SYMPOSIUM ON MULTIPLE FORMS OF ENZYMES AND CONTROL MECHANISMS[1]

I. Multiple Forms of Enzymes[2]

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Introduction

In the past few years, considerable attention has been focused on the heterogeneity of enzymes catalyzing the same function. It is the purpose of this paper to review some of the developments in this field, and to attempt an analysis of the significance of multiple forms of enzymes in the regulation of cellular activity and growth. In this review, I will summarize principally investigations of pyridine nucleotide-linked dehydrogenases, although other enzymes will be discussed. No attempt will be made to include all of the work on multiple forms of enzymes. Examples will be cited which may be of aid in understanding the general problem. (The heterogeneity of enzymes involved in amino acid biosynthesis in microorganisms is discussed in the following paper by E. R. Stadtman.)

Methods for Detecting Multiple Molecular Forms of an Enzyme

There are now a number of experimental approaches which are valuable in detecting the molecular heterogeneity of enzymes. These include electrophoretic migration, resin chromatography, fingerprint patterns, amino acid composition, amino acid sequence, physical properties, immunochemical characteristics, and catalytic properties. If one is fortunate and can extensively purify and crystallize the multiple forms of enzymes which catalyze the same function, then amino acid composition and sequence studies are possible. Fingerprinting of peptides after digestion with specific proteolytic enzymes has also been found to be of value in showing differences between two similar catalysts. A number of physical criteria have proven to be useful parameters in elucidating the nature of the heterogeneity of enzymes; for example, ultracentrifugal analysis, temperature stability, optical rotatory dispersion data, as well as fluorescence and polarization of fluorescence analysis. All these methods have been applied successfully in comparative investigations. The immunological methods have been found to be potent tools in showing the dissimilarities and relationships of enzymes, and this has been particularly true when an antibody can be obtained to one of the purified forms. Examples of this technique will be cited later in this presentation.

What types of evidence imply that more than one form of an enzyme is present in one cell type or tissue? Previous to the last decade, this was suggested by physical separation, that is,

[1] This symposium was held at the Annual Meeting of the American Society for Microbiology, Kansas City, Mo., 9 May 1962, under the sponsorship of the General and Physiology Divisions, with N. O. Kaplan as convener.

[2] This is publication no. 195 of the Graduate Department of Biochemistry.

by fractionation with ammonium sulfate, alcohol, or acetone, and by the other classical methods of protein purification. Today a great deal of emphasis is placed on chromatography on various resins, as well as on various electrophoretic methods. These techniques usually measure charge differences and, under certain conditions, size differences. When a crude extract is applied for electrophoretic examination or diethylaminoethyl (DEAE)-cellulose chromatography, more than one migrating form of the enzyme usually can be detected. In fact, a number of crystalline enzymes have been found to contain a number of electrophoretic forms, even though the preparations, on ultracentrifugal analysis, showed only one peak. Markert and Møller (32) introduced the term "isozymes" to identify these various forms. Important questions, therefore, are: What is the nature of isozymes, and are they significant genetically and functionally? It is the purpose of this paper to try to throw some light on these questions.

Another method of demonstrating differences between enzymes with similar function is by their catalytic characteristics. For example, we have used the comparative rates of reaction of nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP) analogues to show differences between fundamentally related enzymes. This has proven to be of significance in a study of evolution and classification of dehydrogenases (21, 24). Table 1 gives data from such a study in which we examined the lactic dehydrogenases of some animals and compared the ratio of reaction of 3-acetylpyridine adenine dinucleotide (AcPyAD) with that of the natural

TABLE 1. *Ratio of reaction of 3-acetylpyridine adenine dinucleotide–nicotinamide adenine dinucleotide of various animal muscle lactic dehydrogenases*

Group	Species	Ratio
Crustaceans	Lobster	12
	Green crab	17
	Crayfish	10
Arachnids	Tarantula	0.12
	Scorpion	0.16
	Wolf spider	0.06
Fish	Mackerel	0.7
	Sea robin	1.0
	Trout	1.7

TABLE 2. *DNA base composition and malate dehydrogenase of the genus Bacillus*

Organism	G + C*	NAD / EPKAD
	%	
B. subtilis 166	43	16.9
B. subtilis ATCC 9789	43	14.8
B. subtilis var. *aterrimus*	43	22.5
B. subtilis var. *niger*	43	17.0
B. cereus	33	>100
B. cereus var. *mycoides*	33	>100
B. alvei	33	>100
B. cereus megaterium	34	>100
B. thuringiensis	34	>100
B. circulans	35	0.58
B. megaterium	37	1.1
B. licheniformis	46	10.5
B. macerans 1	50	0.84
B. macerans 2	50	0.70

* Guanosine plus cytosine.

coenzyme. The three crustaceans (lobster, crab, and crayfish) have relatively similar enzymes, as do the three members of the arachnid group. There are also close relationships among the fish enzymes. This technique is of value not only in showing relationships among animals but also among microorganisms. Table 2 shows a comparison of the malic dehydrogenases of some *Bacillus* species. The rate of reaction of ethyl pyridyl ketone adenine dinucleotide (EPKAD) is compared with that of NAD. [See Anderson et al. (1, 2) for a description of the properties of EPKAD.] It is of interest that the organism—*cereus megaterium*—which was kindly sent to us by Jackson Foster and which he considered to be an intermediate between the two types, is by its malic dehydrogenase very closely related to *cereus* and not to *megaterium*. Julius Marmur and his associates of our laboratory have made a detailed study of the base composition of the various *Bacillus* species and have found that the deoxyribonucleic acid (DNA) composition closely correlates to the type of malic dehydrogenase. In a similar manner, Susan Horwitz of our laboratory has been able to distinguish the mannitol phosphate dehydrogenases of the coli-aerogenes group from that of the *Bacillus* group (Table 3). From such studies, we feel that the pyridine nucleotide analogues can be used as tools to show heterogeneity among enzymes from different

