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Malate Dehydrogenases. I. A Survey of Molecular Size Measured by Gel Filtration*

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ABSTRACT: The molecular sizes of some diphosphopyridine nucleotide linked malate dehydrogenases (including representatives of several major groups of animal, plant, and microbial species) have been determined by gel filtration. The elution volumes of the malate dehydrogenase activity in crude, cell-free extracts were identical with those of the partly purified enzymes and of the purified, crystalline enzymes. The elution volumes of malate dehydrogenases (mitochondrial and/or supernatant) from all animal and plant sources examined were equal, as were those of the enzymes from several microbial species; however, significantly smaller elution volumes, corresponding to higher molecular weights, were obtained for malate dehydrogenases of certain Gram-positive bacteria in the order *Eubacteriales*. Crystalline proteins, typical of small and large forms of malate dehydrogenase, were dissociated into enzymatically inactive subunits by treatment with acid, urea, or guanidine·HCl; partial reactivation was obtained by dialysis or dilution of the dissociating agent.

While the molecular weights of the diphosphopyridine nucleotide linked dehydrogenases range from 20,000 (dihydrofolate reductase) to more than 300,000 (beef liver glutamate dehydrogenase), little variation is usually found in specific dehydrogenases from one animal or tissue source to another, *e.g.*, the triosephosphate (Allison and Kaplan, 1964) and lactate dehydrogenases (Wilson *et al.*, 1964). On the other hand, the values published for the molecular weights of malate dehydrogenases from different sources would seem to indicate much greater variation in the molecular size of this enzyme.

Comparative studies on molecular weights of proteins are often narrow in scope because the techniques available are tedious and/or require relatively large amounts of highly purified material. The advent of gel filtration has provided a simple, rapid, and relatively accurate means of determining relative molecular sizes of proteins with molecular weights as high as 300,000.

Granath and Flodin (1961) demonstrated an excellent correlation between the molecular weight of a series of dextrans and their gel filtration elution pattern. Further investigations by Whitaker (1963) and Andrews (1964, 1965) showed that gel filtration could also be used for the estimation of molecular weights of proteins; however, more recent studies (Ackers, 1964; Siegel and Monty, 1966) indicate that the elution volume of a protein is a function of its molecular size expressed as the Stokes (molecular) radius.

We have used the gel filtration technique to survey malate dehydrogenases in both purified crystalline preparations and in crude cell-free extracts. Our survey indicates that the malate dehydrogenases of most animals, plants, and microorganisms are of equal molecular size, but in certain bacteria these enzymes are significantly larger.

Materials and Methods

Sephadex G-100, G-150, and G-200 and Dextran Blue were purchased from Pharmacia Fine Chemicals; horse heart cytochrome *c* from the Sigma Biochemical Co.; oxaloacetic and L-malic acids from Nutritional Biochemicals Corp.; nitro blue tetrazolium and phenazine methosulfate from Mann Research Laboratories; chicken ovalbumin from Pentex Inc.; DPNH[1] from

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[1] Abbreviations used: DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; MDH, malate dehydrogenase.

