Mannitol-1-phosphate dehydrogenase of *Escherichia coli*

Chemical properties and binding of substrates

Theodore CHASE, JR.

Department of Biochemistry and Microbiology, Cook College–New Jersey Agricultural Experiment Station, Rutgers–The State University, New Brunswick, NJ 08903, U.S.A.

Mannitol-1-phosphate dehydrogenase was purified to homogeneity, and some chemical and physical properties were examined. The isoelectric point is 4.19. Amino acid analysis and polyacrylamide-gel electrophoresis in presence of SDS indicate a subunit M_r of about 22000, whereas gel filtration and electrophoresis of the native enzyme indicate an M_r of 45000. Thus the enzyme is a dimer. Amino acid analysis showed cysteine, tyrosine, histidine and tryptophan to be present in low quantities, one, three, four and four residues per subunit respectively. The zinc content is not significant to activity. The enzyme is inactivated (> 99%) by reaction of 5,5'-dithiobis-(2-nitrobenzoate) with the single thiol group; the inactivation rate depends hyperbolically on reagent concentration, indicating non-covalent binding of the reagent before covalent modification. The pH-dependence indicated a pK_a greater than 10.5 for the thiol group. Coenzymes (NAD⁺ and NADH) at saturating concentrations protect completely against reaction with 5,5'-dithiobis-(2-nitrobenzoate), and substrates (mannitol 1-phosphate, fructose 6-phosphate) protect strongly but not completely. These results suggest that the thiol group is near the catalytic site, and indicate that substrates as well as coenzymes bind to free enzyme. Dissociation constants were determined from these protective effects: $0.6 \pm 0.1 \mu\text{M}$ for NADH, $0.2 \pm 0.03 \text{ mM}$ for NAD⁺, $9 \pm 3 \mu\text{M}$ for mannitol 1-phosphate, $0.06 \pm 0.03 \text{ mM}$ for fructose 6-phosphate. The binding order for reaction thus may be random for mannitol 1-phosphate oxidation, though ordered for fructose 6-phosphate reduction. Coenzyme and substrate binding in the E·NADH–mannitol 1-phosphate complex is weaker than in the binary complexes, though in the E·NADH⁺–fructose 6-phosphate complex binding is stronger.

INTRODUCTION

Mannitol-1-phosphate dehydrogenase (D-mannitol 1-phosphate:NAD⁺ oxidoreductase, EC 1.1.1.17) was first reported from *Escherichia coli* by Wolff & Kaplan (1956a,b), and has also been purified from *Aerobacter aerogenes* (Liss *et al.*, 1962), *Bacillus subtilis* (Horwitz & Kaplan, 1964) and *Streptococcus mutans* (Brown & Banks, 1977), as well as *Aspergillus niger* (Niehaus & Kiser, 1981) and reported from other fungi (Ueng *et al.*, 1976; Hult *et al.*, 1980), where it participates in the mannitol cycle for NADPH regeneration. The enzyme catalyses the reversible oxidation of mannitol 1-phosphate to fructose 6-phosphate (mannitol being a symmetrical molecule, the primary phosphate is properly a 1-phosphate). The enzyme is distinct from sorbitol-6-phosphate dehydrogenase also found in enterobacteria (Wolff & Kaplan, 1956b; Liss *et al.*, 1962). The *Aero. aerogenes* and *E. coli* enzymes are B-specific in hydride transfer (do Nascimento & Davies, 1975; Alizalde *et al.*, 1976).

Klungsoyr (1966) has purified the *E. coli* enzyme, which he found to comprise up to 2% of the soluble protein of the organism when grown on mannitol, and has studied (Klungsoyr, 1967) the inhibition of the reaction by adenine nucleotides. He reported the enzyme to have an M_r (determined by analytical ultracentrifugation) of only 25000. Other known NAD-linked dehydrogenases generally have subunit M_r values in the range 35000–40000, though *Neurospora malate*

dehydrogenase has been reported to have subunits of M_r 13 500 (Munkres, 1965). Furthermore, they are almost always dimers or tetramers, and some observations have suggested that only one-half the bound substrate and coenzyme may be converted into products at once (Harada & Wolfe, 1968; Bernhard *et al.*, 1970). If mannitol-1-phosphate dehydrogenase is a monomer of M_r 25000, the essential features of nicotinamide nucleotide-linked oxidation and reduction could be studied without interference from site-site interactions; if it is a dimer of monomer M_r 12 500, it would be even more attractively simple for studies of the participation of individual residues in catalysis. A small bacterial enzyme is particularly attractive for study of the effects of biosynthetically incorporated amino acid analogues (Browne *et al.*, 1970; Chase, 1972).