TABLE 3. *D-Mannitol 1-phosphate dehydrogenase in Enterobacteriaceae and genus Bacillus**

Organism	N-6-OH ethylamino D / NAD	NHXD / NAD
Escherichia coli B	0.66	0.83
Erwinia carotovora	0.65	0.86
Aerobacter aerogenes	0.73	0.71
Salmonella typhimurium	0.68	0.82
B. subtilis 168	0.70	0.16
B. subtilis var. *niger*	0.73	0.07
B. natto	0.68	0.15
B. subtilis var. *aterrimus*	0.75	0.13

*Data of Susan Horwitz of this laboratory.

organisms catalyzing the same function, even in crude extracts. I wish to emphasize that when these differences in catalysis are found, differences in immunological and physical properties can also be detected. This type of approach has also been of value in showing similarities and dissimilarities among multiple forms of dehydrogenases found in one cell type or tissue.

HEXOKINASE

The remainder of this report will deal with a discussion of the multiple forms of an enzyme in one organism.

Recently, Colowick and his associates (17) reported that anion chromatography on DEAE cellulose of six times recrystallized yeast hexokinase resulted in the separation of six forms of the enzyme (Fig. 1). The forms can be divided into two main fractions, I and II. Fraction I was eluted by the pH gradient, whereas the second fraction was not eluted unless a sodium chloride gradient was introduced. Within the limits of experimental error, the specific activities, kinetic constants, affinities of the reactants, and physical properties of all the fractions are the same. Nevertheless, when an individual fraction is rechromatographed, it appears as a single peak at the expected position.

Treatment of the crystalline hexokinase with a multitude of chemical reagents, such as butanol, cysteine, glutathione, glucose, thiosulfate, sulfite, and cystine, failed to alter the chromatographic patterns. Proteolytic enzymes, however, have been found to completely convert fraction I into fraction II. This has been accomplished either by trypsin or chymotrypsin in the presence of glucose. Although both the chymotrypsin- and trypsin-treated enzymes are eluted by the NaCl gradient as fraction II, the treatments appear to result in molecular forms which are separable chromatographically. The Colowick group also demonstrated that autolysis of the yeast extract can result in the conversion of fraction I to fraction II, although the rate of this conversion appears to be quite slow and may not account for the observed presence of the various forms in the short time period involved in the extraction of the yeast.

The significance of the various forms of the yeast hexokinase is not clear. There is no question that the various forms can be intraconverted with proteolytic enzymes. Nevertheless, the Colowick group still believes that the two main types may exist within the yeast cell, since short-term extraction results in the presence of the multiple forms. However, it is apparent

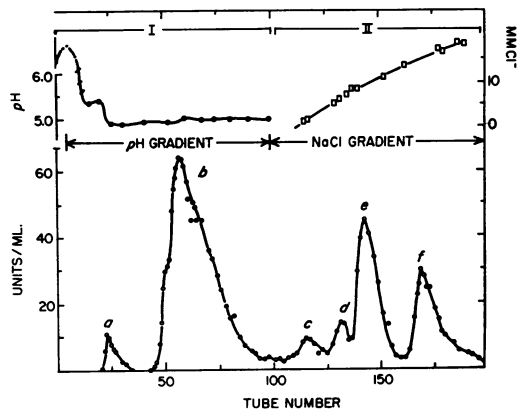

FIG. 1. *Anion-exchange chromatography of yeast hexokinase on DEAE cellulose using gradient elution. Key:* ○, *hexokinase activity (units/ml);* ●, *pH;* □, *chloride ion concentration in effluent. Hexokinase, 21.5 mg in 50 ml of buffer (5 × 10⁻³ M succinate, pH 6.0) crystals (6×), 696 units/mg, placed on a column (9 × 60 mm). Tubes 0 to 4 contained the effluent collected during the addition of the enzyme. A pH gradient was established by placing in pH 5.1 buffer (5 × 10⁻³ M succinate, 10⁻⁴ M versene) in a mixing vessel (600 ml) and running in buffer at pH 4.8 (5 × 10⁻³ M succinate). The pH of the effluent fell to 4.8 more abruptly than expected in this experiment. NaCl gradient 0 to 50 mM, pH 4.8 (5 × 10⁻³ M succinate). An air pressure bulb was used to increase the flow rate to approximately 0.2 ml per min. Approximately 4 ml per tube were collected. From Kaji et al. (17).*

that the various molecular forms are so closely related that no genetic differences can be invoked. The yeast hexokinase represents a good example of multiple forms in which the only differences that can be detected are charge differences.

MULTIPLE FORMS OF ENZYMES APPEARING AS ARTIFACTS OF PURIFICATION

Studies with ribonuclease (42) and glyceraldehyde phosphate dehydrogenase (4) have shown that multiple forms of an enzyme can appear as artifacts during the course of purification. In an elegant study, Margoliash and Lustgarten (31) showed that the presence of relatively numerous species of mammalian cytochrome *c* represents only conformational variants of a single native structure arising as modifications resulting from the purification procedure. The studies with cytochrome *c* again provide a clear-cut demonstration that the various postulated molecular forms on an enzyme are of significance only when it is first ruled out that the forms either cannot conceivably or do not in fact arise one from the other because of the nature of the manipulation of the preparations.

LACTIC DEHYDROGENASE

I now turn to another example of multiple forms of an enzyme, in which the nature of the various forms is distinctly different from that of the yeast hexokinase and the cytochrome *c*. These are the mammalian lactic dehydrogenases, with which our laboratory has been deeply concerned. An extremely large number of reports by many investigators have indicated that in the mammal there are five forms of the lactic dehydrogenase which can be identified either by starch-block electrophoresis or by DEAE cellulose chromatography. Figure 2 shows the position of the various lactic dehydrogenase forms in the rat on starch-grain electrophoresis. The various bands are real, reproducible entities and are not artifacts resulting from the preparative procedures.

