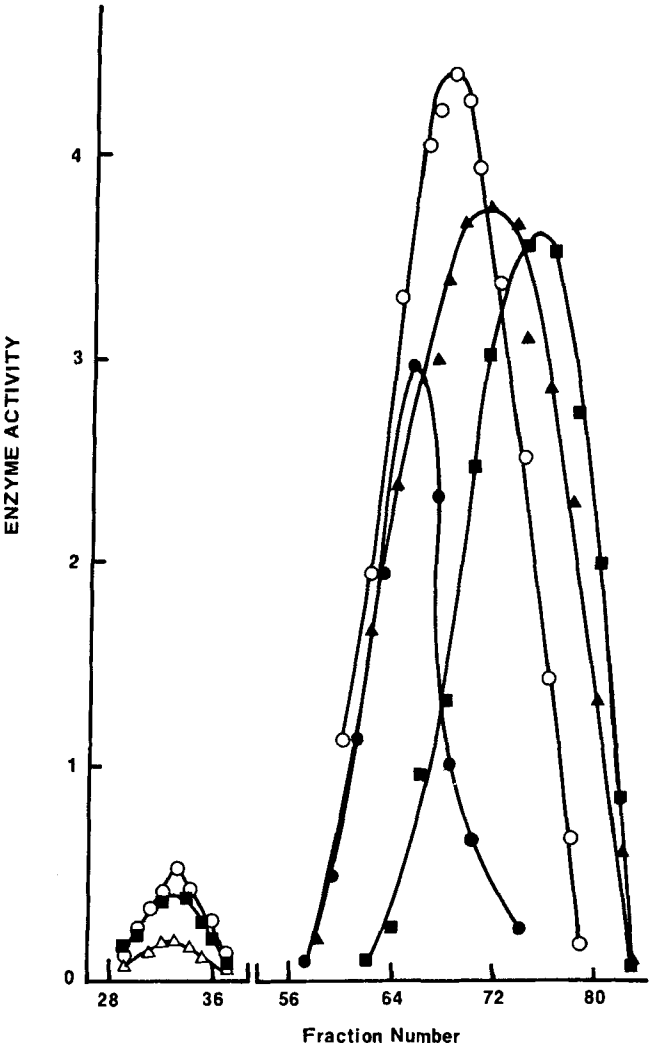


FIGURE 3. Elution profile for supernatant from a lysed spheroplast preparation of *E. coli* from an agarose gel column. ■, phosphofructokinase; ○, phosphoglucose isomerase; ●, glyceraldehyde-3-phosphate dehydrogenase; ▲, triosephosphate isomerase. (After Gorringe, D. M. and Moses, V., *Biochem. Soc. Trans.*, 6, 167, 1978.)

The picture in mammalian tissues is somewhat different (as seen in the subsequent pages), but intensive studies have again lead to the concept of the complexing of these components, with, in this case, a plating onto structural components containing actin-like proteins.[7] Possibly the different subcellular locations in eucaryotes and procaryotes may be related to the phylogenetic divergences of actin distribution.

These findings on the "glycolytic complex", along with the indications of assemblages in other soluble pathways,[113] are significant in relation both to the technical aspects of microlocalization of isozymes and also to interpretations of their function within the cell. From the point of view of methodology we may say that considerable advances in our understanding of association phenomena have occurred in recent years, and that such advances justify a wider consideration of the occurrence of macromolecular assemblages in future investigations of enzyme localization. In relation to their function, there would

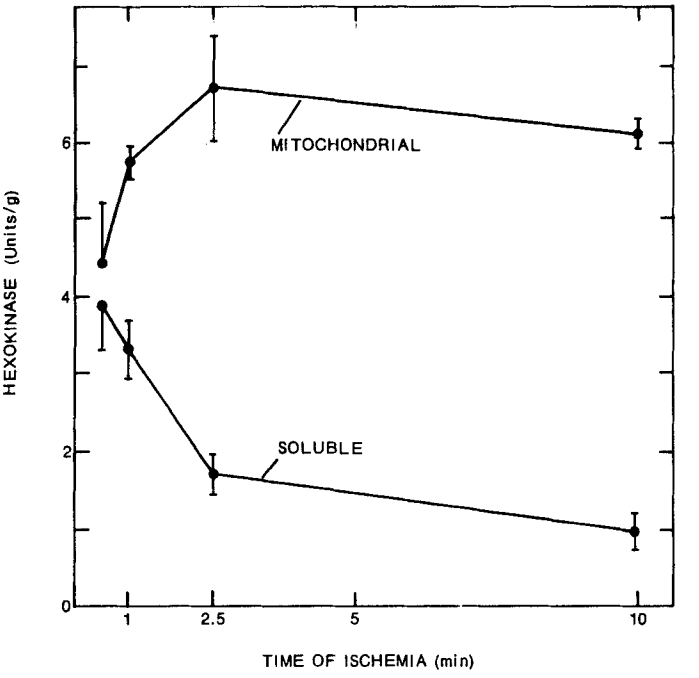


FIGURE 4. Redistribution of brain hexokinase between the mitochondrial and cytosol fractions during ischemia. (After Knull, H. R., Taylor, W. F., and Wells, W. W., *J. Biol. Chem.*, 249, 6930, 1974.)

appear to be real possibilities of advantage in respect of reduced transit times of the substrates and increased possibilities for control, but many significant questions on their metabolic significance still remain to be answered.

In moving to consider the interactions of individual cytosolic enzymes with structure, a small selection of the most widely investigated enzymes has been chosen. The first of these is hexokinase.

B. Hexokinase

Hexokinase (E.C.2.7.1.1.) is a major component of the glycolytic system and possesses well-defined binding properties.[18,19,34,38] This enzyme occurs in most tissues as three electrophoretically distinct forms (types I, II, and III), and in addition liver contains a glucokinase, sometimes referred to as type IV hexokinase.[35-37]

Although commonly referred to as a "soluble" enzyme, essentially all the hexokinase activity in brain is found in the mitochondrial fraction, and a sizable proportion of the activity in other tissues is also bound to the mitochondrial membrane. Of the particulate activity in brain, approximately half is in overt form (that is, the activity can be assayed directly with no pretreatment of the particles) and the remainder is in latent form, the activity becoming assayable only after treatment of the particles with membrane-disrupting techniques which act to solubilize the enzyme.[19,38] The particulate-bound latent state of hexokinase corresponds to type I, while the particulate-bound overt form may consist of both types I and II.[37]

Such biphasic interactions between membranes and enzymes offer intriguing opportunities for metabolic regulation as mentioned previously, and one particular possibility that arises in the present instance is related to a difference in the catalytic properties of the soluble and bound forms. Newsholme et al.[39] have noted that the soluble

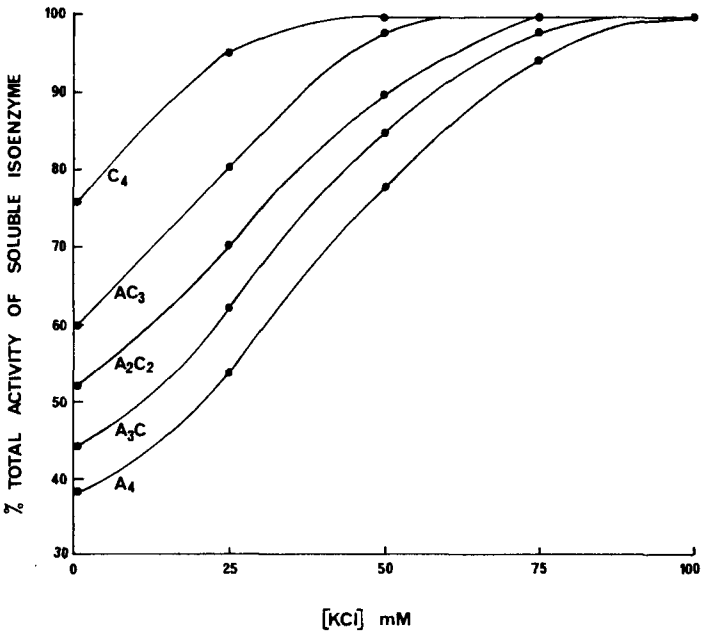


FIGURE 5. The elution of the isoenzymes of aldolase from rat brain by increasing salt concentration. (After Clark, F. M. and Masters, C. J., *Int. J. Biochem.*, 6, 132, 1975.)

form of hexokinase is more susceptible to inhibition by glucose-6-phosphate than the bound form, and it has also been suggested that the compartmentation of hexokinase may influence the ultimate fate of glucose-6-phosphate, which is located, of course, at the junction of three major metabolic pathways. It is notable, too, that the hexokinase partitioning between the soluble and mitochondrial fractions of brain has been observed to vary according to the energy status of this tissue. Ischemia, for example, leads to an increased activity in the mitochondrial fractions[40] (Figure 4). Insulin and glucose concentrations also exert a marked effect on the distribution of activity between the multiple forms and different pools of activity of this enzyme.[36] Observations such as these provide a satisfying confirmation of the presence of a distribution equilibrium of tissue isozymes which is intimately involved in the regulation of carbohydrate metabolism.

A further aspect of the regulation of the particle-bound/soluble equilibrium of hexokinase has been addressed by the recent studies of Katzen and Soderman.[37] They have provided evidence that sulphydryls which are not implicated in the catalytic activities of the enzyme are involved in the binding to membrane components — the type II isozyme in particular was readily solubilized by the inhibition of these groups. Hence, yet another factor which may cause the soluble/particulate equilibrium of isozymes in cell extracts to differ from the natural intracellular distribution is introduced and requires consideration as an artifactual possibility.

C. Aldolase

Within vertebrate species three distinct types of fructose-1,6-bisphosphate aldolase (E.C.4.12.13) activity have been recognized: A type is the classic muscle-type activity, B type predominates in liver, and C type has been isolated from brain. All these enzymes are tetrameric, and five-membered sets of isozymes (i.e., the A-C set and A-B set) are commonly found in vertebrate tissues.[22] It is with aldolase that the most intensive

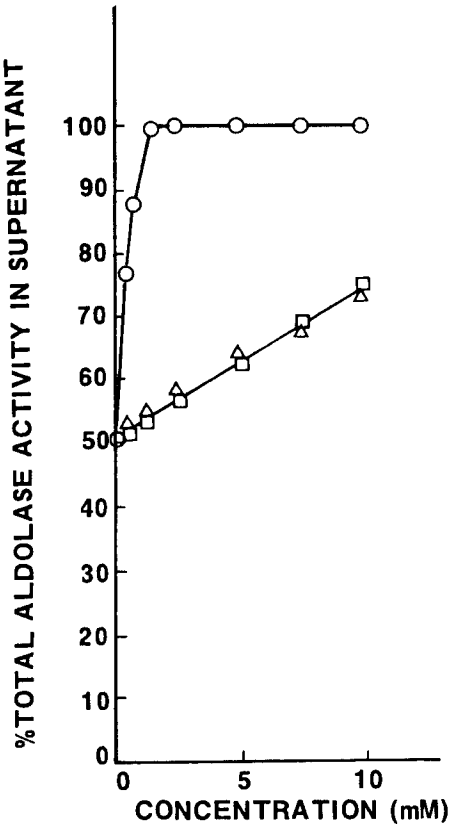


FIGURE 6. Influence of different phosphorylated fructose derivatives on desorption of particulate aldolase in rat brain homogenates. ○, F8P; △, FIP; □, F6P. (After Clarke, F. M. and Masters, C. J., *Int. J. Biochem.*, 7, 359, 1976.)

microlocalization studies of a "soluble" isozyme system in recent years have been undertaken, and these data may be considered with benefit as an example of the multidisciplinary methodologic approach that is required in these investigations if the derived information is to be meaningful in a cellular context.

As one point of interest, it may be noted that, whereas the majority of subcellular fractionations have been concerned with liver, a challenging requirement for studies in other major tissues remains. Brain, for example, is morphologically a much more complex tissue than liver, and it is evident that the problems of enzyme localization and interaction are compounded by this fact. Taking this tissue as an example, it may be noted that one of the first points which was established in regard to the distribution of aldolase was that a considerable quantity of enzyme (present as an A-C isozyme set) was identified as binding to the particulate material in sucrose homogenates. In studying the nature of this binding, it was noted that aldolase was readily removed by salt and pH gradients with a markedly preferential release of C-type subunits over A-type subunits[21] (Figure 5).