The present paper reports studies on the chemical properties of the enzyme, and on the binding of substrates to it, as measured by protection against reaction with DTNB. Preliminary versions of some aspects have been reported (Chase & Lucek, 1974).

MATERIALS AND METHODS

Materials

Bovine serum albumin (fraction V), β -lactoglobulin, Coomassie Blue G, (bovine) haemoglobin, NAD⁺ (grade III), NADH, 2-amino-2-methylpropane-1,3-diol, piperazine hexahydrate, phenazine methosulphate, *p*-

Abbreviation used: DTNB, 5,5'-dithiobis-(2-nitrobenzoate).

Nitrotetrazolium Blue, $\alpha\alpha'$ -bipyridyl, 1,10-phenanthroline monohydrate, protamine sulphate and sodium diethyl-dithiocarbamate were obtained from Sigma Chemical Co. Tris and DTNB were products of Calbiochem. $(\text{NH}_4)_2\text{SO}_4$ (special enzyme grade), cytochrome *c* and ovalbumin were purchased from Mann Research Laboratories (now Schwarz-Mann); '*m*-' (4,7-)phenanthroline was purchased from K & K Chemicals. Chymotrypsinogen, chymotrypsin, deoxyribonuclease and ribonuclease A were obtained from Worthington Biochemical Corp. Glycyl-L-tryptophan and 3-(2-aminoethyl)indole hydrochloride were purchased from Nutritional Biochemical Co.; the latter was converted into the free base as described by Liu & Chang (1971). Ninhydrin and methanesulphonic acid [4 M solution containing 0.2% 3-(2-aminoethyl)indole, packaged under N_2] were obtained from Pierce Chemical Co. Sephadex G-75 and QAE- (quaternary aminoethyl-)Sephadex A-50 were products of Pharmacia Fine Chemicals, and microgranular DEAE-cellulose DE-52 was a Whatman product. Coomassie Blue R and Amido Black were obtained from Colab Laboratories. Other chemical were reagent grade. Distilled water was deionized by passage through a mixed-bed ion-exchange resin.

Mannitol 1-phosphate for routine assays was prepared from mannitol and POCl_3 as described by Klungsoyr (1966, 1967); for assay, the barium salt was converted into the sodium salt with Na_2SO_4 , BaSO_4 being removed by centrifugation. Mannitol 1-phosphate for binding studies was a product of Sigma Chemical Co. (prepared by reduction of mannose 6-phosphate).

Growth of organism

A tryptophan-requiring strain of *E. coli* (lacking tryptophan synthetase and tryptophanase; Browne *et al.*, 1970) was used as source of the enzyme, since it was desired eventually to study the effects of incorporation of tryptophan analogues into the enzyme (Chase, 1972). This strain (ΔAC9) was generously supplied by Dr G. D. Hegeman. Cells were grown in a New Brunswick Scientific Co. fermenter at 35 °C on the medium of Mayers & Spizizen (1954), with substitution of mannitol (6 g/l) for glucose and with supplementation with ferric ammonium citrate (1.14 mg/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.4 mg/l), L-tryptophan (20 mg/l) [or DL-tryptophan (40 mg/l)] and yeast extract (0.5 g/l). This strain required glycine, as well as tryptophan, for rapid growth. Cells were harvested with a Sharples continuous centrifuge and stored at -20 °C.