During the past few years, we have compared a large number of vertebrate heart and skeletal muscle extracts with respect to their lactic dehydrogenase activities as characterized by their rates of reaction with coenzyme analogues (Table 4; 21). As can be seen, the heart and muscle lactic dehydrogenases of one species

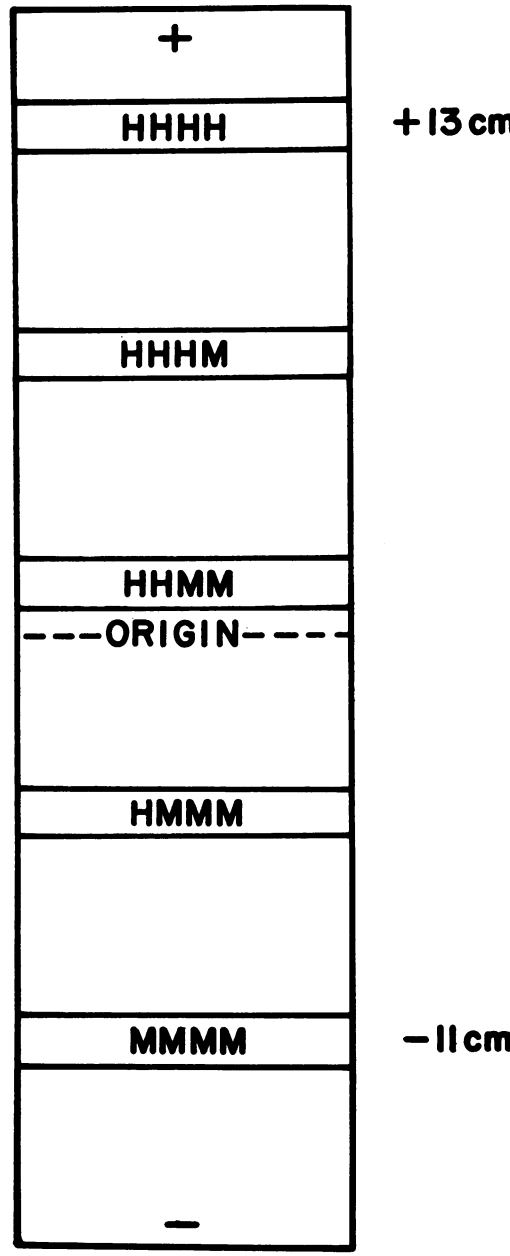

FIG. 2. *Electrophoretic migration of various lactic dehydrogenase forms of the rat on starch grain. From Fine et al. (14).*

are very different, and the heart catalysts of different species are more closely related to each other than are the heart and muscle dehydrogenases of one species (21, 24). There is also a relatively close relationship among the skeletal muscle enzymes of the different vertebrates.

We have been able to crystallize a number of heart and muscle lactic dehydrogenases from different animals and to study their properties (8, 34, 33). A comparison of the crystallized heart and breast muscle lactic dehydrogenases of the chicken is given in Table 5. The two enzymes give different electrophoretic mobilities; only one form with each crystalline preparation can be observed on electrophoresis. The two enzymes also differ with respect to their amino acid composition (particularly striking is their difference in histidine content), their stability, and their catalytic characteristics, as indicated by their affinities for pyruvate as well as their reaction with the coenzyme analogues (8). The two lactic dehydrogenases also give different fingerprinting patterns after tryptic digestion. Antibodies made in the rabbit against the heart dehydrogenase show no cross reaction with the crystalline muscle enzyme, when tested by double diffusion in agar, by quantitative inhibition, by precipitation of enzyme activity, or by quantitative complement fixation. The same is true for antibodies against the crystalline muscle lactic dehydrogenases; the antibodies are absolutely specific and do not cross-react with the enzyme from heart.

TABLE 4. *Rate of thionicotinamide adenine dinucleotide$_{(L)}$-3-acetylpyridine adenine dinucleotide$_{(H)}$ activity in crude extracts of different vertebrate hearts and skeletal muscles**

Animal	Skeletal muscle	Heart
Turkey	0.48	5.3
Chicken	0.50	5.2
Pigeon	0.63	5.0
Tuna	0.22	8.2
Toad	1.60	6.2
Green frog	1.62	6.8
Rabbit	0.73	5.4
Human	2.2	5.7
Mouse	1.7	5.6
Rat	1.2	5.8

* See references (8, 23) for significance of ratios.

From a consideration of the properties of the two lactic dehydrogenases, it is evident that they are entirely different moieties. Several years ago, we advanced the concept that there are two main types of lactic dehydrogenase in most animals, one which predominates in heart and the other in skeletal muscle (21, 24). Now a question arises concerning the relationship of these two principal types to the five forms observed on electrophoresis.

I will now summarize recent data from our laboratory which have led us to advance the following hypothesis for the occurrence of the five forms. Appella and Markert (3) have reported that four subunits are present in the crystalline beef heart lactic dehydrogenase. A. Pesce and R. D. Cahn have been able to demonstrate by ultracentrifugal analyses that both the chicken heart and muscle enzymes consist of four subunits. They have also detected the presence of four subunits in the beef muscle lactic dehydrogenase. Four subunits are also indicated by the fact that approximately 4 moles of coenzyme are bound per mole of enzyme (44), as well as by the fact that there are four essential SH groups present in the native enzymes (10). From the fingerprint data and other evidence, it appears that the subunits in each pure enzyme type are identical. However, the subunits in the heart enzyme are different in primary structure from those of the muscle enzyme; we have some fairly substantial evi-

TABLE 5. *Comparison of chicken breast muscle and chicken heart lactic dehydrogenase (LDH)**

Determination	Breast muscle LDH	Heart LDH
Electrophoretic migration at pH 7.0	−	+
Optimal pyruvate concentration (pH 7.5)	1.7×10^{-3} M	4×10^{-4} M
Histidine residues per mole of enzyme	64	26
Tyrosine residues per mole of enzyme	19	32
$\frac{NHXDH_{(L)}}{NADH_{(H)}}$†	0.50	3.22
Destroyed at 55 C for 10 min (%)	100	0

* Data taken from Cahn et al. (8), Brand et al. (5), Wilson et al. (49), and Pesce et al. (34).
† See references (8, 14) for significance of ratios.