These data are consistent with the existence of an electrostatic interaction between the enzyme and the associated structural components. Aldolase A carries a more anodic charge at physiologic pH than aldolase C or the AC isozymes, and this order of charge diminution parallels the individual binding propensities of the isozymes. In regard to the

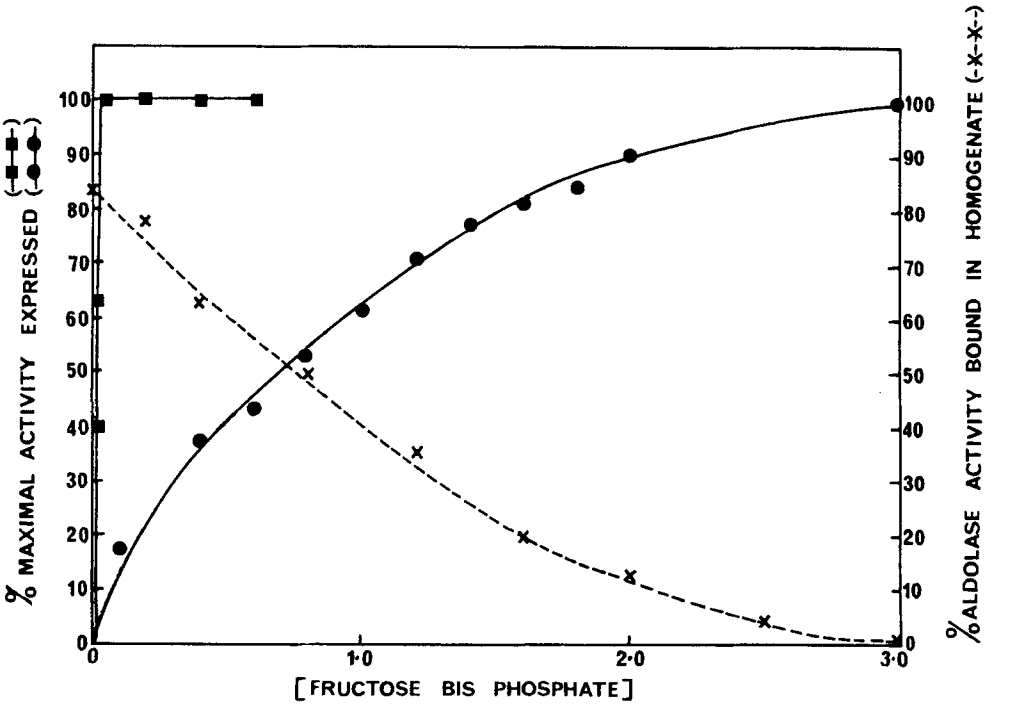


FIGURE 7. Fructose bisphosphate saturation curves for aldolase activity from rat brain in the free soluble form (■) compared to the activity of whole homogenate (●). The declining curve (x) represents activity of whole homogenate which was extractable from the particulate fraction. See Reference 5.

membrane contribution, it would be anticipated that binding of the isozymes was occurring with a binding domain which contained both negatively charged groups and a degree of structural complementarity.

Adding interest to this situation and setting it apart from a general nonspecific binding is the degree of aldolase attachment in this and other tissues,[12,41] and the property of specificity in the degree of desorption brought by certain function-dependent metabolities. The substrate of this enzyme, fructose-1,6-bisphosphate, for example, exerts a pronounced effect on the desorption process, causing a considerable enhancement of release at very low concentration (Figure 6). Such data would suggest the participation of further, more subtle interactions in the binding of the enzyme, involving the active site either directly or indirectly.

In relation to the physiologic correlations of this binding, a question remains as to what happens to the activity of the aldolase when it binds to the membrane. To provide some insight to this query, the effects of increasing FDP concentration on the activity of the soluble enzyme, and by comparison on the aldolase activity of a rat brain homogenate system, have been studied and are shown in Figure 7. The activity response curves are markedly different in these two situations. At low FDP levels the catalytic activity of the bound enzyme was markedly reduced by comparison with the soluble systems, and in the biphasic system more than 50 times the level of FDP than in the soluble system was required for maximal expression of catalytic activity.

Previous studies on the binding of aldolase to cellulose phosphate[42] are relevant to these observations in that they have pointed to a masking of the active site of this enzyme by adsorption, with desorption of the enzyme from the model particulate phase by FDP

reestablishing the ready accessibility and catalytic activity of the aldolase. A similar mechanism in the cellular environment would provide a ready basis of explanation for the differences in activity which are observed in nerve tissue homogenates between aldolase in the free and bound states. Also, the existence of an equilibrium between free and bound forms of aldolase, each form having intrinsically different catalytic properties with the equilibrium being dependent on metabolites, presents the ingredients of a control system for glycolysis; indeed several studies have noted high FDP levels in brain, consistent with a limitation of flux for this enzyme of lowest maximal catalytic capacity in this tissue.[5,7]

In attempting to define further the subcellular localization of the bound enzyme within the complicated detail of cellular structure which exists in a tissue such as brain, differential gradient centrifugation and microscopic examination of the fractions are indicated as logical subsequent steps. These procedures have revealed that each of the primary fractions exhibited appreciable aldolase activity, but that the microsomal fraction was of special interest since it exhibited a specific activity which was more than half as great again as that in the original homogenate.[21] Subfractionation of this primary fraction by gradient centrifugation identified the source of the bound enzyme as one particular microsomal membrane fraction.

A membrane type having been identified and purified in this way, more detailed information can be derived on the nature of the adsorption phenomena contributing to this microlocalization and on the kinetic parameters of the enzyme in the membrane-bound state. In the present instance, for example, the membranes have been isolated and treated with specific reagents such as phospholipases and ribonucleases in order to clarify the role of specific groups in the adsorption process, and the numbers and interactions of the binding sites have been deduced by analysis of kinetic properties.[43] Thus, even in such a complex tissue as brain it has been possible to reach a reasonably sophisticated level of understanding in regard to the molecular interpretation of microlocalization by the aldolase isozymes.

In skeletal muscle the subcellular localization of aldolase isozymes is much more simply prescribed. The predominant isozyme is the A_4 form, and histochemical observations and binding studies have localized this activity at the I band of the contractile unit[44,45] (see subsequent section of this review).

In mammalian liver the major form of aldolase is the B_4 isozyme, and the intracellular location of this activity has also been investigated. Foemmel et al.[46] have presented evidence that rat liver aldolase is for the most part associated with the elements of the endoplasmic reticulum. In particular they quoted data on the subcellular distribution and cytoimmunology of liver aldolase. Subsequently, however, Arion and Lange[47] reexamined the distribution of this isozyme and came to the conclusion that the previous reports referred to an artifactual binding to ribosomal nucleic acids. They concluded that aldolase-B was not associated with the endoplasmic reticulum *in situ*.

D. Lactate Dehydrogenase

Lactate dehydrogenase (LDH.E.C.1.1.1.27) commonly occurs as five isozymes by the combination of A and B subunits into tetrameric structures.[22] These isozymes are usually separated under *in vitro* conditions by means of their charge of differences, and it would seem very probable that charge characteristics also influence the location of the isozymes under *in vivo* conditions.

There are several reports in the literature that suggest an association of this enzyme with nuclear, mitochondrial, or microsomal fractions, but the data usually stop short of a demonstrable functional association. Evidence of the preferential association of LDH-B_4

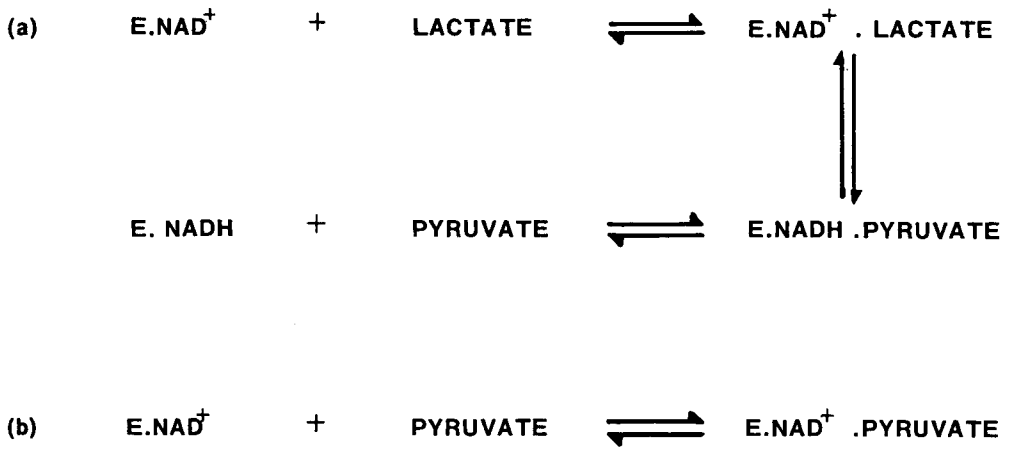


FIGURE 8. (a) Reaction sequence for the enzyme-catalyzed conversion of lactate to pyruvate via ternary complexes of enzyme-coenzyme substrate. (b) Formation of an abortive ternary complex.

with mitochondria is particularly strong, and in this case the positioning makes good physiologic sense in relation to the particular kinetic characteristics of this isozyme and indications of a metabolic association.[48-50] The latter workers, for example, noted that repeated washing of mitochondria failed to abolish aerobic oxidation of lactate, and that such oxidation occurred internally rather than externally. LDH-B_4 was found to be localized within the intermembrane space of liver mitochondria, and evidence was adduced of an energy-dependent transfer of reducing equivalents with the enzyme systems of the inner membrane and matrix. LDH-C_4, the lactate dehydrogenase isozyme that is specific for testis and sperm, has also been reported to be associated with a special type of mitochondria in cells of the spermatogenic line, in the mitochondrial sheath of spermatozoa, and in the cytoplasm of sperm.[51,52]

More recent data on the particulate associations of LDH isozymes are also worthy of further consideration at this point. Ehman and Hultin[20] have made the interesting observation that whereas LDH-A_4 binds readily to the particulate matter in skeletal muscle, LDH-B_4 binds little if at all. Furthermore, under their experimental conditions almost complete inhibition of the soluble enzyme by high concentrations of pyruvate was observed, whereas there was no detectable inhibition of bound LDH. Pyruvate inhibition of lactate dehydrogenase occurs through the formation of an abortive ternary complex involving the enzyme, the oxidized form of nicotinamide adenine dinucleotide and pyruvate,[49] (Figure 8). Ehman and Hultin's data show clearly that inhibition of bound LDH-A_4 by NAD^+ is almost totally eliminated. They favor as an explanation the view of Griffin and Criddle,[53] who suggested that in order for the ternary complex to form, LDH had first to dissociate into its monomeric subunits, and they also argued that the particulate association may preserve the tetrameric integrity of the enzyme, thus preventing the formation of an inactive complex. Other kinetic possibilities which follow from the binding of LDH-A_4 to cell ultrastructure include diffusional restriction of the substrate, localization of pH effects, charged repulsion of substrate, and conformational changes in the enzyme,[54] but the major point is that the alteration in kinetic properties may be readily construed in terms of biologic advantage to the cell.

E. Creatine Phosphokinase

Creatine phosphokinase (CPK, ATP, creatine N-phosphotransferase, E.C.2.7.3.2) exists as three major isozymes in vertebrate tissues.[55] In muscle the major isozyme is

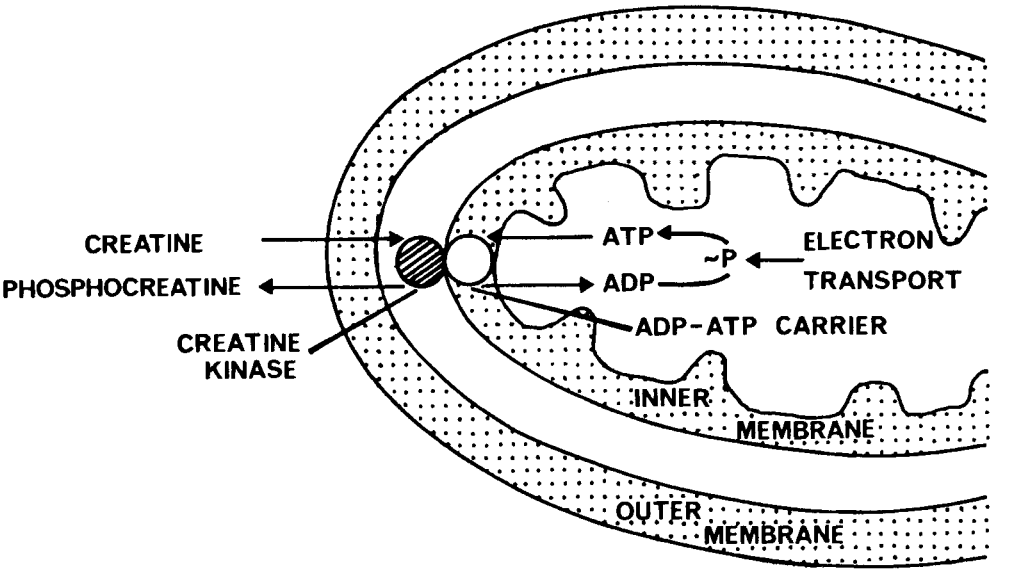


FIGURE 9. Diagrammatic illustration of the positioning and possible function of mitochondrial creatine kinase. (After Jacobus, W. E. and Lehninger, A. L., *J. Biol. Chem.*, 248, 4803, 1973.)