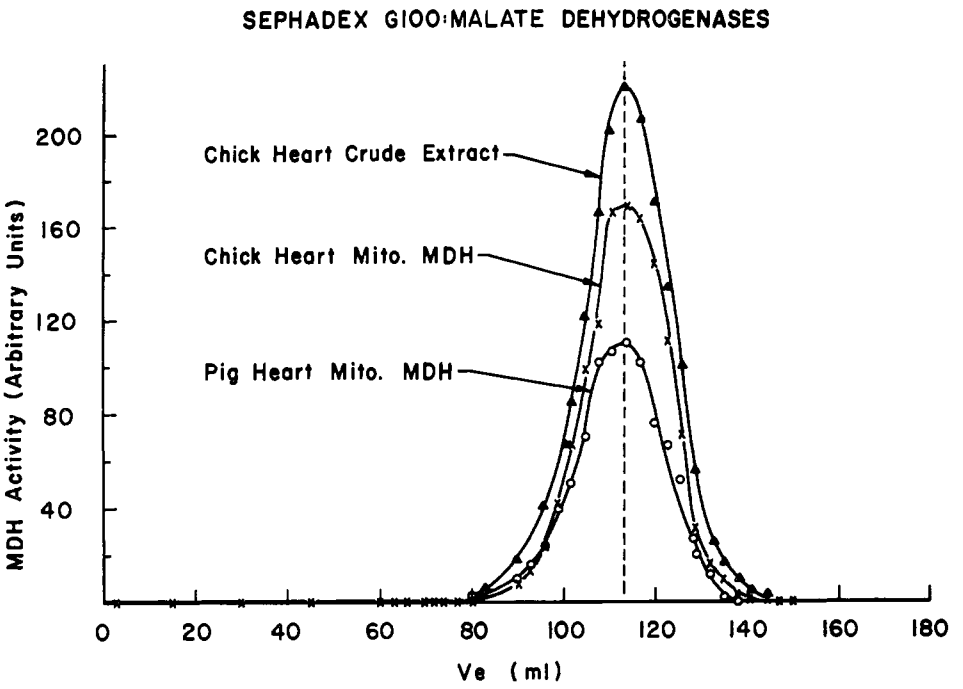

FIGURE 1: Gel filtrations of crystalline chicken heart mitochondrial and pig heart mitochondrial malate dehydrogenases, and a crude chicken heart extract on a Sephadex G-100 column (2.8 × 50 cm).

P-L Biochemicals Inc.; ribonuclease and bovine serum albumin from Armour Pharmaceutical Co.; hydrolyzed starch from Connaught Laboratories; and guanidine·HCl from Eastman Kodak. The crystalline malate dehydrogenases from chicken, pig, tuna, and ostrich hearts were prepared in this laboratory by methods to be presented elsewhere. Bacterial cultures were obtained from the Brandeis University Department of Biochemistry stock culture collection or from the American Type Culture Collection. All chemicals were of reagent grade.

Crude animal and plant extracts were prepared from frozen tissues. The tissue was homogenized in hypotonic (0.01 M potassium phosphate, pH 7.5) medium in a tissue grinder, frozen, and thawed several times, and the cellular debris was removed by centrifugation at 50,000*g* for 20 min. This supernatant liquid was used as the source of the enzymes. This method was found to be adequate for the solubilization of both supernatant and mitochondrial forms of malate dehydrogenase. In some cases, the enzymes were concentrated by ammonium sulfate precipitation.

Bacterial extracts were prepared from cells grown on Davis and Mingioli's (1950) minimal, synthetic medium with glucose, malate, or citrate as the sole carbon source or in Difco's Brain Heart Infusion broth. Growth factors were added only when necessary. The bacteria were harvested by centrifugation and sonicated in 0.1 M potassium phosphate buffer, pH 7.5, for 2–15 min. The sonicates were centrifuged for 20 min at 50,000*g* and this high-speed supernatant was used as the source of the enzyme.

Starch gel electrophoreses were run at pH 7.0 with potassium phosphate–citrate buffer, as described by Fine and Costello (1963) and stained specifically for malate dehydrogenase as described by Thorne *et al.* (1963).

Malate dehydrogenase activity was assayed by following the initial rate of oxidation of DPNH spectrophotometrically in a 3-ml cuvet with a 1-cm light path. The cuvet contained 0.03 ml of DPNH (10 mg/ml), 0.05 ml of neutralized oxaloacetate (13 mg/5 ml), enzyme, and sufficient potassium phosphate buffer (0.1 M, pH 7.5) to make a total volume of 3.0 ml. One enzyme unit is defined as the amount of enzyme required to cause a change in absorbance at 340 mμ of 1.00/min.

Sephadex gel filtration columns (of various diameters but 50 cm in height) were prepared and washed, and the samples were applied and eluted in 0.05 M Tris–0.1 M KCl, pH 7.5, as described by Andrews (1964). Aliquots, usually of 3.0 ml or less, were collected in a Gibson fraction collector. The columns were calibrated with crystalline proteins. A standard curve for each column was prepared by plotting the logarithms of published molecular weights of the proteins against the elution volumes of the proteins measured either by enzymatic activity or absorbance at characteristic wavelengths.

Dissociation of malate dehydrogenases was carried

TABLE I: Malate Dehydrogenases of Low Molecular Weight (60,000) by Gel Filtration.