FIG. 3. *Schematic representation of various forms of lactic dehydrogenase (LDH).*

dence indicating that the synthesis of the two types of subunits is controlled by different genes. We have indicated that the subunit in the heart enzyme is H, and the pure enzyme would then be designated as H_4 (see Fig. 3). The muscle subunit has been termed M, and the muscle protein would have four M subunits. When both M and H genes are active in the same cell, three types of hybrid enzymes are formed, consisting of both M and H subunits in different combinations (H_3M_1, H_2M_2, H_1M_3). Thus, if M and H subunits differ in net charge at a given pH, five electrophoretically distinct lactic dehydrogenase bands should be observed. In mammals, the pure M type is always the most negatively migrating form and the H lactic dehydrogenase is the most positively migrating form. If these two types migrate sufficiently far apart, then the three intermediate or hybrid lactic dehydrogenases can be clearly seen, as shown with a number of mammals (8). In the chicken, at pH 8.7, the two forms move relatively close together, and under these conditions it is difficult to recognize the hybrids. However, if the electrophoresis is carried out at pH 7.0, the three hybrids can then be identified. Hence, the detection of the intermediates depends on the migratory characteristics of the two types of subunits. We have found instances where the M and H forms of one animal are very similar in their electrophoretic migration, yet are very different in other properties.

The hypothesis predicts that there should be a regular progression of enzymatic properties from the heart lactic dehydrogenase through the hybrids to the muscle form. This is illustrated in Table 6 by a comparison of the rates of activity of reduced NAD (NADH) and of its hypoxanthine analogue, reduced nicotinamide hypoxanthine dinucleotide (NHXDH; 8). The hybrid ratios are intermediate between the two pure types. Furthermore, it is possible to predict the ratio of the hybrids from the values of the two pure types, if one assumes that each subunit contributes equally and independently to the properties of the tetramer.

A second postulate of the hypothesis should be a prediction that all three hybrids are precipitable with antibodies against each pure enzyme type. As indicated in Table 7, with the highly specific antibodies against the crystalline chicken heart and chicken muscle enzymes, we have been able to show that both antisera can completely precipitate all three hybrids (8). It is evident that, if a molecule possesses one H or one M subunit, the entire molecule can be precipitated by either antibody. Quantitative techniques, however, have indicated that the hybrid forms give only partial cross reactions with the specific anti-M and anti-H antibodies. There is also a difference in the amount of antibody required for precipitation. For example, less of the anti-M antibody is required to precipitate the M_3H_1 hybrid than is required to precipitate the other two hybrids. The data reported here, as well as other evidence, strongly suggest that the five forms of the lactic dehydrogenase represent the two pure genetic types and the three intermediate hybrids.

I would like to discuss briefly the possible significance of the lactic dehydrogenase hybrids. It is our opinion that the hybrids are formed at

TABLE 6. *Comparison of reduced nicotinamide hypoxanthine dinucleotide–reduced nicotinamide adenine dinucleotide ratios of different forms of lactic dehydrogenase (LDH) from chicken*

LDH	Found	Predicted
M_4	0.50	—
M_3H_1	0.99	1.18
M_2H_2	1.98	1.86
M_1H_3	2.50	2.54
H_4	3.22	—

TABLE 7. *Reaction of various forms of chicken lactic dehydrogenase (LDH) with antibodies of H_4 and M_4*

LDH	Anti-chicken H_4	Anti-chicken M_4
H_4	+	0
H_3M_1	+	+
H_2M_2	+	+
H_1M_3	+	+
M_4	0	+

random when both genes are operating in one cell, in the same manner as genetic recombinants. Our data, as well as those of other workers, indicate a binomial distribution. Hence, what appears to be of importance is the relative activities of the two genes responsible for the synthesis of the two different types of subunits in a given tissue. This is clearly illustrated in studies on differentiation. In the early chick embryo, the breast muscle contains almost completely H type lactic dehydrogenase (8). As the embryo develops, there is a shift from the H type lactic dehydrogenase to the M type. It is important to emphasize that the muscle gene is first expressed by the formation of hybrids and then by the pure muscle type in the late embryo. Furthermore, the appearance of the hybrids follows the prediction of random distribution of the subunits; that is, the first hybrid to appear is the H_3M_1 type, then the M_2H_2, then the M_3H_1, and finally the M_4 pure type.

Since the subunits act as independent catalytic entities in the hybrids, and because of the randomness of their distribution (8, 14), it is our guess that the hybrids do not have any particular physiological significance *per se* within the cell. What appears to be of importance is the relative amounts of the two types of lactic dehydrogenase subunits. That the subunits are combined as a tetramer may be owing to the fact that the subunits are unstable and that interaction of four units leads to a structure which stabilizes them.

The M and H types of lactic dehydrogenase appear to be under the control of separate genes (8). They also appear to have functional differences (8, 22, 23, 49). The concept that one gene controls more than one lactic dehydrogenase form appears to be erroneous (48), and certainly there is no need to put the hybrid form of lactic dehydrogenase under the control of separate genes.

The nature of the multiple forms of animal lactic dehydrogenases is certainly different from that observed with the yeast hexokinases or the mammalian cytochrome *c*. We also wish to caution that, when one observes five forms of an enzyme, hybrids, such as are found in the lactic dehydrogenase, are not necessarily involved. For example, we recently observed five distinct electrophoretic forms of malic dehydrogenase from *B. subtilis*. However, these forms turned out to be identical with respect to their catalytic activities as well as other properties. It is our impression that to understand the genetic and physiological significance of multiple forms of an enzyme one must first clarify by a variety of methods the nature of the heterogeneity.