CPK-A2 (also called MM); in brain CPK-B2 (or BB) predominates, and is also present in heart, kidney, and other tissues; while CPK-AB (or MB) is the major isozyme of heart muscle in many species.

CPK is another enzyme the easy extractability of which has led to a general classification as "soluble". Reports of a bound form of the enzyme date from as far back as 1964,[56-58] however; in recent years there has been considerable interest in the particulate association of the creatine kinase isozymes and their role in cellular energy transport.

It now seems clear that an appreciable proportion of creatine kinase activity exists within mitochondria and that a tissue specificity is evident in regard to this localization. Whereas heart, skeletal muscle, and brain mitochondria contain appreciable creatine kinase activity, mitochondria from liver, kidney, and testis contain essentially none.[59,59a] There is also evidence that mitochondrial CPK possesses characteristics distinct from the AB isozyme set and that the enzyme is bound to the outside of the inner mitochondrial membrane.[59,114] Consequently, this phenomenon has found application in the determination of the sidedness of submitochondrial particles (Figure 9). Jacobus and Lehninger[59] have proposed that the presence of mitochondrial creatine kinase activity may serve to maintain an optimal rate of respiration and channel high-energy phosphate to the contractile system via the mitochondrial and sarcoplasmic isoenzymes. The mitochondrial isozyme appears to be specially effective in this regulation, because of its strategic location in the inner membrane and its high affinity for ATP. A close functional coupling with ATP:ADP translocase may allow efficient conversion of ATP and creatine phosphate.

The A_2 isozyme of creatine kinase has also been shown to be intimately associated with the plasma membrane of heart cell, where it may ensure effective rephosphorylation of ADP produced during the active transport of cations across the cell membrane.[60]

Considerable interest has also been directed recently toward the association of creatine kinase isozymes with the M-line region of the myofibrillar contractile apparatus.

Morimoto and Harrington[61] isolated a protein from the M line of chicken muscle which was subsequently identified as CPK-A_2 by Eppenberger and his group (Turner and Eppenberger[62]). Unlike chicken skeletal muscle, chicken heart contains almost exclusively the B_2 isozyme, and about 2% of this binds tightly to the Z-line region. Since chicken heart myofibrils function without CPK at the Z line, neither of the bound forms of CPK seem essential for muscle function in general. It has been proposed that these characteristics may reflect a structural as well as an enzymatic role for this protein.[63,64]

F. Glyceraldehyde-3-Phosphate Dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase; E.C.1.2.1.12) occurs as multiple forms of activity in a variety of plants, fish, birds, insects, and microorganisms.[23] In mammalian tissues, however, the enzyme appears to be electrophoretically homogeneous, although it has been reported that the enzyme form in mammalian muscle may be electrophoretically and kinetically distinct from that in mammalian liver.[65]

Binding of this enzyme activity to particulate tissue fractions with significant consequent alterations in the kinetic parameters has been observed in a variety of species,[7,115] and these characteristics and those of lactate dehydrogenase (see previous section) have been combined in one hypothesis of the role of interactions in controlling glycolysis. Hulthin[66] has suggested that at low NADH levels in resting muscle, lactate dehydrogenase is bound and relatively inactive, and that consequently NADH oxidation takes place via the respiratory chain. When oxygen becomes depleted in contracting muscle, though, the NADH levels build up and have the effect of solubilizing lactate dehydrogenase, which can then act to reoxidize NADH more efficiently than does the bound enzyme. This mechanism would allow for the maintenance of glycolysis under anaerobic conditions, while at the same time minimizing the competition for oxidation of coenzyme between lactate dehydrogenase and the respiratory chain. Ehmann and Hultin[20] have also shown that bound lactate dehydrogenase is less sensitive to inhibition by high concentrations of NAD than the soluble form of the enzyme, so that even though the bound form is inherently less active, it can still function even in the presence of the high levels of NAD expected in resting muscle.

IV. BIOLOGICAL IMPLICATIONS OF INTERACTIONS

A. Modification of Classical Views of Metabolic Regulation

In the last two decades much of the emphasis in metabolic control has centered on the self-regulating properties of enzyme systems. A "classical" view of metabolic regulation by appropriate regulatory circuits has emerged, with such circuits being composed of an allosteric enzyme interacting with a "signal" metabolite to produce an appropriate range of control possibilities (Figure 10).

While such visualizations have provided an elegant picture of control possibilities, recent comment has pointed out that many of these classical concepts may require reexamination in the light of the realities of the cellular microenvironment.[5] It should be more widely recognized, for example, that many of the laboratory conditions which are commonly employed in enzymological investigations bear very little relation to the conditions under which the enzymes act *in vivo*. Model analytical systems usually employ unnatural enzyme and substrate concentrations and give little or no cognizance of the biphasic nature of the cellular microenvironment or the modifications of the kinetic characteristics of the enzymes in such gel-like conditions. In consequence, there would

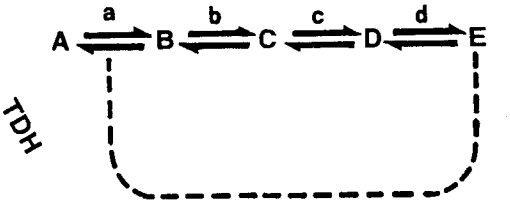


FIGURE 10. Generalized representation of a regulatory circuit involving feedback inhibition by the end product of the metabolic sequence. The metabolites constituting the pathway (A, B, C, D, E) are interconverted by the relevant enzymes (a, b, c, d), with the end product exerting an inhibitory influence on the first enzyme of the sequence.

appear to be a clear compulsion on biological scientists to move toward a position which gives more recognition to the cellular realities of enzyme action.

Failure to observe allosteric effects with an enzyme *in vitro,* for example, does not necessarily preclude the reaction catalyzed by the enzyme as a point of control in biological systems. To take one example, it has been demonstrated in the previous sections that many enzymes which display classical Michaelis-Menten kinetics in dilute aqueous solution, may occur in cellular systems in a form largely adsorbed to cellular structure. It is also known, though, that this association may be released by physiological increments in substrate concentration. It is very pertinent to note, then, that with this combination of characteristics available, such biological systems may display allosteric kinetic characteristics and hence provide a feedback system in which the available activity of the enzyme may be regulated in response to cellular conditions. Or, in other words, enzymes catalyzing reversible reactions may under cellular conditions provide a biphasic analogy of the response commonly associated with *in vitro* studies of regulatory enzymes (Figure 11).

Interactions between enzymes and subcellular structure also give cause to remember that other classical control parameters, namely the Michaelis constant and dimensions of maximum velocity of enzyme activity, may also be significantly altered in the cellular situation. Binding of the enzyme to structure may significantly alter both of these characteristics, and in many cases the total activity available in the cellular microenvironment may be very different from that which is extractable in soluble form under *in vitro* conditions.

Again, in relation to the changes in concentration of signal metabolites during perturbations of metabolic flux, a perusal of the literature makes it clear that the metabolic responses *in vivo* are often quite different in composition and localization to those ascribed on the basis of *in vitro* experiments and the classic regulatory circuits. This is an important anomaly that requires explanation, and it would seem that one possible solution may lie in the recognition that an apposite conjunction of regulatory enzymes may occur in the cellular microenvironment. For example, if phosphofructokinase and aldolase interact to control the concentration of fructose-1,6-bisphosphate in a conjoint fashion, the sandwiching influencing would allow for far wider fluctuations of this regulatory metabolite than would be possible by control at either enzyme independently, such a response could then provide an appropriate regulatory signal to the many areas of metabolism in the cell which are influenced by FDP concentrations.[5]

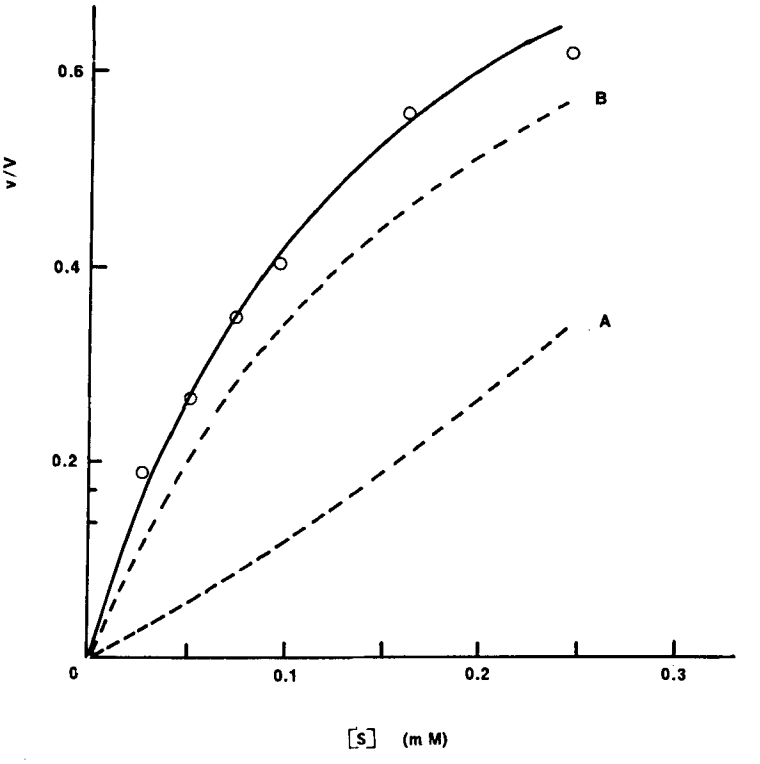


FIGURE 11. The effect of substrate on the reversible adsorption of aldolase to cellulose phosphate. The continuous line and experimental points refer to activity in the absence of adsorbent. Curve A is based on the assumption that adsorbed aldolase possesses no activity, and curve B on a model in which the adsorbed form of the enzyme retains three fully active sites. (After Masters, C. J., *Curr. Top. Cell. Regul.*, 12, 75, 1977.)

Taking all of these features of biological control in mind, there is an obvious need for continuing critical appraisal of regulatory requirements in the light of the conditions which prevail in the cellular microenvironment and in the context of the present review, it is to be emphasized that interactions between soluble enzymes and cellular structure may contribute significantly to these considerations.[116-121]

B. Ambiquitous Enzymes

On close examination of the literature, substantial indications of the role of enzyme partitioning come to the fore. Several investigators have reported that the subcellular distribution of some enzymes may not be invariant, but rather may be a dynamic characteristic which alters in response to the changing metabolic needs of the cell. As far back as 1937, for example, Oparin[1] reported that invertase occurred in both soluble and particulate forms in plants, and that these different forms differed in their function. He suggested that variation in the soluble-particulate distribution of the enzyme might occur in response to changes in the metabolic needs of the cell. Siekevits[2] has also proposed that reversible binding of enzymes to cellular membranes might influence catalytic activity and be ". . . involved in the regulation of the course of substrate metabolism"; while more recently Masters et al. have consistently pointed to the regulatory possibilities inherent in the reversible association of glycolytic enzymes with subcellular structure.[5-7]

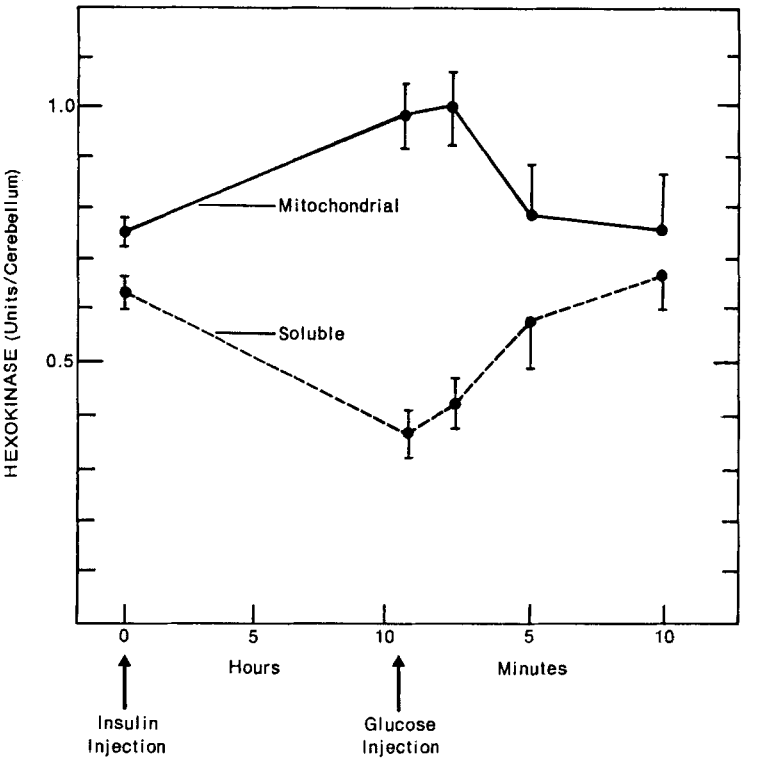


FIGURE 12. The redistribution of brain hexokinase activity between soluble and mitochondrial bound forms after insulin and glucose injections. (After Knull, H. R., Taylor, W. F., and Wells, W. W., *J. Biol. Chem.*, 249, 6930, 1974.)