Crystalline Enzymes	Partially Purified Enzymes and Crude Extracts
Pig heart mitochondrial MDH	Beef liver
Pig heart supernatant MDH	Hedgehog (*Erinaceus europenas*) heart
Chicken heart mitochondrial MDH	Garter snake (*Thamnophis sirtalis sirtalis*) heart
Chicken heart supernatant MDH	Bullfrog (*Rana catesbiana*) heart
Ostrich heart supernatant MDH	Horseshoe crab (*Limulus polyphemus*) heart
Tuna heart mitochondrial MDH	Oak silkworm (*Antherea perneyi*)
Tuna heart supernatant MDH	Honey bee (*Apis mellifera*)
P. shermanii[a] MDH	Periwinkle (*Littorina littorea*)
N. crassa[b] MDH	Cellular slime mold (*Polysphondylium pallidum*)
E. coli MDH	*Euglena gracilus*
	Saccharomyces cerevisiae
	Neurospora crassa
	Potato (*Solanum tuberosum*)
	Milkweed (*Ascelpias syriaca*)
	Cauliflower (*Brassica oleracea* var. *botrytis*)

[a] Gift from Dr. H. G. Wood (see Allen *et al.*, 1964). [b] Gift from Dr. K. Munkres (see Munkres and Richards, 1965).

TABLE II: Malate Dehydrogenases of Low Molecular Weight (60,000) by Gel Filtration.

Bacterial Extracts		
Family	Species	ATCC No.
Pseudomonadaceae	*Rhodopseudomonas palustris*	*a*
	Xanthomonas pruni	10017
Spirillaceae	*Spirillum serpens*	11330
Rhizobiaceae	*Chromobacterium violaceum*	12472
Achromobacteriaceae	*Alcaligenes faecalis*	8750
Enterobacteriaceae	*Escherichia coli* B	*a*
	Escherichia freundii	8090
	Aerobacter aerogenes	884
	Erwinia carotovora	495
	Proteus vulgaris	8427
Micrococcaceae	*Micrococcus lysodeikticus*	4698
Brevibacteriaceae	*Brevibacterium vitarumen*	10234
	Brevibacterium acetylicum	953
	Arthrobacter tumescens	6947
Corynebacteriaceae	*Cellulomonas biazotea*	486
	Microbacterium lacticum	8180
Actinomycetaceae	*Streptomyces coelicolor*	*a*

[a] Brandeis University, Department of Biochemistry, Stock Culture Collection.

out by diluting the enzyme (600 or more units/ml) (1:10) into 0.1 M β-mercaptoethanol containing 7.6 M guanidine·HCl, 8 M urea, or 0.1 M citric acid. (The guanidine·HCl and urea were previously recrystallized from methanol.) Reactivation was carried out by (a) dilution of the dissociated enzyme (1:50) into 0.1 M β-mercaptoethanol containing 0.1 M sodium citrate, pH 7.0, or (b) dilution (1:30) into the malate dehydrogenase assay mixture described above.

Results

Gel Filtration Studies. When the purified, crystalline malate dehydrogenases of pig or chicken heart mitochondria are filtered through a Sephadex G-100 column, the enzymatic activity of each has the same elution volume. The malate dehydrogenase activity in a sample of crude chicken heart extract gives an identical elution pattern (Figure 1) even though the specific protein cannot be detected by its absorbance at 280 mμ. Similar results are obtained with malate dehydrogenase purified from other animal sources. Thus, it is not necessary to obtain the enzymes in pure form before examining their gel filtration properties.

We have used this technique to survey the malate dehydrogenases from a number of animal, plant, and microbial sources. The elution volume from a Sephadex G-100 column of the malate dehydrogenase activity of each animal, plant, and microbial source listed in Tables I and II is identical, indicating that differences, if any, in size and/or shape between these proteins are relatively small.

Until certain Gram-positive bacteria were included in the survey, it appeared that the molecular size of the DPN-linked malate dehydrogenases had remained constant throughout evolution; but extracts of some of these bacteria contained malate dehydrogenase

TABLE III: Malate Dehydrogenases of High Molecular Weight (117,000) by Gel Filtration.