Alkaline Phosphatase

An interesting study on multiple forms of an enzyme, and one somewhat related in principle to the lactic dehydrogenase investigations described above, is the experiments of Levinthal and his associates on alkaline phosphatases. The *Escherichia coli* enzyme has been purified and found to have, in its native state, a molecular weight of 80,000 (15). The enzyme appears to have two subunits with identical molecular weights of 40,000. These two subunits are linked through disulfide bonds. The purified *E. coli* alkaline phosphatase, although homogeneous in an ultracentrifuge and by Tiselluis electrophoresis, shows on zone electrophoresis several enzymatically active bands (35). The different bands have approximately equal enzymatic properties. Single mutations in the alkaline phosphatase gene result in a change of charge, and affect all bands identically. The same number of bands is observed in the mutant, but the migration of the bands is all shifted. This would suggest that all the bands are the product of one gene.

Levinthal and his co-workers (29) have recently shown that the *E. coli* phosphatase can be reduced in the presence of thioglycolate and 6 M urea. This results in the formation of monomers which are enzymatically inactive. Under appropriate conditions, the monomers can be converted to the dimeric state, with a restoration of enzymatic activity and a return of the original electrophoretic pattern. Two alkaline phosphatases have been purified from pseudorevertants of *E. coli*. One enzyme moves faster than the wild type; the second moves more slowly. When these two mutant phosphatases are mixed in vitro, no new bands appear. However, if the two types of phosphatases are first reduced and then reactivated together, several bands of intermediate mobility appear which have enzymatic activity. These studies suggest that hybrids of the two mutant phosphatases have been formed.

Serratia marcescens possesses an alkaline

phosphatase which is different from the corresponding *E. coli* enzyme by its electrophoretic migration and immunological characteristics, and in the peptide pattern after tryptic digestion (29). When both the *S. marcescens* and *E. coli* genes are present in active form in one cell, both forms of phosphatase are produced. In addition, enzymatically active bands were detected with electrophoretic mobility between the two parent enzymes. These hybrids are not formed when a mixture of the two phosphatases is electrophoresized. However, if the monomers of the two forms are reactivated together, the hybrids are formed. Hence, hybridization can occur both in vivo and in vitro, although the rate of recombination appears to be quite slow in vitro. This result supports the views of Fincham (13) and Brenner (6) that genetic complementation can result from two different structural genes each producing a different monomer.

There is no good explanation as yet of the several bands of phosphatase found by starch-gel electrophoresis, which are apparently the action of one gene. The peptide chain may exist in several alternate states which may be the result of cytoplasmic factors.

This study emphasizes the danger of examining electrophoretic patterns without thorough investigation, since electrophoretic bands may be due to hybrids as well as to cytoplasmic altered proteins.

ESTERASE FROM MAIZE

Schwartz (40) reported that two different alleles of one gene, responsible for the synthesis of an esterase in maize, will produce two forms of the enzyme, as indicated by differences in electrophoretic migration. When both these alleles are present, a hybrid form of the enzyme is found which has an intermediate electrophoretic migration between the two homozygous esterases. From a comparison of the intensities of various types of crosses, it has been suggested that the enzyme consists of two units, the subunits being identical in the homozygote. Schwartz (41) has recently suggested that the enzyme is synthesized as a dimer, since he has found that the enzyme can be extracted from ribosomal particles. He interprets this finding as indicating that the free monomer is not formed. However, the studies of the Levinthal group on the phosphatase indicate that monomers can be converted to dimers.

FUNCTION OF NAD AND NADP ENZYMES CATALYZING THE SAME REACTION

In considering multiple forms of an enzyme, it is of interest to review the work of Kornberg and Pricer that was carried out some 10 years ago on the isocitric dehydrogenases of yeast (26). There are two isocitric dehydrogenases, one NADP specific and the other NAD specific. The end products of the reaction, however, are the same: α-ketoglutarate and CO_2. The NAD enzyme has an absolute requirement for 5′ adenosine monophosphate (AMP), whereas the NADP enzyme is not influenced by this nucleotide. No evidence has been obtained for oxalosuccinate as intermediate with the NAD enzyme. It is indeed evident that the NAD and NADP enzymes are completely different proteins.

The fact that two forms of pyridine nucleotide, NADP and NAD, exist in nature makes possible the occurrence of two types of enzyme in which the same over-all reaction can be carried out, but whose functional characteristics are dissimilar. Some time ago, we obtained evidence that the two forms of the pyridine nucleotide had generally different functions, illustrated in Fig. 4. That is, NADH, which is generated by the NAD-specific dehydrogenases, is oxidized in the electron-transfer pathway to form adenosine triphosphate (ATP; 20). The reduced NADP (NADPH), we believe, is used primarily as the reducing power in synthetic reactions. We have also described an enzyme, which we have called pyridine nucleotide transhydrogenase (18, 19, 43, 47), capable of promoting an oxidation of NADPH by NAD to give reduced NAD (Fig. 4). In Table 8, we have compared the distribution of

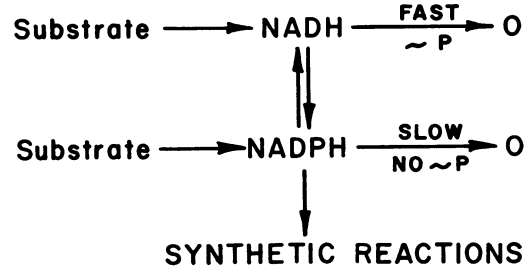

FIG. 4. *Pathways of electron transport. From Kaplan et al. (20).*

NADP and NAD isocitric dehydrogenases with that of transhydrogenase. Yeast and *Neurospora* have both types of isocitric dehydrogenase, but no detectable NADPH-NAD transhydrogenase. On the other hand, *Pseudomonas* species and *Azotobacter* contain not only NADP isocitrate dehydrogenase but also have a high level of transhydrogenase. Liver and kidney mitochondria of the rat have large amounts of NADP isocitrate dehydrogenase and transhydrogenases, but practically no NAD-linked enzyme (see Table 8). Brain has, relatively, a higher proportion of the NAD system and much lower levels of the transhydrogenase. From the data, there appear to be two major ways by which isocitrate oxidation can yield NADH, either directly by the NAD-linked enzyme or by a combination of the NADP-specific enzyme and the transhydrogenase. John Lowenstein of our laboratory has suggested that the NAD-linked enzyme becomes functional in the whole yeast when the level of ATP is low and that of 5′ AMP high, since the mononucleotide is essential for the activity of the enzyme. Therefore, it seems possible that the regulation of the NAD enzyme may be an important factor in the over-all respiration of the yeast cell and that the NADP and NAD dehydrogenases have distinctly different functional roles.