John Wilson has coined the term "ambiquitous" to describe the situation where the distribution of an enzyme between soluble and particulate forms may vary with the metabolic status of the cell. He nominates hexokinase as a prime example of this behavior.[10]

As we have noted previously, although commonly referred to as a "soluble" enzyme, essentially all the hexokinase activity in brain is found in the mitochondrial fraction, and a sizeable proportion of the activity in other tissues is bound to the mitochondrial membrane.

The soluble form of hexokinase is more susceptible to inhibition by glucose-6-phosphate than the bound form, and it has been suggested that the soluble-particulate distribution is regulated by the influence of various metabolites (e.g., glucose-6-P, ATP, P_i) on this equilibrium. It has been shown, for example, that the *in vivo* levels of glucose-6-P, ATP, and inorganic phosphate vary during times of increased glycolytic flux in brain, and in such a manner that the soluble-particulate distribution might be expected to shift toward increased amounts of the particulate form. Based on such observations, it was predicted that increased amounts of particulate enzyme (which was considered to be the more active form on the basis of kinetic considerations) would be found during times of glycolytic stress.[18-18b]

In a test of this hypothesis, Knull et al.[40] (Figure 12) have produced data which clearly indicate a rapid and reversible shift to increased proportions of the particulate enzyme

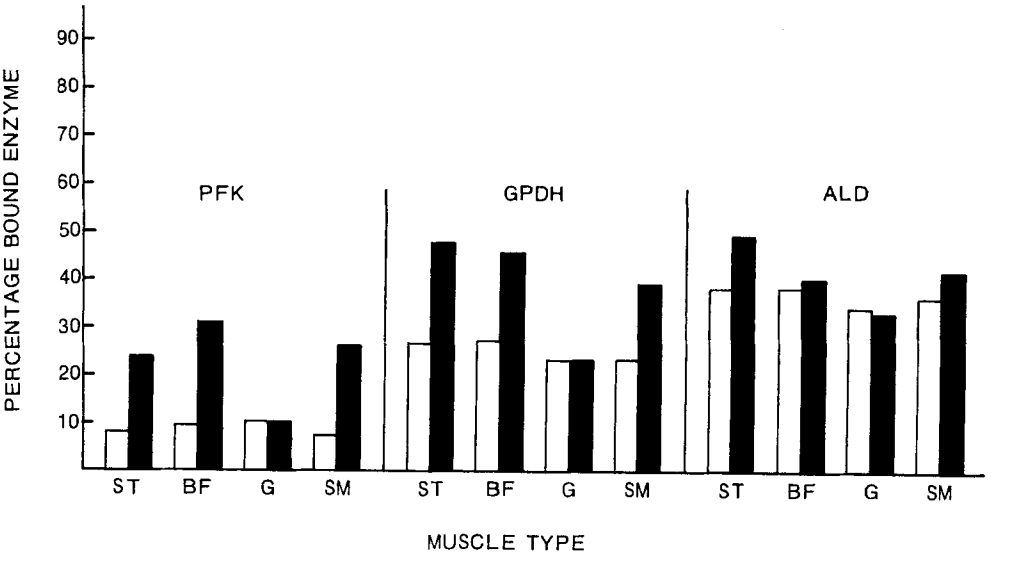


FIGURE 13. The extent of binding of certain glycolytic enzymes to muscle structure before and after electrical stimulation. Unstimulated animals are represented by the open histograms; stimulated animals by the shaded histograms. ST, semitendinosus muscle; BF, biceps femoris; G, gluteus; SM, semimembranosus. (After Masters, C. J., *Proc. Aust. Biochem. Soc.*, 12, Q17, 1979.)

when the glycolytic rate of mouse and chick brain is increased by ischemia or insulin treatment. These results would seem to provide firm support for the concept that changes in intracellular distribution do occur *in vivo* in response to altered energy status in the brain, and are consistent with the previously advanced hypothesis[18] in which the mitochondrial enzyme was proposed to be the more active form.

Another example of ambiquity in an *in vivo* situation is that provided by certain glycolytic enzymes in mammalian tissues. When muscles are stimulated, a quite considerable redistribution of enzyme activity occurs between the soluble/particulate fractions. As indicated in Figure 13, for example, when one hind limb of a sheep was electrically stimulated, the proportion of certain enzymes in these two fractions was significantly altered by comparison with that in the control limb. The extent of these alterations was dependent on a number of factors, such as the extent of stimulation and the individual muscle type involved, but the overall effect was an increased binding of phosphofructokinase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase, in particular, [11,11a] Similarly, when the effect of anoxia and ischaemia on the distribution of glycolytic enzymes in perfused rat heart was studied, marked increases in binding were observed which were especially dramatic in the case of aldolase activity.

Further examples of redistribution of enzyme localization with physiological stimuli are also evident in the literature. From all of these experiments, it should be quite apparent that changes in the extent of binding of enzymes to structure occur with alteration of the functional status of tissues. Clearly, then, ambiquity is well established on an experimental basis, and in view of the possible regulatory ramifications, further investigations of the details of these phenomena are warranted, and indeed highly desirable.

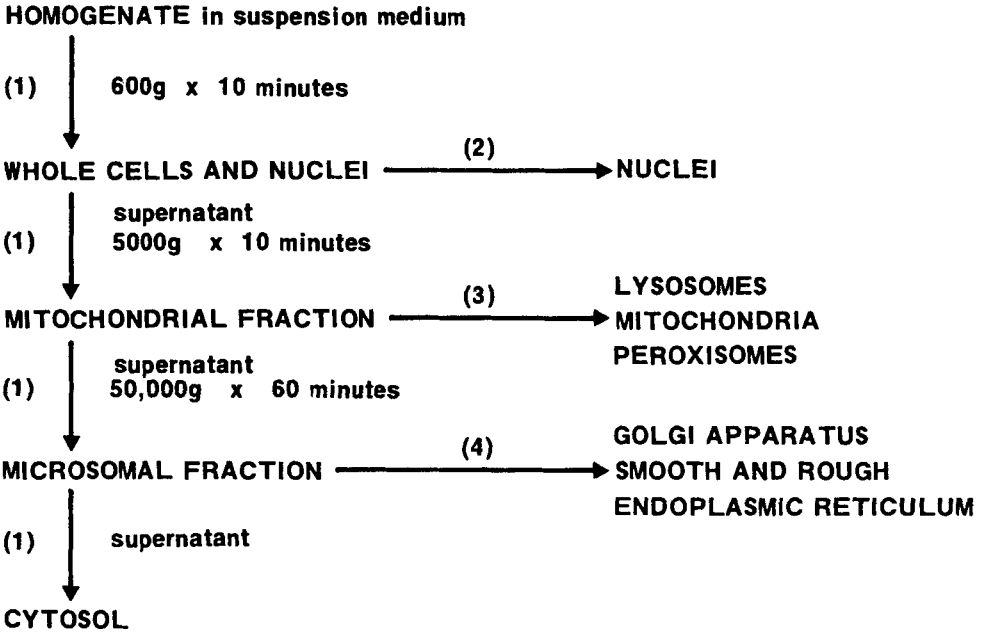


FIGURE 14. Generalized scheme illustrating the main steps involved in the separation of subcellular organelles from liver homogenate by differential centrifugation. References: Hogeboom,[107] Hogeboom et al.,[108] Trouet et al.,[109] Leighton et al.,[110] Delevati et al.,[111] Goldstone et al.[112]

V. ANALYTICAL ALTERNATIVES FOR THE STUDY OF INTERACTIONS

A. Differential Centrifugation

As should be evident from the previous discussion, one of the major hindrances to the ready acceptance of ambiquity into general considerations of cellular control has been the difficulty associated with the unequivocal demonstration of the reality and extent of the interactions between soluble enzymes and subcellular structure. Hence, a reasonably broad consideration of the scope of the available analytical alternatives is a necessary element in any review of the present status of this research topic.

The most widely used technique employed in the study of the subcellular localization of enzymes is undoubtedly differential centrifugation (Hogeboom, 1955), where tissues are homogenized and their organelles separated on the basis of their different size-shape-density characteristics (Figure 14). Such cell fractionation studies have proved of immense value to cell biologists, but unfortunately at this time it is still not possible to completely obviate the possibility of artifactual modification of cell morphology and enzyme distribution during the separatory process. Apart from the trauma associated with homogenization, one of the greatest difficulties arises from the necessary choice of a suspension medium for the cell components. Ideally this medium should so approximate the soluble phase of the cytoplasm that the cellular constituents remain unaltered; however, the perfect medium has not yet been devised. Aqueous media, for example, are subject to the criticism that their use may lead to the loss of water-soluble components from the particulate fractions, the transfer of substances between the soluble phases, and the retention of such substances by adsorption to the membranes and organelles. Sucrose

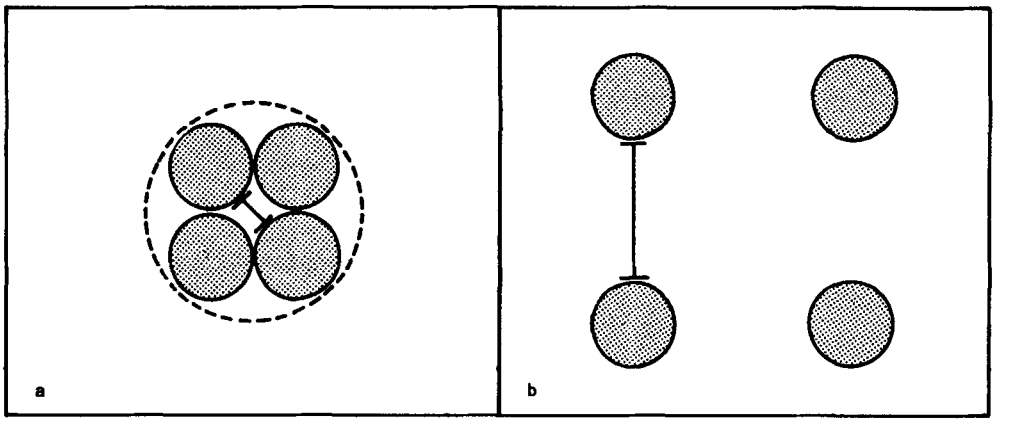


FIGURE 15. Diagrammatic representation of associated (a) and nonassociated (b) states of macromolecules in a cellular environment, and the expression of the differences between these states by physicochemical parameters measuring relative size, intermolecular distances, or binding.

solutions have been widely used in subcellular fractionations and help to minimize some of these deficiencies, as do isopycnic fractionations and the use of nonaqueous isolation media, but all of these procedures suffer to some degree from such limitations.

Particularly difficult problems occur in relation to the definition of subcellular localization in the case of the so-called "soluble" enzymes, i.e., the enzymes which are normally characterized as components of the cytosol. The enzyme forms contained in this fraction are probably the most susceptible to redistribution, and consequently the decision as to whether associations between such enzymes and particulate matter are artifactual has proved to be an extremely refractory problem. What is often required is a multifaceted approach, with additional methodologies to complement the classical centrifugal separations, and the particular emphasis of attack varying with the nature and extent of the individual problem. While the evidence from any single technique may not be considered compelling, a consensus of appropriate approaches must have considerable impact in the interpretation of physiologic correlations.

In order to emphasize this point, some of the relevant methodologies and sample applications are considered in this section.

B. General Characteristics of Aggregates in Cellular Extracts

Just as the standard approach to subcellular fractionation studies has utilized the differences in size and shape of organelles in their separation and analyses by differential centrifugation, so the majority of tests for the presence of interactions between the components of cellular extracts have used physicochemical parameters of aggregating characteristics. These methodological approaches include a diverse group of techniques, but essentially the procedures are directed toward similar, central elements of the problem; these focal aspects are displayed in a diagrammatic manner in Figure 15.

While the weak interactions that are involved in these supramolecular assemblies are noncovalent in nature, these complexes may nevertheless be stable under biological conditions, and as a result of this association, the components are situated in relatively close proximity one to the other. Basically then the analytical problem is one of demonstrating this degree of proximity, ordering or binding between the component molecules.