Species	ATCC No.	Species	ATCC No.
Bacillus subtilis	(168w)[a]	*Bacillus pantothenticus*	14576
Bacillus cereus	2[a]	*Bacillus lentus*	10840
Bacillus licheniformis	243[a]	*Bacillus firmus*	14575
Bacillus pumilus	7061	*Bacillus badius*	14574
Bacillus megaterium	4531	*Bacillus pulvifaciens*	13537
Bacillus stearothermophilus	2184[a]	*Bacillus sphaericus*	14577
Bacillus laterosporus	64	*Bacillus polymyxa*	842
Bacillus pasteurii	11859	*Bacillus coagulans*	7050
Bacillus brevis	10068	*Bacillus subtilis* (nonsporogenic)	168S
Brevibacterium linens	8377	*Sarcina ureae*	13881
Corynebacterium tritici	11402	*Sarcina lutea*	272
Corynebacterium flaccumfasciens	68867	*Pediococcus cerevisiae*	8081
Butyribacterium rettgeri	10825	*Neisseria subflava*	11076

[a] Brandeis University, Department of Biochemistry, stock culture collection.

TABLE IV: Physical Properties of Malate Dehydrogenases.

MDH Source	$s^0_{20,w}$ ($\times 10^{-13}$)	$D_{20,w}$[a] ($\times 10^7$)	Mol Wt[b]	$\bar{v}$[c]	Reference
Beef heart supernatant	5.1	9.1 (FD)	52,000 ($s:D$)	0.74 (A)	Englard and Breiger (1962)
Beef heart acetone powder	4.3	6.45 (FD)	62,000 ($s:D$)	0.74 (A)	Siegel and Englard (1961)
Ox heart mitochondria	4.44	—	—	—	Thorne (1962)
Horse heart mitochondria	4.36	—	—	—	Thorne (1962)
Beef heart mitochondria	4.6	6.6 (NR)	65,000 ($s:D$)	0.75 (A)	Grimm and Doherty (1961)
Pig heart mitochondria	3.6	8.47 (FD)	40,000 ($s:D$)	0.74 (A)	Wolfe and Neilands (1956)
Pig heart mitochondria	4.32	4.9–5.5 (E)	68–70,000 (E)	0.74 (Am)	Thorne and Kaplan (1963)
Neurospora crassa mitochondria	4.77	8.30 (NR)	54,000 (A)	0.739 (Am)	Munkres and Richards (1965)
Chicken heart mitochondria	4.3	5.44 (E)	67,000 (E)	0.74 (A)	Kitto (1966)
Chicken heart supernatant	4.27	—	66,500 (Y)	0.74 (A)	Kitto (1966)
Escherichia coli	4.4	—	62,500 (Y)	0.74 (A)	Murphey *et al.* (in prepn)
Bacillus subtilis	6.7	5.5–5.7 (E)	117,000 (Y)	0.74 (A)	Murphey *et al.* (in prepn)
Bacillus subtilis	—	—	146,000 (Y)	0.74 (Am)	Yoshida (1965a)
Bacillus stearothermophilus	—	—	118,000 (Y)	0.74 (A)	Murphey *et al.* (in prepn)

[a] Diffusion coefficient determined by: FD = free diffusion; E = Ehrenberg (1957); NR = not reported. [b] Molecular weights determined by: $s:D$ = Svedberg equation; E = Ehrenberg (1957); A = Archibald (1947); Y = Yphantis (1964). [c] Partial specific volume ($\bar{v}$): A = assumed; Am = calculated from amino acid analysis.

activity with a significantly smaller elution volume. This smaller elution volume would indicate a larger molecular size. The bacterial species examined and found to have this larger malate dehydrogenase are listed in Table III. Similar differences in elution patterns were obtained using Sephadex G-150 and G-200 gel filtration columns.

If the Sephadex column is calibrated with proteins of known molecular weight according to Andrews (1964), the elution volumes of these enzymes would correspond to molecular weights of 67,000 and 115,000 (Figure 2). The former value is in agreement with molecular weights obtained by ultracentrifugation studies on the crystalline malate dehydrogenases of pig heart (Thorne, 1962; Thorne and Kaplan, 1963) and with other investigators of the enzymes from higher animals (see Table IV). It should be pointed out, however, that the calibration of the gel filtration

columns according to molecular weight is an empirical method. Siegel and Monty (1966) have also recently discussed an empirical calibration of the columns according to the Stokes (molecular) radius of the protein standards. Whatever physical parameters may be involved in separation of proteins by gel filtration, the data provided in Figure 2 demonstrate that the smaller form of malate dehydrogenase can be readily separated from proteins with molecular weights of 87,000 (creatine kinase and alcohol dehydrogenase) and of 44,000 (ovalbumin).