TABLE 8. *Distribution of nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP) isocitric dehydrogenases (IDH) and pyridine nucleotide transhydrogenases*

Source	NADP IDH	NAD IDH	Transhydrogenase
Yeast	+	+	0
Neurospora	+	+	0
Pseudomonas fluorescens	+	0	+
Azotobacter vinelandii	+	0	+
Rat*			
Kidney	1,900	6	107
Liver	520	6	44
Brain	85	28	4

* Data from Vignais and Vignais (47).

TABLE 9. *Properties of intramitochondrial and extramitochondrial isocitric dehydrogenases (IDH) of rat liver**

Determination	Intramitochondrial IDH	Extramitochondrial IDH
Distance of migration toward cathode on electrotrophoresis (cm)	6.0	0.5
Nicotinamide adenine dinucleotide phosphate–thionicotinamide adenine dinucleotide phosphate ratio	2.4	5.3
Reaction with antibody to intramitochondrial IDH (%)	75	0

* Data from Lowenstein and Smith (30).

TABLE 10. *Comparison of methods for the extraction of glutamic dehydrogenases from yeast cake**

Method of extraction	Enzymatic activity†	
	NADP-linked enzyme	NAD-linked enzyme
Homogenization with glass	1,160	378
Frozen and thawed (four times)	1,260	79
French press	1,605	1,125

* Data of Mary D. Doherty of this laboratory.
† Enzymes assayed at pH 7.6. Expessed as units/g of cells.

Recently, Dr. Lowenstein has been able to demonstrate two NADP-linked, isocitrate-linked dehydrogenases in rat liver (Table 9; 30). One is found in the soluble part of the cell, and the other in the mitochondria. They are different entities, as indicated by immunological, catalytic, and electrophoretic criteria. It will be of considerable interest to learn the nature of the differences in the two types and the mechanism of their synthesis.

In mammals, the glutamic dehydrogenase can react with both NADP and NAD. Recently, evidence has been obtained that two glutamic dehydrogenases exist in yeast as well as in *Neurospora*, one NADP specific and another NAD specific. Mary D. Doherty (11) of our laboratory has been able to crystallize the NADP enzyme from yeast. She has also extensively purified the NAD-linked catalyst.

In examining the relative levels of enzyme, it is important to consider the methods of extraction. This has been particularly true with the yeast glutamic dehydrogenases (Table 10). The NAD catalyst is extremely difficult to solubilize, and reproducible results were only obtained by use of the French press. It is of con-

TABLE 11. *Effect of various supplements of glutamate in the minimal medium on the activity of the NADP- and NAD-dependent glutamic acid dehydrogenases after 48 hr of growth**

Glutamate concn	Specific activity of	
	NAD enzyme	NADP enzyme
M		
0.005	112	492
0.01	203	211
0.03	857	68
0.05	897	62

* Data from Sanwal and Lata (38).

TABLE 12. *Effect of glutamate in media on the nature of glutamate dehydrogenase in Fleishmann's yeast**

NH_4SO_4	Glucose	Glutamate	NADP enzyme	NAD enzyme
			units	*units*
+	+	+	300	21
+	−	+	12	840

* Data of Mary D. Doherty of this laboratory.

siderable interest that the NADP enzyme has a much greater affinity for NH_3 than does the NAD dehydrogenase. This suggests that the NADP protein may be more geared for glutamate synthesis than is the NAD enzyme. Further evidence of the functional difference of the two glutamic dehydrogenases may be found in the very recent report of Sanwal and Lata (38, 39, 37) on *Neurospora*. Their data, represented in Table 11, show that the level of glutamate in the medium markedly influences the relative concentrations of the two enzymes. Glutamate appears to stimulate the synthesis of the NAD-linked catalyst and repress the NADP enzyme. M. D. Doherty has made similar observations with yeast grown on glutamate (Table 12). It would seem that the NAD enzyme is more directed toward the oxidation of glutamate, since it arises when glutamate is in the growth medium. These data would also indicate that when glutamate is present in the medium the synthesis of the amino acid is repressed, since the NADP enzyme may be involved in this synthesis. This would be in line with Dr. Doherty's observations on the NADP enzyme's higher affinity for ammonia. Hence, the phenomena of induction and repression may be of considerable significance in regulating the metabolic patterns of a cell in which different enzymes catalyzing the same over-all reaction are involved. Kato et al. (25) have also recently reported that a glutamate medium induces a NAD-linked glutamic dehydrogenase in *Piricularia oryzae*, and that the NADP enzyme is not formed under these conditions. The studies with the glutamic dehydrogenases give additional support to the view that when a system is directed toward oxidation NAD is involved, and that NADP is more concerned with anabolic reactions.

MULTIPLE FORMS OF MALIC DEHYDROGENASE

I would now like to turn to another example (in animals) which further illustrates the role of more than one molecular type catalyzing the same reaction. Two forms of malic dehydrogenase have been found in mammalian tissues; one is present largely in the mitochondria and the second in the soluble cytoplasm. These two types differ in their electrophoretic migration, their behavior on DEAE-cellulose chromatography, their kinetic constants, and their reaction with NAD analogues; they also show dissimilar physical characteristics in the purified state (9, 12, 45, 16).