As we have already seen, differential centrifugation provides one widely applied approach to the problem of size and shape differences in cellular extracts, and in theory

might be applied to some of these analyses. Although there are difficulties in applying this technique to soluble-particulate interactions (see above), there still remains the significant question of interactions between soluble components. It has been reasoned by de Duve,[28] for example, that the reality of a glycolytic complex might be tested by examining the sedimentation characteristics of the individual glycolytic enzymes in a cytosol fraction, but when he applied this test to rat liver, he was unable to detect the presence of any multienzyme aggregates, and concluded that the individual enzymes of the glycolytic sequence were molecularly dispensed in this tissue.

By contrast, when Clarke and Masters[67] studied the differential centrifugation of the glycolytic enzymes in concentrated myogen preparations of skeletal muscle, they were able to demonstrate a definite complexing between a number of glycolytic enzymes. Typical sedimentation profiles from some of these experiments are illustrated in the accompanying diagram, with the main point of interest being the significant second boundary which occurs in parallel for each glycolytic enzyme (Figure 16). The size of this complex is sufficient to encompass an association of all the glycolytic enzymes, but the stability of the complex is dependent upon the presence of solubilized structural components, and is very sensitive to factors such as pH, ionic strength, the concentrations of proteins and metabolites, and the isoenzymic composition.

Overall, then, the picture has emerged of an association of glycolytic enzymes in tissues in the form of a complex which is plated onto structural components containing contractile proteins. The fact that the complex is so sensitive to the methods applied to the preparation of the tissue extract, however, serves to emphasize the care which is necessary in the involved experimental techniques.

In addition to tests of association by centrifugal characteristics, it might also be expected that exclusion chromatography could be used as an indication of the size of any aggregates which may be present, and indeed several applications of this technique to the demonstration of interactions are in evidence. For example, in the demonstration of the presence of a multienzyme aggregate with glycolytic activity in extracts from *E. coli,* Gorringe and Moses studied the elution of lysate from an agarose gel column and observed a high molecular weight ($\sim 1.15 \times 10^6$) fraction which contained all the relevant enzyme activities.[68] They were also able to demonstrate a degree of aggregation in the fraction representing individual enzyme activity by this methodology when this latter fraction was concentrated.

Another means of demonstrating the presence of interactions is by estimations of the intermolecular distance between individual components. It should be readily apparent that association implies closer structural relationships than the average (Figure 15) and hence techniques such as chemical cross-linking, using bifunctional reagents of defined length, could well be expected to be applicable to problems of association in conditions approximating to those of the cellular environment.[69,70]

To take a specific example of the use of such nearest neighbor analysis in this field, and one where both of these previous techniques were combined in the testing of specific protein-protein interaction, the work of Fahien et al.[71] may be cited. These workers investigated the possibility of complex formation between the glutamate dehydrogenase and mitochondrial malate dehydrogenase of bovine liver. By incubation of these two enzymes with the bifunctional cross-linker, dimethyl 3,3′-dithiobispropionimidate and subsequent chromatography on Sephadex G-200, these workers were able to provide strong evidence for complex formation in the presence of palmitoyl CoA.

The alterations in size and surface characteristics attendant upon the association of individual cellular components into complexes (Figure 15) may also be evident in the relative partitioning between immiscible phases composed of different aqueous polymer solutions. In its simplest form, for example, the behavior of individual components and

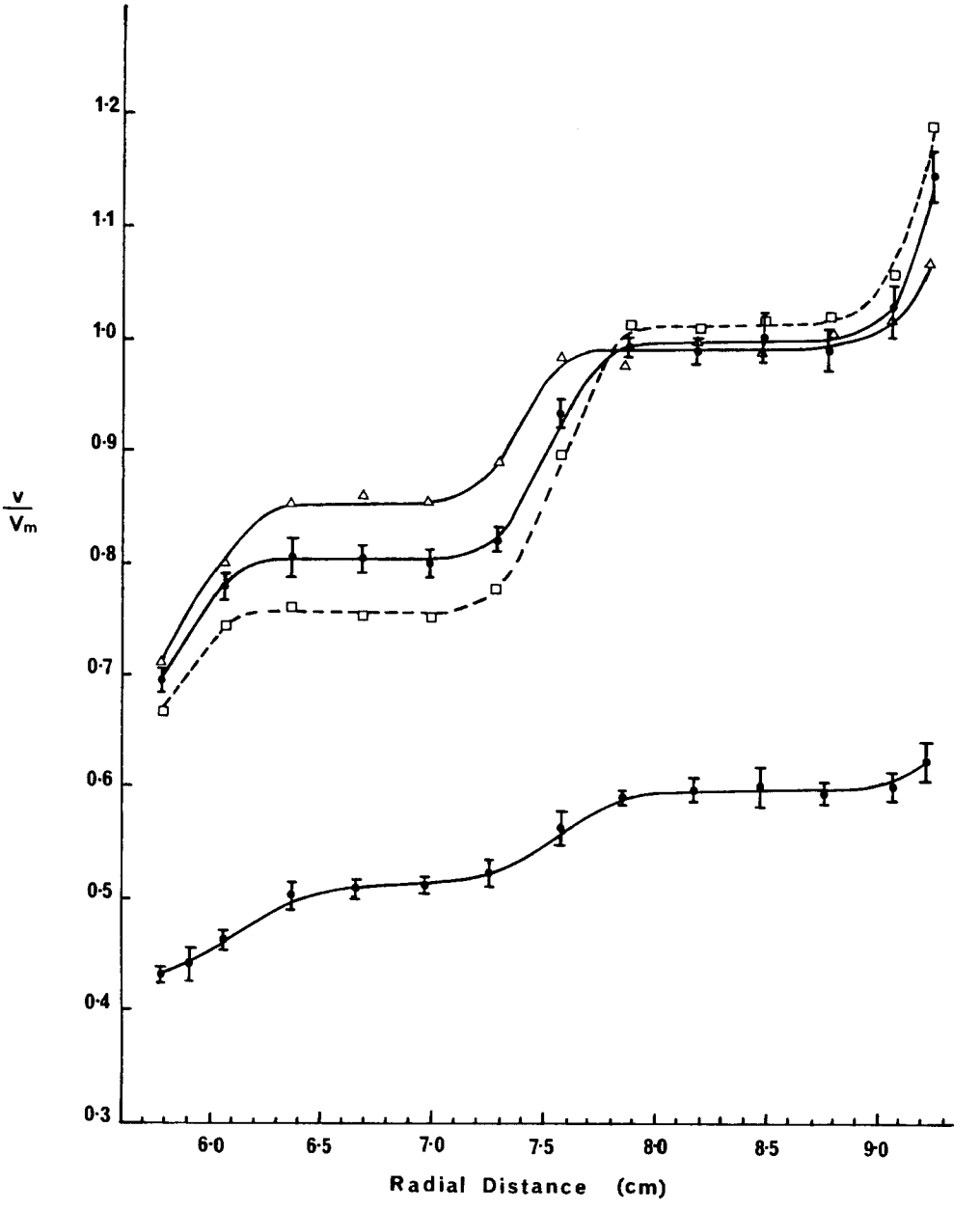


FIGURE 16. The sedimentation of enzyme activity in a myogen preparation from sheep semitendinosus muscle. ●, aldolase; □, pyruvate kinase; △, lactate dehydrogenase; ○, phosphofructokinase. (After Clarke, F. M. and Masters, C. J., *Biochim. Biophys. Acta,* 358, 193, 1974.)

their mixture vis-a-vis partitioning in 14% polyethylene glycol has been used as a measure of interaction.[71,76,77] More refined studies of weak interactions between proteins may be studied by their mutual influence on the partitioning in biphasic systems such as those developed by Albertsson,[80] and where single-batch procedures do not provide sufficiently sensitive indications of partitioning differences, multistage procedures such as countercurrent distribution may allow a finer resolution. Backman and Johansson,[81] for example, have used a system of 60 transfers to demonstrate that a

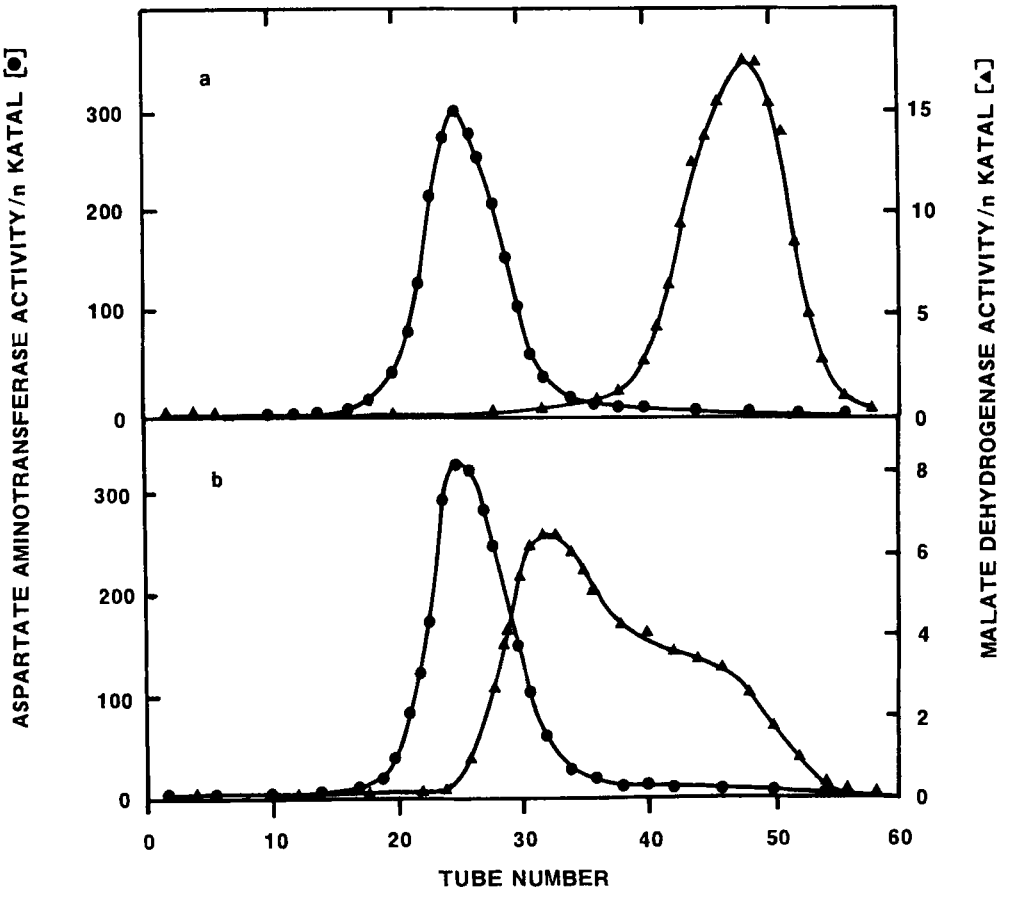


FIGURE 17. Countercurrent distribution curves of soluble alanine aminotransferase (●) and soluble malate dehydrogenase (▲), (a) separately and (b) when both present. (After Backman, L. and Johansson, G., *FEBS Lett.*, 65, 39, 1976.)

specific interaction existed between the cytoplasmic forms of alanine aminotransferase and malate dehydrogenase (S-AAT, S+MDH), and between the mitochondrial forms of these enzymes (m-AAT, mMDH), but that no interaction existed between a cytoplasmic and a mitochondrial form of these enzymes (Figure 17). It may be noted that the water content and the enzyme concentration in these aqueous biphasic systems may be very similar to that in the cell *in vivo,* and that the systems provide a milieu which is more favorable for complex formation than dilute aqueous solutions.

One other parameter which is potentially indicative of association is the degree of binding. Clearly the extent of binding interaction between components may be measured by the normal Scatchard procedures where purified and high-affinity components are available. As ancillary approaches, though, other useful techniques are now becoming available which may be of special or general utility. Petersen, for example, has made an accurate estimation of the binding constant of the cytochrome c - cytochrome aa_3 complex by an interesting extension of the technique of aqueous two-phase partition[82] (Figure 18).