Figure 3 demonstrates that an artificial mixture of the two forms of malate dehydrogenases in crude cell-free extracts can be resolved by Sephadex G-100 columns. Further evidence for separation is provided by the difference in catalytic properties between these two particular enzymes. Each enzyme exhibits substrate inhibition, but the enzyme from *Bacillus subtilis* has a higher substrate inhibition ratio ($DPNH_{(L)}$:$DPNH_{(H)}$ 4.0) than the enzyme from *Polyspondyllium pallidum* (1.4). Ratios of 3.9 and 1.3 were obtained in fractions from the leading and trailing edges of the peak, respectively.

Starch Gel Electrophoresis Studies. The partially purified enzymes and the crude extracts listed in Table I were also examined by starch gel electrophoresis. Multiple electrophoretic forms of malate dehydrogenase were observed in each case, but no evidence of multiple molecular weight species was obtained in the gel filtration studies. The supernatant and mitochondrial malate dehydrogenases of pig, chicken, and ostrich have been obtained in pure form in this laboratory and have identical molecular weights by ultracentrifugation studies.

Each of the crude cell-free extracts of bacteria listed in Tables II and III were examined by starch gel electrophoresis. Only one electrophoretic form was observed in each case. Growth of these bacteria on a complex medium (brain heart infusion broth) or on a synthetic medium with glucose, citrate, or malate as the sole carbon source yielded slightly different amounts of malate dehydrogenase, but did not alter the gel filtration or electrophoretic properties of the enzyme.

Ultracentrifuge Studies on Purified Enzymes. In order to study the enzymes by more classical techniques and to see if the larger form of the enzyme was either an artifact of the Sephadex column or due to the association of the enzyme with some other component of the crude cell extract, we purified the enzymes of *B. subtilis* and *Bacillus stearothermophilus*. The enzyme from *Escherichia coli* was also purified as a representative of the microbial enzymes of smaller molecular size. (Procedures of purification are being published elsewhere.) Repeated crystallizations of the *B. subtilis* and *E. coli* enzymes were required to achieve maximal specific activities; however, we did not recover sufficient enzyme from the *B. stearothermophilus* preparation to permit crystallization of this protein. The maximal specific activities of the *B. subtilis*, *B. stearothermophilus*, and *E. coli* malate dehydrogenases obtained under

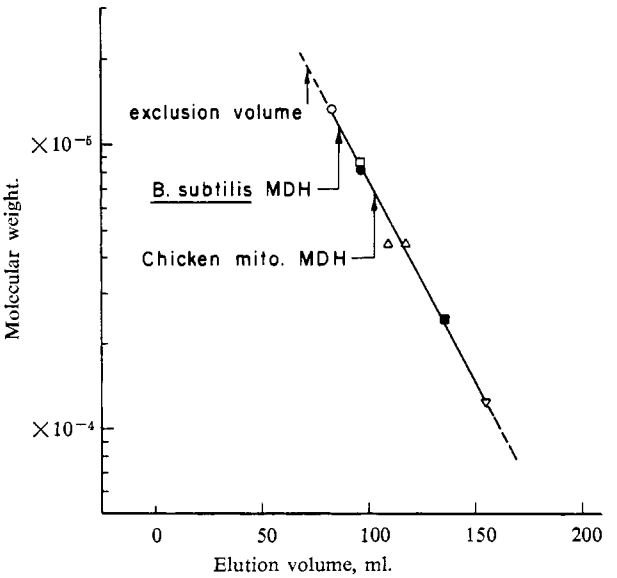

FIGURE 2: Calibration of Sephadex G-100 column (2.2 × 53 cm) according to Andrews (1964). Dextran Blue (arrow), beef heart (H_4) lactate dehydrogenase (○), chicken muscle creatine kinase (●), horse liver alcohol dehydrogenase (□), ovalbumin (△), soybean trypsin inhibitor (■), and cytochrome *c* (▽).

our usual conditions of assay were 640, 122, and 542 μmoles of DPNH oxidized/min per mg of protein, respectively. Each of the enzymes was homogeneous in the ultracentrifuge. Following electrophoresis at pH 7.0, each yielded single, coincident spots on slices of starch gel stained for protein and malate dehydrogenase activity. The elution volume of each enzyme was checked several times with calibrated Sephadex G-100 columns and did not change throughout the purification procedures.