Recently, in our laboratory (46), it has been found that the purified mitochondrial malic dehydrogenase can give a number of different forms on starch gel. These multiple forms migrate rather close together and are distinctly different from the soluble enzyme (see Fig. 5). The distribution patterns of the various mitochondrial forms are not influenced by age, the tissue of origin, or the method of purification.

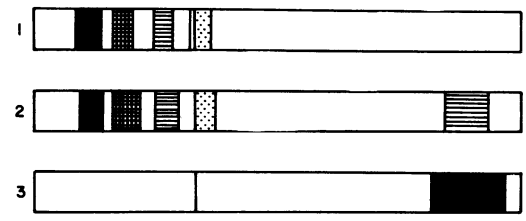

FIG. 5. *Electrophoretic pattern observed after starch-gel electrophoresis (20 hr at pH 7.0). (1) Purified pig heart mitochondrial malic dehydrogenase; (2) crude extract of a single acetone-dried pig heart; (3) purified pig heart supernatant malic dehydrogenase. From Thorne et al. (46).*

After elution from gel, these mitochondrial components give relatively the same catalytic criteria, but they differ collectively from the cytoplasmic enzyme. Hence, the relationship of the different mitochondrial forms to each other is certainly different from that of the cytoplasmic enzyme. This illustrates the point that we have tried to emphasize: that electrophoretic forms cannot be compared without knowledge of other criteria. The various multiple forms of the mitochondrial malic dehydrogenase are, in a sense, similar to the various electrophoretic forms of bacterial phosphatases controlled by a single gene. They may be more clearly illustrative of what Markert and Møller (32) have termed "isozymes."

A comparison of the substrate affinities of the soluble and mitochondrial malic dehydrogenases

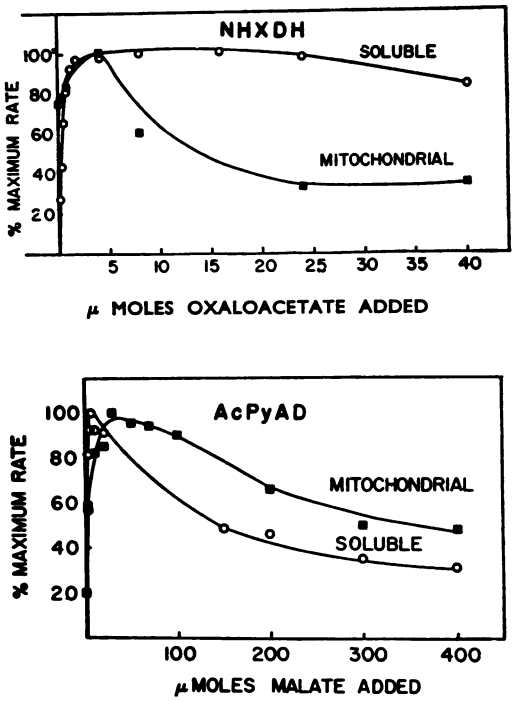

FIG. 6. *(upper) Effect of oxaloacetate concentration on the malic dehydrogenase of soluble and mitochondrial fractions of rat liver using reduced nicotinamide hypoxanthine dinucleotide (NHXDH) as pyridine nucleotide. (lower) Effect of malate concentration on the malic dehydrogenase of soluble and mitochondrial fractions of rat liver using 3-acetylpyridine adenine dinucleotide (AcPyAD) as pyridine nucleotide. From Kaplan (22).*

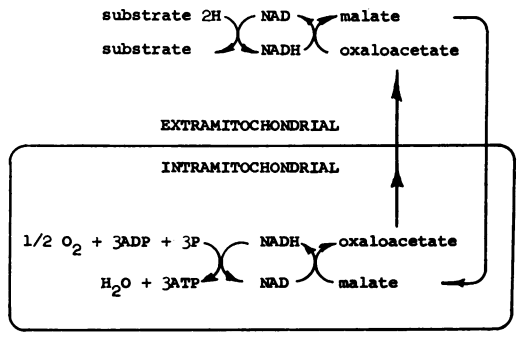

FIG. 7. *Scheme for the role of soluble and mitochondrial malic dehydrogenases.*

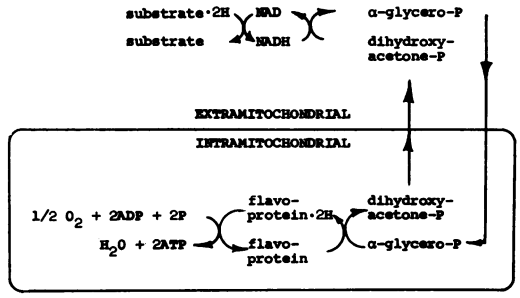

FIG. 8. *Role of glycerol phosphate dehydrogenase in reduced pyridine nucleotide oxidation.*

shows some striking differences, which may be of significance physiologically. As illustrated in Fig. 6, oxaloacetate shows a strong inhibitory effect on the mitochondrial enzyme, whereas high levels of malate tend to produce a greater inhibition of the rate of the soluble malic dehydrogenase. NHXDH was used as the coenzyme for the data obtained in Fig. 6, since a more marked difference can be observed between the two enzymes when the reduced analogue is the reductant than when NADH is. AcPyAD gives a more pronounced difference than the natural coenzyme, when malate is the substrate.