Again, Powell and Morrison[83] (Figure 19), have used the technique of affinity chromatography to demonstrate that enzymes involved in the biosynthesis of certain aromatic amino acids may undergo reversible interactions. To determine if there were any interactions between these enzymes, one member was adsorbed specifically onto an

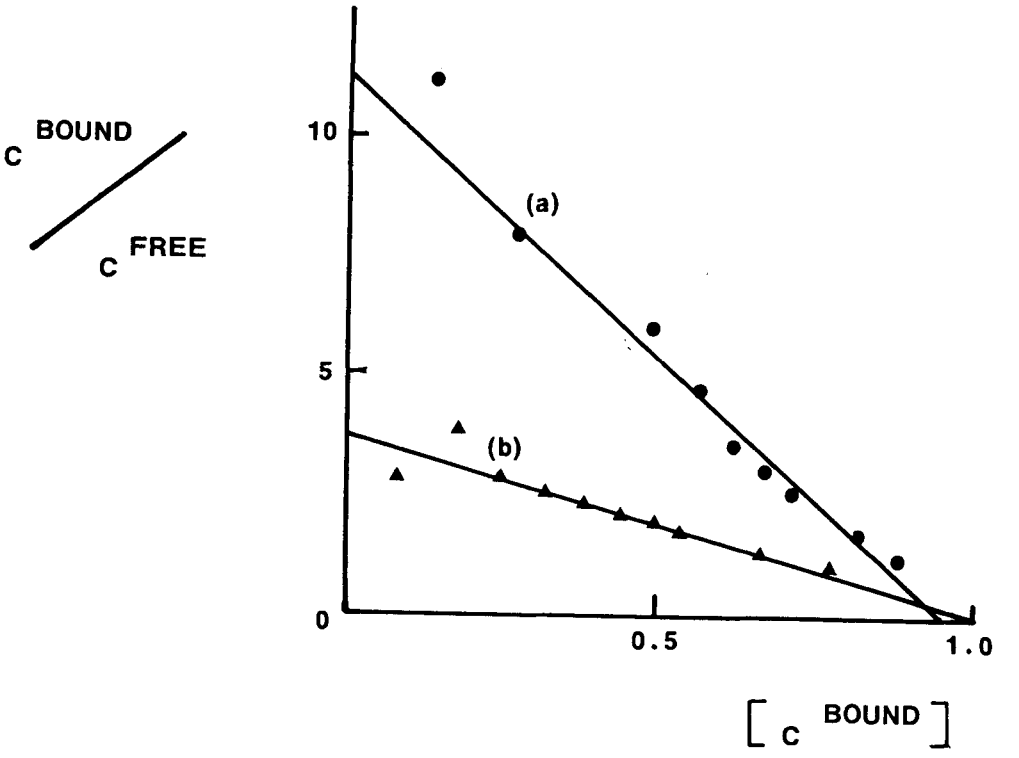


FIGURE 18. Scatchard plot of cytochrome C binding by cytochrome aa_3. The assay was carried out in a two-phase dextran/polyethylene glycol system. Cytochrome aa_3 partitioned in the top phase, with cytochrome C between the bottom phase (free form) and the top phase (bound form). (After Petersen, L. C., *Biochem. Soc. Trans.*, 6, 1274, 1978.)

affinity column, and a study made of the retention or retardation of the other enzymes on the passage through the column. It was shown that the aromatic amino acid transferase (E.C.2.6.1.57) reacts specifically with chorismate mutase-prephate dehydrogenase (E.C.5.4.99.5 and E.C.1.3.1.12) in the absence of reactants, and with chorismate mutase-prephenate dehydrogenase (E.C.4.2.1.51) in the presence of phenylpyruvate. Tyrosine caused dissociation of the aminotransferase:mutase dehydrogenase complex, whereas dissociation of the aminotransferase-mutase dehydratase complex occurred on omission of phenylpyruvate.

In a similar fashion, the technique of affinity electrophoresis would seem well suited to the study of interactions between individual macromolecules in complex biological mixtures.[84]

Hopefully, then, one of the major hindrances to the acceptance and further understanding of the scope and function of the interactions between enzymes and subcellular structure — namely the limitations of available methodology — is being overcome, and the selection of appropriate procedures from those mentioned above may lead to an increased understanding and definition of these interactions.

C. Histochemistry

A valuable complementary approach to the problems of interactions and enzyme localizations within the cell is the application of histochemical techniques, especially in those instances where it is important to relate enzyme location *in situ* to morphologic detail. This latter objective is often extremely difficult to achieve by other procedures.

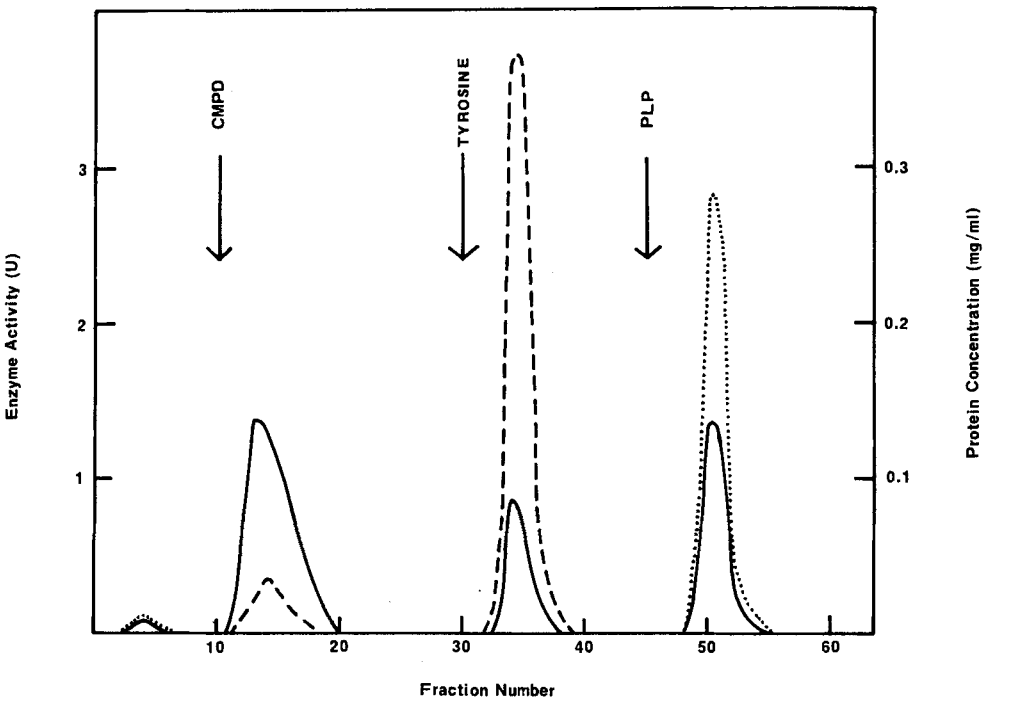


FIGURE 19. Interaction of chorismate mutase-prephenate dehydrogenase (CMPD) with aromatic amino acid aminotransferase bound to a column of pyridoxamine phosphate. The sequence from left to right represents binding of CMPD to the aminotransferase, removal of excess CMPD activity, specific elution of CMPD by tyrosine, and subsequent elution of aminotransferase activity by pyridoxal phosphate (PLP): protein concentration (——); CMPD activity (– – –); aminotransferase activity (· · · ·). (After Powell, J. T., and Morrison, J. F., *Biochim. Biophys. Acta.*, 568, 467, 1979.)

A limitation to the histochemical definition of the microlocalization of soluble enzymes, however, is often caused by the extent of diffusion of reactants and enzyme during the staining of tissue sections. Using aqueous media in order to link the substrates of the dehydrogenase with tetrazolium dyes, for example, may lead to the great majority of enzyme being lost from the section within the staining period and may result in a diminution or distortion of the original isozyme distribution. Such undesirable effects may be minimized by using a gelled film to contain the histochemical reagent.[85] To take an example of a specific application of this approach, the distribution of the multiple forms of LDH activity in mammalian preimplantation ova and oocytes might be considered with benefit. This is a situation of considerable ontogenetic significance which has been reviewed recently.[86] Extraordinarily high levels of activity of particular lactate dehydrogenase isozymes have been reported in the ova of some experimental mammals, and considerable contemporary interest has centered on the question of the microlocalization of this enzyme. Brinkworth and Masters[87] (Figure 20) have applied a histochemical technique to the solution of this problem, and by the use of differential inhibitors and different substrate concentrations have examined the comparative distribution of the different types of LDH activity in the contiguous cell types of reproductive tract tissues. It seems clear from these results that at least some murine strains do not exhibit the massive intrinsic quantities of activity in the sole form of LDH-B_4 which have been proposed as characteristic of this species. Rather, the histochemical data lead to the conclusion that much of the LDH activity associated with preimplantation ova may derive from accumulative adsorption of enzyme from the oviducal fluid or be present in the cell in

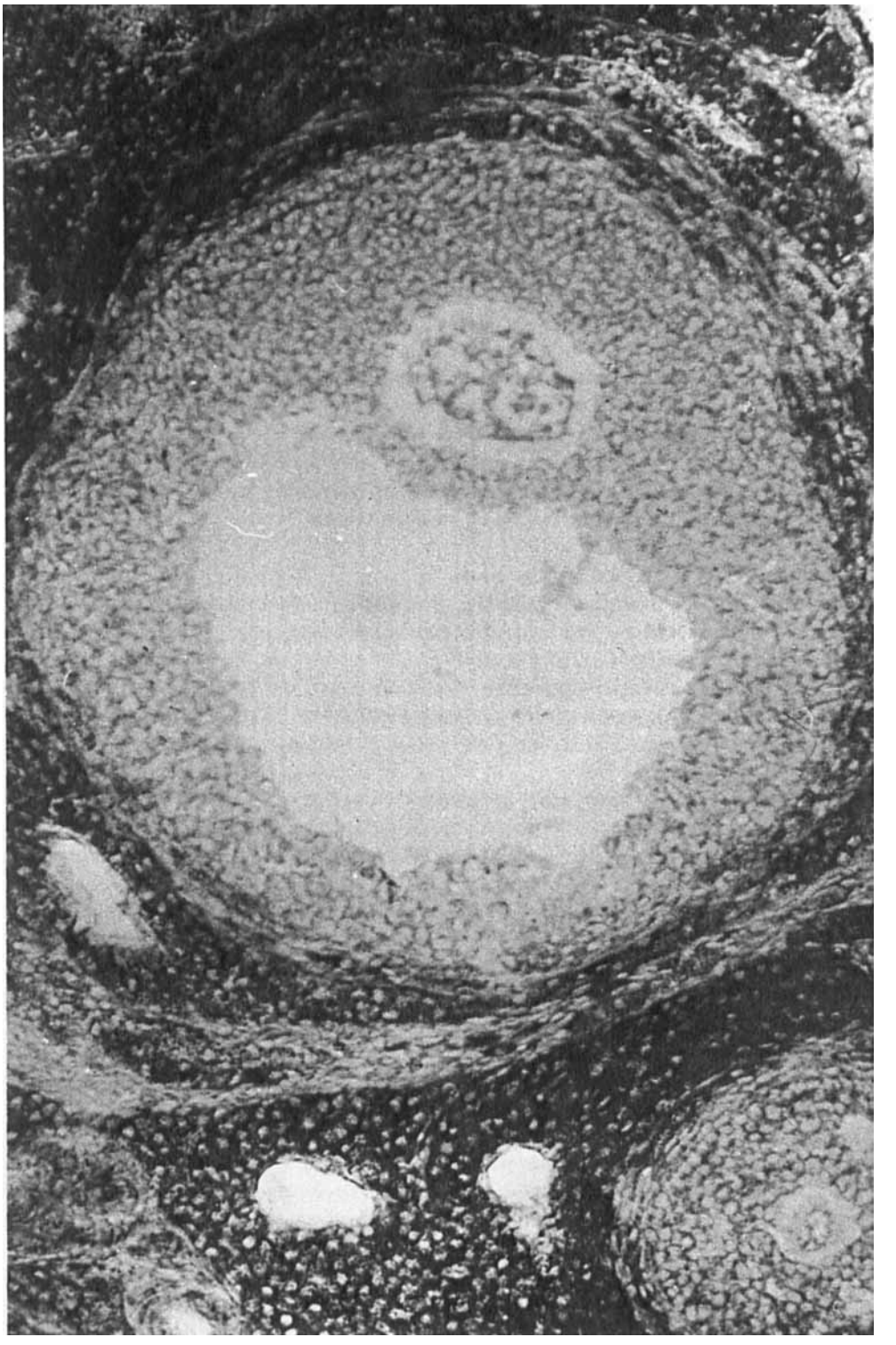


FIGURE 20. Histochemical staining for lactate dehydrogenase activity in a graafian follicle of rat ovary. The intensity of staining varies from indications of high activity in interstitial tissue to comparatively low levels in the oocyte and follicular cells. (See Brinkworth, R. I., and Masters, C. J., *Mech. Ageing Dev.*, 8, 69, 1978.)

covert form; additionally, there are indications that A-type activity is of widespread occurrence in the oocytes and ova of mammalian species in contrast to earlier reports.