The data we have obtained from the ultracentrifuge studies on these and other malate dehydrogenases (to be reported in detail elsewhere) indicate that the enzymes with the larger elution volumes from Sephadex G-100 columns have a weight-average molecular weight of 62,000 and the enzymes with the smaller elution volumes are not artifacts of the preparation or of the gel filtration process, but have molecular weights of 117,000 (see Table IV).

Reversible Dissociation of Purified Malate Dehydrogenases. Since the larger molecular form of malate dehydrogenase appeared to be twice the size of the smaller, we examined the possibility that the larger form was composed of subunits. We found that both light and heavy forms of the enzyme can be dissociated into subunits, either by acid or treatment with high concentrations of urea or guanidine hydrochloride. The *B. subtilis* enzyme has an $s^0_{20,w}$ of 6.7 S in the native state. It is converted by dialysis against 0.1 M β-mercaptoethanol at pH 2.0 into material which is enzymatically inactive and moves as a single peak in

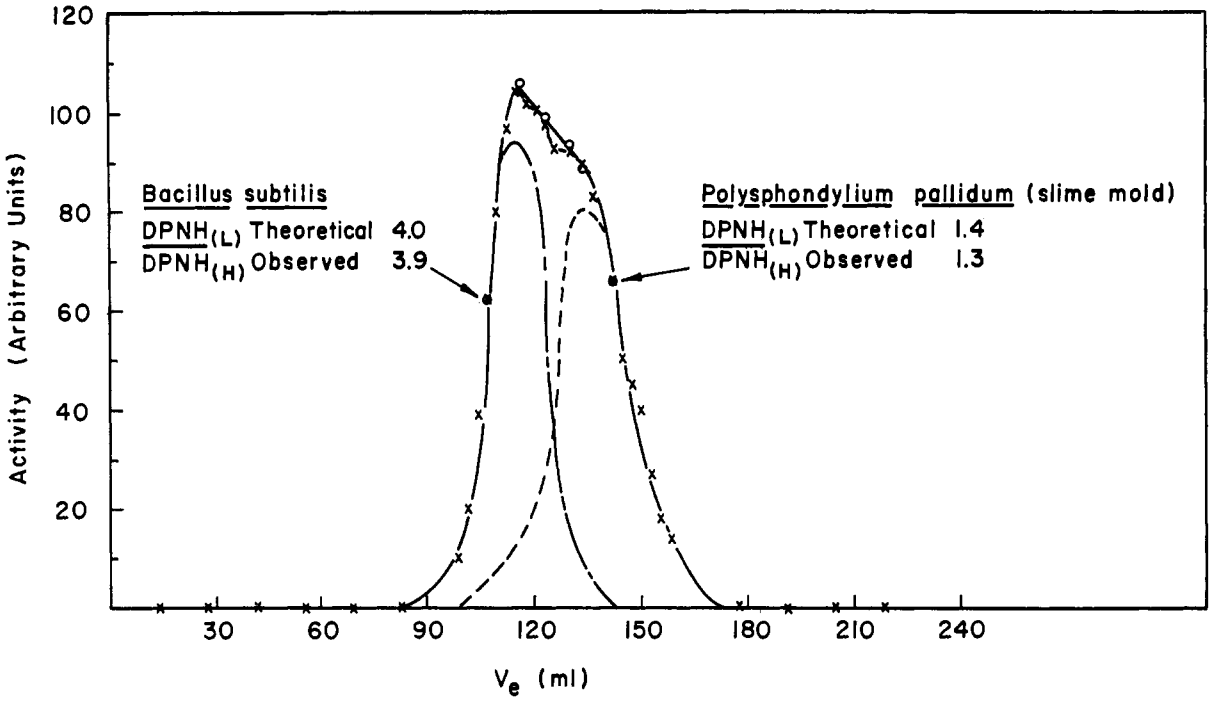

FIGURE 3: Sephadex G-100 gel filtrations of malate dehydrogenases in crude extracts of *B. subtilis* (———), *P. pallidum* (– – – –), and a 1:1 mixture of the two extracts (experimental points, ×——×; calculated points, O——O). Substrate inhibition ratios ($DPNH_{(L)}$:$DPNH_{(H)}$) are the relative initial rates of DPNH oxidation at low (3×10^{-4} M) and high (1×10^{-2} M) oxalacetate concentrations.

the ultracentrifuge with a sedimentation coefficient of 1.77 S.