From a consideration of the data on substrate inhibition it would appear that the mitochondrial enzyme is oriented toward oxidation of malate, whereas the function of the soluble malic dehydrogenase is directed toward a reduction of oxaloacetate. The structural differences in the two catalysts may prevent oxaloacetate reduction in the mitochondria and malate oxidation in the soluble fraction of the cell. We have presented a scheme which utilizes the two malic dehydrogenases for coupling the oxidation of

TABLE 13. *Specificity of vic glycol dehydrogenase of Aerobacter aerogenes with different coenzyme analogues**

Coenzyme	Glucose-grown	Glycerol-grown
Nicotinamide adenine dinucleotide	100	100
Nicotinamide hypoxanthine dinucleotide	12	252
3-Acetylpyridine adenine dinucleotide	73	4
Pyridine 3-aldehyde adenine dinucleotide	55	20

* See references 27 and 28. In all cases the substrate was propylene glycol.

soluble NADH with phosphorylation (Fig. 7; 22). The essential points of this cycle involve oxaloacetate and malate as a shuttle between the soluble and mitochondrial pyridine coenzymes. The soluble NADH is oxidized by oxaloacetate to form malate; the hydroxy acid then penetrates the mitochondria and is oxidized to oxaloacetate by the mitochondrial malic dehydrogenase and the mitochondrial-bound NAD. The oxaloacetate would then re-enter the soluble cytoplasm and be reduced by another molecule of NADH. The mitochondrial NADH could then be oxidized by the respiratory chain enzymes and this could be coupled with the formation of ATP.

A similar shuttle system has been described (36, 7) for the soluble and mitochondrial α-glycerol phosphate dehydrogenases (Fig. 8). This involves, as can be observed, a soluble NAD-linked α-glycerol phosphate dehydrogenase which converts dihydroxy acetone phosphate into the α-glycerol phosphate at the expense of NADH. The α-glycerol phosphate is then oxidized by a second α-glycerol phosphate dehydrogenase in the mitochondria, which is a flavoprotein. This oxidation would again lead to the formation of ATP. However, 2 moles of ATP would be produced per mole equivalent of soluble NADH, as compared with 3 moles of ATP that can be formed per mole of soluble NADH by coupling the two malic dehydrogenases.

GLYCOL DEHYDROGENASES

Finally, I would like to cite the study with *vic* glycol dehydrogenases of *Aerobacter aerogenes*, carried out in our laboratory several years ago by Marvin Lamborg. *A. aerogenes* produces two types of NAD-linked dehydrogenases capable of oxidizing glycerol and other diols (27, 28). From the reaction with the coenzyme analogues, it can be seen that the enzyme formed when the organism is grown on glucose is different from that synthesized when glycerol is the carbon source (Table 13). These differences in analogue specificities have been substantiated by immunochemical as well as by physical studies. Hence, glucose appears to repress one enzyme, and glycerol the other. It would seem reasonable to assume that the glycerol-induced enzyme is geared toward glycerol oxidation; the role of the glucose-induced enzyme is not clear.

TABLE 14. *Nicotinamide adenine dinucleotide–nicotinamide hypoxanthine dinucleotide ratio of glycol dehydrogenase in different organisms when grown on glucose or glycerol*

Organism	Glucose-grown	Glycerol-grown
Aerobacter		
8724	33.0	5.4
884	0.63	1.5
8329	No enzyme	0.89
Escherichia coli K-12	2.0	1.8
Acetobacter suboxydans	0.63	0.62

It is of interest that different strains of *Aerobacter* have different characteristics in synthesizing the diol dehydrogenases when grown either on glucose or glycerol. (Table 14). As can be seen from the ratio of the reaction of NAD to nicotinamide hypoxanthine dinucleotide (NHXD) and with glycerol as substrate, there is a marked difference between the various organisms listed. The greatest difference occurred on the two different carbon sources, as with strain 8724, the organism used for the data in Table 13. Strain 8329 shows no enzyme when grown on glucose but produces a great deal of enzyme when grown on glycerol. It is of interest that *E. coli* and *Acetobacter suboxydans* apparently produce the same enzyme when grown either on glucose or glycerol. Therefore, it would seem that the phenomenon of having two enzymes promoting the same function is not a universal characteristic of all organisms.

SUMMARY

I wish to emphasize that there are other examples in the literature of multiple forms of enzymes. It is my opinion that when multiple forms are found, which show different catalytic and immunological characteristics, they will turn out to be significant genetically as well as physiologically. To appreciate clearly the significance of different forms of an enzyme, we must establish the nature of their nonidentity. We must look critically at the methods by which heterogeneity has been detected; we must be aware of the pitfalls of these methods. No single method is adequate. Although it is evident that different molecular forms of an enzyme from one organism having dissimilar catalytic properties may be extremely significant, it is not clear what the implications are of multiple molecular forms of an enzyme whose only detectable differences are their electrophoretic mobility and their behavior on resin chromatography. Whether they are artifacts formed during the preparation or in the method of analysis, or even as accidents of synthesis within the cell due to cytoplasmic factors, their significance is certainly different from those molecular forms whose catalytic activities are different. They may also represent different alleles which are controlled by one gene or duplicate genes. The role of cytoplasmic factors in determining the final structure of enzymes certainly is worthy of considerable investigation.

The finding that hybrids possess subunits of two different forms of one enzyme is of considerable importance to the geneticist and may lead to some clarification of the observations on multiple forms. It has been suggested that forms of an enzyme with different charges may be important in the localization of a given enzyme in different parts of the cell. No evidence of this has yet been established; however, this possibility is certainly worthy of consideration. It should be pointed out that enzymes catalyzing the same function may be different in various parts of the cells, but these differences go beyond only charge dissimilarities and represent catalytic differences which are of importance in the functional relationship of the cell (e.g., malic dehydrogenase). Perhaps one of the most exciting areas of future biological research lies in an understanding of the factors governing the repression and derepression of enzymes catalyzing the same over-all chemical reaction as well as in an understanding of their evolutionary function. From a survey of the studies already carried out, it seems reasonable to predict the importance of multiple forms of an enzyme in microorganisms as well as in multicellular organisms.

ACKNOWLEDGMENT

Some of the unpublished work reported here was aided in part by grants from the National Science Foundation (G-14612) and from the American Cancer Society (P-77E, The Thomas S. Miller Memorial Grant for Cancer Research).

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