The application of similar principles (i.e., the use of histochemical techniques involving gelled reacting solutions and differential inhibitors) to many other systems of multiple enzyme forms would seem in principle to be feasible and to allow a considerable advance in our understanding of the intra- and intercellular disposition of multiple enzyme forms. It is also possible to combine the advantages of the histochemical approach with the selectivity possible in immunochemical techniques. When a system of multiple enzyme forms contains different types of subunits, for example, it should be possible to identify the localization of each type separately in a tissue section by means of conjugation with the appropriate fluorescent antibody and subsequent examination under a fluorescence microscope.[88,89] Such a study has been recently carried out by Hintz and Goldberg,[52] who have analyzed the cellular distribution of LDH-C_4 in mouse testis sections using a fluorescence-labeled anti-C_4 antibody. Their results demonstrated that specific fluorescence was restricted to cells within the seminoferous tubules of the testis, particularly those germinal cells beyond the stage of late pachytene primary spermatocytes. Moreover, Eppenberger and co-workers[62,63] have also applied this immunohistochemical technique to specifically localize the A_2 enzyme of creatine phosphokinase on the M-line region of chicken muscle.

D. Interactions of Purified Components

In many areas of biological chemistry, purification of the individual components and subsequent study without the complications of naturally occurring ancillary involvements is often considered to be the appropriate response to the difficulties inherent in studying molecular interactions in the cellular situation. On this basis, once it has been established that an enzyme is closely associated with a particulate fraction within the cell, a logical next step would be to study the nature of the interaction between the purified components.

While there may be no unusual difficulty involved in the purification of the enzyme contribution to such biphasic interactions, the difficulties of membrane purification are often considerable, if not insuperable. Procedures for the isolation of membranes from the cell and their separation from other membranes without modification of structure require a delicate balance between the rigors of the isolation procedure and the inherent instability of the membrane to be isolated, and in few if any instances have the *in vivo* characteristics of membrane enzyme actions been faithfully reproduced *in vitro*.

There is one case, however, where the particulate involvement in enzyme binding has been studied in a system of purified components in some detail and may be cited as an example of the potentialities of this approach. As noted previously, histochemical observations of muscle have revealed a location of the glycolytic enzymes at the site of the actin filaments,[44] and a comparison of the binding properties of the main structure proteins has confirmed that F-actin is the main component in this interaction.[13] Actin is, of course, a single defined protein, normally a part of the particulate material in muscle, but it is not subject to the difficulties of purification encountered with many membranous entities. Hence, the interaction with other purified components can be studied in some detail in *in vitro* systems.

While study of the binding activities with F-actin have done much to advance our qualitative understanding of these association phenomena, it is necessary to emphasize that cellular conditions may differ markedly from the simple *in vitro* systems used in these early experiments, and in particular that other muscle proteins may appreciably influence these binding properties. The thin filaments of muscle, for example, are complexes of F-actin, tropomyosin, and troponin; consequently a much more satisfactory *in vitro* model than actin alone is the use of reconstituted thin filaments

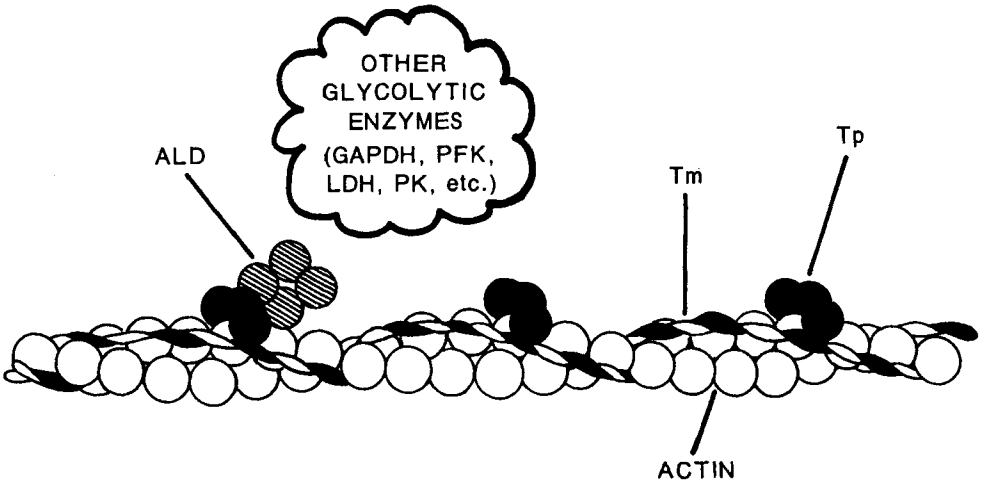


FIGURE 21. Diagrammatic representation of the binding of glycolytic enzymes to the thin filaments of muscle. Aldolase binds to troponin (Tp). Other glycolytic enzymes also have binding sites on the thin filament. Tm, tropomyosin.

(F-actin-troponin-tropomyosin). By means of such systems, binding curves have been derived that are significantly different from those with F-actin alone — showing that aldolase-A_4 is adsorbed at two nonequivalent sites and is appreciably modified in its kinetic characteristics by such binding.[90] A number of glycolytic enzymes have been shown to bind to reconstituted thin filaments under conditions of "physiologic" ionic strength and protein concentration.

It should also be mentioned that the interactions between purified glycolytic enzymes and reconstituted thin filaments have visualized by means of electron microscopy.[91] The demonstration of lattices, or regular repetitions in the interaction between reconstituted thin filaments and individual isozymes, has allowed an accurate dimensional analysis of the relative positions of these components, and opened promising possibilities for further advances in the definition of such complex multifactorial interactions. Dimensional considerations, for example, indicated that this cross-linking occurred via aldolase-A_4 by a junction with the troponins on adjacent filaments; experiments with subunit preparations of troponin have indicated that troponin I and troponin-T subunits are required for the formation of the lattice, whereas troponin-C seems to be excluded from participation in cross-bridge formation (Figure 21).

Despite these previous lines of evidence, the adsorption of glycolytic enzymes to particulate fractions has not been universally accepted as physiologically meaningful, because of the commonly observed dependence of this binding on ionic strength. In this connection the work of Masters et al. (Tables 4 and 5) on the influence of a number of factors on the binding of glycolytic enzymes to thin filaments of muscle is relevant. Under low ionic strength conditions, significant enzyme adsorption was observed in mixtures of both high and low protein concentration. Increasing the ionic strength to 0.15 certainly abolished adsorption in mixtures of low protein concentration, but mixtures of high protein concentration still displayed significant adsorption at this ionic strength (Table 4). Subsequently, a survey of the adsorption of all the individual glycolytic enzymes under conditions of high ionic strength and high protein concentration was made. The majority of glycolytic enzymes displayed significant adsorption to reconstituted thin filaments

Table 4
EFFECT OF IONIC STRENGTH AND PROTEIN CONCENTRATION ON ADSORPTION OF GLYCOLYTIC ENZYMES TO F-ACTIN AND F-ACTIN-TROPOMYOSIN-TROPONIN

Adsorbent	Myogen concentration (mg/mℓ)	Pyruvate kinase	
		5 mM KCl	150 mM KCl
F-Actin	3.5	13.2 ± 0.9	0.0
	35.0	24.5 ± 0.8	15.6 ± 1.0
F-Actin-Tropomyosin-troponin	3.5	17.1 ± 0.7	0.0
	35.0	32.9 ± 1.1	22.7 ± 0.9

Table 5
ADSORPTION OF GLYCOLYTIC AND NONGLYCOLYTIC ENZYMES TO F-ACTIN-TROPOMYOSIN-TROPONIN UNDER HIGH IONIC STRENGTH CONDITIONS

Enzyme	Press juice (% bound)
PFK	87.0 ± 2.0
PK	55.5 ± 1.7
ALD	40.0 ± 1.1
CK	0.0
AK	0.0
MDH	0.0
GPDH	13.0 ± 1.0
AAT	17.0 ± 1.5
LDH	22.0 ± 1.2

Note: PFK, phosphofructokinase; PK, pyruvate kinase; ALD, aldolase; CK, creatine kinase; AK, adenylate kinase; MDH, malate dehydrogenase; GPDH, glyceraldehyde phosphate dehydrogenase; AAT, aspartate aminotransferase; LDH, lactate dehydrogenase.

(F-actin tropomyosin-troponin), clearly establishing that interaction of these enzymes with structural proteins may occur under conditions of physiological ionic strength and protein concentration. While all the glycolytic enzymes displayed a significant degree of binding, a wide divergence in the relative degree of adsorption of individual enzymes was noted. Some like phosphofructokinase, pyruvate kinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate isomerase, and lactate dehydrogenase had a large proportion of their total activities bound, whereas only a small percentage of total activities of triosephosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and hexokinase were bound (Table 5).

The interactions of aldolase with the purified regulatory proteins of rabbit skeletal muscle have also been investigated by analytical ultracentrifugation and moving-boundary electrophoresis, with the results indicating that aldolase possesses multiple binding sites for these muscle proteins. Again quantitative binding studies under high ionic strength conditions have demonstrated the relative binding parameters of these systems. Aldolase bound to F-actin alone with an affinity constant of 12×10^{-6}M and with a stoichiometry of one aldolase per 14 actin monomers. With F-actin-tropomyosin as adsorbent, the affinity increased twofold, with two aldolases binding for each 14 actin

monomers. With F-actin-tropomyosin-troponin, the affinity of enzyme binding was about six times that of F-actin alone, and was calcium sensitive. The stoichiometry of binding was 4:14 in the absence of calcium and 3:14 in the presence of calcium.[92,93]

Hence, the study of the interactions between the soluble enzymes and the structural elements of muscle may be said to have progressed to a satisfying level of detail, when analyzed at the level of the purified components of these binding reactions.

VI. CONCLUDING COMMENTS

It should be evident from the previous discussion that a substantial body of evidence is now available to support the biological reality of interactions between the enzymic components of the cytosol and the structural elements of cells. It should also be clear that the characteristics of such binding have been studied in some detail in both *in vivo* and *in vitro* systems, and that the kinetic parameters of the enzymes may be significantly modified by these interactions with structure. In addition to substantial alterations in the Km and Vmax of bound enzymes, alterations in the characteristic allosteric properties of regulatory enzymes have also been reported, as well as alterations in the extent of binding in response to changes in the metabolic state of a tissue. These findings when considered *in toto* appear to indicate that these interactions display the scope for a major contribution to metabolic regulation, and open up many interesting possibilities such as metabolic channeling, the segregation of competing pathways, reduction of the transient time in metabolic sequences, and coordinate regulation.

All of these features of our present knowledge of these interactions point to an increasing interest in this area. A thorough understanding of such binding would seem to be fundamental to any detailed understanding of the many structural and functional implications of these phenomena, but in pursuing these goals, enzymologists may have to abandon some of the ingrained conclusions derived from the *in vitro* studies of uniphasic systems. For example, many of the classical methods of extracting and fractionating enzymes and subcellular structure may not be favorable, and indeed may act to preclude the isolation of organized multienzyme systems — hence the continued references to the importance of innovative technologies in future investigations. Nevertheless, the relationships between the complex infrastructure of the cell and the dynamic metabolic processes in the cell undoubtedly pose some of the most exciting prospects available to experimentalists and theoreticians involved in cellular biology at this time.

Allied with the apparent need for an increasing appreciation of the metabolic importance of such interactions is the desirability of directing increased attention towards the status of enzyme multiplicity in these phenomena. It has not yet been established, for example, whether or not a multiplicity of pathways catalyzing similar functions may exist in a cell or tissue, in the same manner that isoenzymes are distributed with high specificity, or whether such multiple metabolic alternatives play a significant role in cellular regulation. It must be conceded as possible, for example, that the high degree of multiplicity which is observable in the individual glycolytic enzymes, and their demonstrably different adsorption characteristics, may lead to the presence of a number of discrete systems of glycolytic components in individual tissues, and the effective partitioning of these functional systems between different subcellular or tissue compartments.[7,11] Ureta has recently suggested that there may be up to 10 separate polyisozymic complexes associated with glucose metabolism in some tissues[94] (Figure 22). Such considerations stress the need for continuing studies at this level of organization in order to achieve a satisfying comprehension of the manner in which the cell may utilize these characteristics of multiplicity and interaction in determining its remarkably versatile metabolic capabilities.