Similarly, at pH 2.0 and below, the light forms of malate dehydrogenases ($s^0_{20,w}$ = 4.3 S) can be converted into material with a sedimentation coefficient of 1.79 S which is devoid of malate dehydrogenase activity. Upon dialysis against 0.1 M sodium citrate at pH 2.8 in the presence of 0.1 M β-mercaptoethanol, two peaks were observed with sedimentation coefficients of 4.0 and 1.79, corresponding to native and dissociated protein, respectively. Upon standing, the native protein was slowly converted into the form with the lower sedimentation coefficient.

Dialysis or dilution of either of the acid-treated enzymes into 0.1 M β-mercaptoethanol buffered with 0.1 M sodium citrate, pH 7.0, lead to partial reactivation of enzymatic activity. In a similar manner, enzymatic activity can be regained by dilution of guanidine hydrochloride or urea-treated high and low molecular weight enzymes. The best yields obtained have been of the order of 75%, suggesting that the process is completely reversible (Table V). The reconstituted low molecular weight enzymes appear to be identical with the native proteins in molecular weight and catalytic and immunological properties (Chilson *et al.*, 1965, 1966); further studies on the reversible dissociation of the high molecular enzymes will be reported elsewhere.

TABLE V: Reversible Dissociation of Malate Dehydrogenases by Guanidine·HCl.[a]

Source of Enzyme	% Reactivation after 5 hr
B. subtilis	31
B. stearothermophilus	75
B. subtilis + *B. stearothermophilus*[b]	58

[a] Dissociation of purified enzymes as described in Methods. Reactivation by dilution into sodium citrate as described in Methods. [b] Dissociated enzymes mixed (1:1) before reactivation.

Discussion

This survey by gel filtration has shown that the malate dehydrogenases from a wide variety of sources belong to one of two groups based on their molecular size. Within the experimental limits of the technique, no differences can be detected among the enzymes in any one group. The gel filtration properties of the enzymes in any group appear to be independent of heterologous protein concentrations since the elution

volumes of the enzymatic activities in crude cell extracts are identical with the elution volumes of the purified crystalline enzymes.

Weight-average molecular weights of malate dehydrogenases published since 1956 range from 40,000 to 70,000; the various physical parameters reported are tabulated in Table IV. It is evident that there is general agreement on sedimentation coefficients of these proteins at infinite dilution, but significant differences exist in the diffusion coefficients and/or the $s^0:D$ ratios used in calculating the molecular weights by the Svedberg equation. The present study suggests that these differences probably reflect either the methods used in the determinations or microheterogeneities in the size distributions of the protein preparations.

Evidence obtained from hybridization studies, peptide maps of tryptic digests, and coenzyme-binding studies suggest that the lower molecular weight malate dehydrogenases are composed of two identical subunits or possibly two pairs of nonidentical subunits (Kitto, 1966). The reversible dissociation of both forms of malate dehydrogenases by guanidine, urea, and acid as reported here and by others (Munkres, 1965a,b; Yoshida, 1965a,b; Chilson *et al.*, 1965, 1966) suggest that the larger forms of the enzyme could be stable tetrameric aggregations of the same fundamental structure found in the dimeric forms of the enzyme.

Multiple molecular weight forms of catalytically active enzymes are not hitherto unknown, *e.g.*, glutamate dehydrogenase (Fisher *et al.*, 1962), alcohol dehydrogenases, nucleases (Chesbro *et al.*, 1966), and hyaluronate lyase (Abramson and Friedman, 1966). The ease and precision of gel filtration plus the advantage of being able to deal with enzymes in crude extracts should facilitate studies of such variation in molecular size of other enzymes.

Such studies may be useful in determining phylogenetic relationships, particularly among species of microorganisms. Our finding that each of the 17 *Bacillus* species examined possesses the larger form of malate dehydrogenase leads us to predict the presence of this form in all *Bacillus* species, but obviously this form of the enzyme is not restricted to members of this genus.

Thus far, we have found no animal, plant, or microbial source with both high and low molecular weight forms of malate dehydrogenase. We also have found no evidence for the existence of a form with an intermediate molecular weight.

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The Reactivity of Thiol-subtilisin, an Enzyme Containing a Synthetic Functional Group*

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ABSTRACT: A synthetic enzyme was prepared by transforming the reactive serine residue in the active site of subtilisin to a cysteine residue. The transformation was accomplished nearly quantitatively by treating the serine residue with phenylmethanesulfonyl fluoride (PMSF), displacing the PMS group with thiolacetate ion, and allowing the acetyl-thiol-subtilisin to deacylate enzymatically. Titration with *p*-mercuribenzoate (PMB) and amino acid analysis indicated the presence of thiol groups and cysteine residues, respectively. Thiol-subtilisin catalyzes the hydrolysis of *p*-nitrophenyl acetate. This reaction, which is inhibited by *p*-mercuribenzoate, is characterized by a presteady-state burst of *p*-nitrophenol followed by a zero-order, steady-state release of *p*-nitrophenol. The steady-state reaction obeys Michaelis–Menten kinetics. Comparison of *p*-nitrophenyl acetate hydrolyses catalyzed by subtilisin and thiol-subtilisin indicates identical k_{cat}/K_m, differing k_{cat} and K_m, similar pK values of k_{cat}, and different pK values of k_{cat}/K_m. The decinnamoylation of subtilisin and thiol-subtilisin derivatives shows similar pK values but the former is much faster than the latter. *trans*-Cinnamoyl-thiol-subtilisin, however, is much more sensitive toward nucleophiles like glycinamide than is the counterpart of the native enzyme. The hydrolyses of acylamino acid esters and amides are not sensibly catalyzed by thiol-subtilisin. Thiol-subtilisin catalyzes certain hydrolytic reactions by a mechanism similar to that of native subtilisin with the exception that the acyl-enzyme is a thiol ester.

The reactions catalyzed by hydrolytic enzymes usually take place through the formation of an intermediate acyl-enzyme. In the most studied hydrolytic enzymes, the hydroxyl group of a serine or the thiol group of a cysteine residue forms an intermediary covalent bond with the substrate. Since these two amino acid residues differ only in an oxygen or sulfur atom of the side chain, it is an interesting problem in enzymology to transform the two types of hydrolytic enzymes into one another. Such a transformation might contribute to a better understanding of the mechanism if the artificial enzyme retains activity.

To perform the transformation, one should find a reaction taking place specifically at the active site of the enzyme. Specific reactions with a complex molecule like a protein are not always feasible but have been demonstrated in a few instances (*e.g.*, Balls and Jansen, 1952; Schoellmann and Shaw, 1963; Lawson and Schramm, 1965).

Recently investigations on small peptides demonstrated the conversion of a serine to a cysteine residue with retention of optical activity (Photaki and Bardakos, 1965; Zioudrou *et al.*, 1965). The reaction was performed in three steps: (1) the reaction of the serine residue with tosyl chloride; (2) the displacement of the sulfonyl compound by thiol acetate ion; (3) the hydrolysis of the thiol ester formed in step 2. This reaction series may be applied to an enzyme if it contains no disulfide bridges, to avoid damaging reactions with thiolacetate through disulfide interchange. Such a hydrolytic enzyme is subtilisin. A preliminary report on the transformation of subtilisin to thiol-subtilisin was previously published (Polgar and Bender, 1966). Here we report some of the characteristics of this synthetic enzyme.

Experimental Section

Subtilisin, Bacterial Proteinase Novo, was purchased from the Novo Pharmaceutical Co., and Nagarse from Teikoku Chemical Industry Co., Ltd. *p*-Nitrophenyl acetate was recrystallized from alcohol–water and melted at 114.5°. *N-trans*-Cinnamoylimidazole, a product of the Aldrich Chemical Co., Inc., was recrystallized four times from dry hexane, mp 134.0°.

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