Further, while most of the emphasis in this article has been directed toward the acute

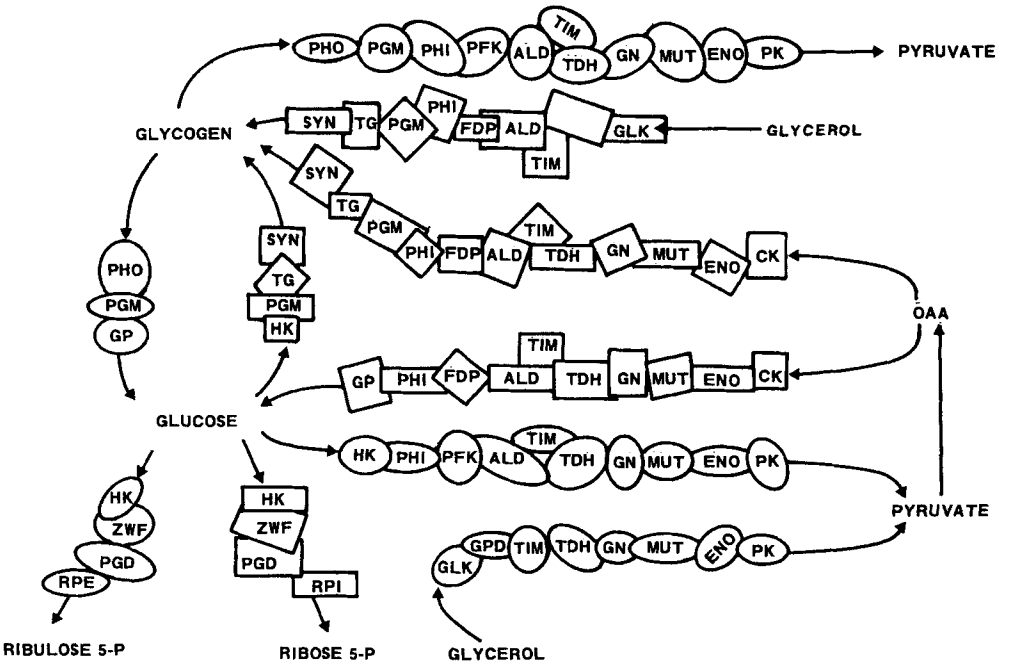


FIGURE 22. Hypothetical polyisozymic complexes in glucose metabolism. Hexokinase (HK); glucose-6-phosphatase (GP); phosphoglucomutase (PGM); UDP-glucose pyrophosphorylase (TG); glycogen synthase (SYN); glycogen phosphorylase (PHO); glucose-6-phosphate dehydrogenase (ZWF); 6-phosphogluconate dehydrogenase (PGD); ribulose-S-phosphate epimerase (RPE); ribulose-S-phosphate isomerase (RPI); phosphofructokinase (PFK); fructose bisphosphatase (FDP); aldolase (ALD); glycerol kinase (GLK); glycerol-3-phosphate dehydrogenase (GPD); triosephosphate isomerase (TIM); glyceraldehyde-3-phosphate dehydrogenase (TDH); phosphoglycerate kinase (GN); phosphoglycerate mutase (MUT); enolase (ENO); pyruvate kinase (PK); oxaloacetate (OAA). (After Ureta, T., in *Topics in Cellular Regulation*, Vol. 13, Academic Press, New York, 1978, 253.)

aspects of the putative role of these interactions in metabolic control, apposite arguments may also be developed in relation to the longer-term influence of interactions on cellular regulation. While it has long been realized that control of the rate of synthesis of enzymes may play an important role in the chronic regulation of metabolic flux, for example, it has only recently become widely recognized that the rate of degradation should be considered as of comparable importance in vertebrate systems.[95] There are now many known instances when an altered level of enzyme has been traced to a variation in the rate of degradation; and hence proteolysis *in vivo* must increasingly be considered as a major determinant of the concentrations of control enzymes. In this respect, it may be noted that conformational changes are known to play a significant role in the initiation of protein degradation, and to be an essential preliminary to the uptake of proteins by lysosomes.[96] Consequently, there is a need to consider interactions of a macromolecule with the cellular environment that may lead to conformational alterations and different rates of proteolysis.

Fructose-1:6-bisphosphate, for example, is known to influence the conformation of the enzyme aldolase, and the question might be asked as to the influence of this alteration on degradation ratio *in vivo*. This point has been specifically studied in *in vitro* systems,[97,98] and it is clear that in the case of purified aldolase binding to the contractile proteins of muscle the susceptibility of aldolase towards proteolytic digestion is significantly altered by comparison with that of the freely dissolved enzyme.

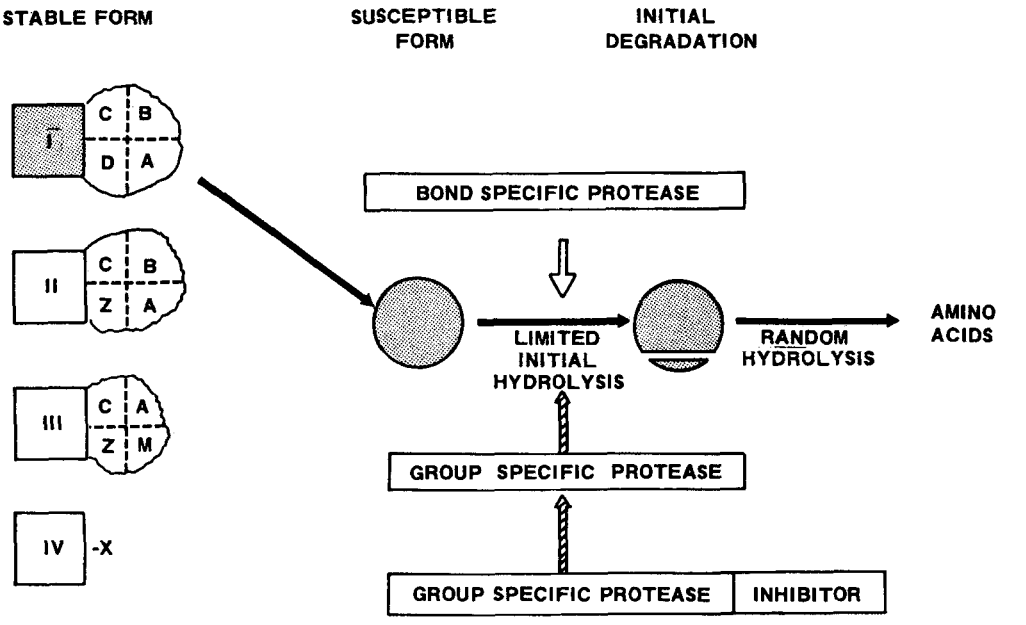


FIGURE 23. Diagrammatic representation of elements in the regulation of intracellular proteolysis. Stable forms of the initial protein substrate are protected by association with polypeptide aggregates, with different polypeptide combinations conferring different specificities to this protection. The stable form of the substrate becomes susceptible to proteolysis when the association with the protecting factor is released, but the rate of proteolysis at this step may also be controlled by the extent of interaction between a protease and its polypeptide inhibitor. (After Don, M. and Masters, C. J., *Int. J. Biochem.*, 11, 551, 1980.)

More generally, the close proximity and relationship of many macromolecules in the cell must exert a considerable effect on turnover in the cellular situation, as may be readily visualized from reference to Figure 1. In the simplest terms it should be clear that interactions between a macromolecule and membrane may well influence the degree of exposure of portions of the molecule which are sensitive to proteolysis and affect protection in this way. On the other hand, membrane binding of a protein may with equal facility be visualized as acting to enhance its rate of degradation. There is an increasing number of examples in the literature where membrane association of a protein appears to precede an involvement with lysosomal proteolysis, and recent theories on the mechanism of control of protein degradation rely heavily on the concepts of interactions between proteolytic enzymes, their inhibitors, and cellular structure[99] (Figure 23).

Additionally, in relation to the chronic influences of degradation on the interactions between soluble enzymes and structure, mention may be made of recent developments in relation to putative control possibilities. In addition to the acute interaction responses referred to in Section IV, there are now indications that longer-term influences on binding may arise in response to degradation and other types of modification to cellular enzymes. Physiological perturbations of cells and tissues have been shown to lead to the proteolytic modification of soluble enzymes, giving rise to markedly different binding characteristics than those which are normally present.[72,98] This behavior raises interesting possibilities of metabolic rechanneling, and points to the desirability of research workers in the field of interactions closely investigating the possibility that biphasic interactions contribute not only to the short-term metabolic response, but also to the longer-term adjustments to variations in the physiological environment.

Finally, a brief comment would seem to be indicated in relation to some of the areas

where rapid developments in our understanding of the interactions between enzymes and cellular structure may be expected. First, now that a qualitative acceptance of the biological reality of these interactions has infiltrated the scientific community, more emphasis may be expected to be directed toward a refinement of the concepts, and a quantitation of the degree of interaction. This will require a due emphasis on the methodological considerations which have been referred to in this review. It is also of interest that much of the quantitative data which is presently available has been derived from study of the contractile proteins of muscle. This information may be more widely applicable than originally perceived, because actin-like proteins are now known to be widely distributed in nonmuscle cells, and to participate in many basic cellular functions.[100] Binding of glycolytic enzymes to contractile proteins has been demonstrated in a wide variety of mammalian tissues, and offers potential advantage in many cell types of allowing the adjacent positioning of energy-producing and energy-consuming processes.

Second, further development of the theoretical background of biological interacting systems is to be expected. Despite an increasing awareness of the existence of reversible interactions between enzymes and cellular structures (e.g., see Table 1 of Reference 72), surprisingly little attention has so far been focused on the problem of defining these interactions in quantitative terms. Arnold and Pette[73] and Arnold et al.[74] have employed an empirical relationship in the form of summed rectangular hyperbolae to describe the binding of glycolytic enzymes to two classes of site on F-actin under conditions of low ionic strength. A similar empirical approach was adopted[92] to describe the interaction of aldolase with just a single class of site on synthetic thin filaments under conditions of higher ionic strength ($I = 0.088$). Kurganov and co-workers[72,75] have attempted to place the quantitative aspects of reversible enzyme adsorption on a firmer theoretical footing, both in regard to thermodynamic and enzyme kinetic consequences. However, a limitation of their theoretical expressions is the implicit assumption, made also in the empirical analyses referred to above, that only one interaction between enzyme and matrix occurs even in instances where the enzyme possesses several binding sites. Should this assumption which may well be a reasonable approximation for systems with a sufficiently low density of matrix sites[42,76,77] not be appropriate, the binding isotherm is no longer describable in terms of the theory developed[78] for the binding of a univalent ligand to a multivalent acceptor. In the event that enzyme and matrix material must both be considered to be multivalent, allowance needs to be made for the formation of complexes in which enzyme forms cross-links betweeen sites on the cellular matrix. Such an approach has been illustrated recently[79] in a reappraisal of the data for the interaction between aldolase and synthetic thin filaments.[92] Finally, from the viewpoint of metabolic control, one of the most important features of the coexistence in equilibrium of adsorbed and free forms of the same enzyme is the possible displacement of the partition equilibrium position by effectors (e.g., see Table 1 of Reference 72). For the situation in which the formation of cross-linked enzyme-matrix complexes may be neglected, quantitative expressions are available for complete elucidation of the system.[76] Although these relationships were developed in the context of quantitative affinity chromatography, they apply with equal validity to any partitioning system in which specific ligand addition leads to enhanced adsorption or desorption of enzyme. Quantitative analysis of the effect of ligand addition in the more general situation is in principle feasible, but must await the development of theory that takes into account enzyme-matrix interactions of the cross-linking as well as of the multiple-binding type.

Third, the chemical nature of interactions between enzymes and subcellular structure may expect increased attention in the near future. The binding of an enzyme to subcellular structure clearly implies a defined molecular basis of the recognition and

interaction of these species, and clarification of the involved groups may be expected in the near future. Recently, for example, Wilson et al[101] have isolated a protein from the outer mitochondrial membrane which appears to be responsible for the specific interaction with hexokinase. Experiments have shown that treatment of the hexokinase with chymotrypsin results in no loss of catalytic activity, but total loss of binding ability. Apparently a restricted N-terminal region of the enzyme, discrete from the catalytic site region, directly interacts with the membrane component. Again, interactions of glyceraldehyde-3-phosphate dehydrogenase with the human erythrocyte membrane have been carefully studied by Steck and his colleagues.[8] These workers have demonstrated that the enzyme binds specifically to a protein ("Band 3") on the cytoplasmic surface of the erythrocyte. Mild tryptic treatment of the membrane release a peptide sequence from the Band 3 protein which interacts with the glyceraldehyde-3-phosphate dehydrogenase. Thus, the binding of these enzymes to the erythrocyte membrane, like the binding of hexokinase to the outer mitochondrial membrane, appears to depend on specific protein-protein interactions. Based on such examples, it would seem likely that specific interactions between enzymes and cellular membranes result from the existence of peptide sequences on the enzyme which permit it to specifically interact with complementary sequences on a particular membrane protein. Thus enzyme possessing different catalytic function may share the common ability to bind to a specific membrane protein by possessing, in addition to their different catalytic regions, a common peptide sequence which interacts with the membrane protein.

Clearly then, many significant questions remain to be answered, both in relation to the factors affecting the interactions of enzymes and structural elements in the cell, and also in relation to the broad biological implications of these phenomena. While many important principles have been established, and there is increasing acceptance of the reality of data in this area, it is still necessary to continue to formulate and test new hypotheses, to give adequate recognition to the biphasic nature of many of these cellular interactions and the potentialities for control by median metabolities, but above all, to endeavor to relate experimental techniques and concepts to cellular conditions. If our concepts of cellular biology are to be meaningful, they should of necessity be based firmly on the physiological realities of the microenvironment.

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