Assay of enzyme activity

The enzyme was assayed essentially as described by Klungsoyr (1966). The assay mixture contained 2-amino-2-methylpropane-1,3-diol/HCl buffer, pH 9.0 (0.05 M), NAD^+ (1.33 mM) and mannitol 1-phosphate (2.0 mM), and bovine serum albumin (0.1 mg/ml) for stability of the enzyme when it was very dilute. The reaction was initiated by addition of enzyme, and increase of A_{340} was monitored in either a Gilford recording spectrophotometer (assay volume 1.0 ml) or a Calbiometer photometer with Heath recorder (assay volume 2.0 ml). Both instruments were thermostatically maintained at 30 °C. The unit is formation of 1 μmol of NADH/min under these conditions, assuming $\epsilon_{340} = 6200 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Determination of protein

Protein concentrations were determined by the methods of Lowry *et al.* (1951) or Bradford (1976), standardized with bovine serum albumin (concentration determined by weight after drying in a vacuum oven). For absolute molar concentrations either amino acid analysis or the method of Klungsoyr (1969), based on Cu^{2+} binding to peptide bonds, was used.

Enzyme purification

Mannitol-1-phosphate dehydrogenase was purified by a modification of the procedure of Klungsoyr (1966), necessitated by the lower specific activity and larger volume of crude extracts. Cells were broken by ultrasonic oscillation, and cell debris was removed by centrifugation to yield crude extract. Nucleic acids were removed with MnCl_2 (0.05 M), and the pH was adjusted to 4.25 to precipitate other proteins. After centrifugation, the supernatant solution was re-adjusted to pH 7, and the enzyme was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (75% saturation) and further fractionated by extraction of the precipitate with 61%-saturated and 49%-saturated $(\text{NH}_4)_2\text{SO}_4$ solutions. Alternatively, other proteins were precipitated at 52.5% saturation in $(\text{NH}_4)_2\text{SO}_4$, and the enzyme was precipitated at 67.5% saturation. Precipitated enzyme was redissolved in a minimum volume of 0.03 M-imidazole/HCl buffer, pH 7.3, dialysed briefly to lower the ionic strength, and filtered upward through a column (5 cm $\times$ 89 cm) of Sephadex G-75 equilibrated with the same buffer. Active fractions were adsorbed directly on a column of QAE-Sephadex A-50, and the enzyme was eluted with a pH gradient (7.3–5.3) in 0.03 M-imidazole hydrochloride/0.04 M-piperazine dihydrochloride (adjusted to pH 3 with HCl and back to pH 5.3 and 7.3 with aq. NH_3 , to keep $[\text{Cl}^-]$ constant). In some purifications, the active fractions were diluted with an equal volume of water, the pH was adjusted to 7.3, and the enzyme was adsorbed on a small column of DEAE-cellulose DE-52 and eluted with an NH_4Cl gradient (0.03–0.20 M) in 0.03 M-imidazole/HCl buffer, pH 7.3. The enzyme is stable for months in solution at 4 °C, and for years as an $(\text{NH}_4)_2\text{SO}_4$ precipitate.

Enzyme characterization

Isoelectric focusing was carried out in an 110 ml apparatus according to the instructions of the manufacturer, LKB Produkter. Polyacrylamide-gel electrophoresis was carried out at 4 °C in the standard Davis (1964) system, with 7.5% polyacrylamide gels. Protein bands were located by staining with Coomassie Blue R [1% in 7% (v/v) acetic acid], Amido Black [1% in 15% (v/v) acetic acid] or Coomassie Blue G [0.04% in 3.5% (v/v) HClO_4]. Mannitol-1-phosphate dehydrogenase activity was located by incubating unfixed gels in the standard assay mix (without bovine serum albumin) supplemented with 0.018% phenazine methosulphate and 0.08% *p*-Nitrotetrazolium Blue (Schachter *et al.*, 1969) until satisfactory bands of formazan appeared (generally 2–3 min), then replacing the assay mixture with 7% acetic acid to fix the gels. Gel electrophoresis for determination of M_r (Hedrick & Smith, 1968) was carried out in 6%, 9% and 12% gels (acrylamide/*NN'*-methylenebisacrylamide ratio 30:0.8) in the Davis (1964) buffer system, with as standards β -lactoglobulin (M_r 17 500), deoxyribonuclease (M_r 31 000), ovalbumin (M_r 45 000) and haemo-

globin (M_r 64 500). Polyacrylamide-gel electrophoresis in the presence of SDS was carried out in the system of Weber & Osborn (1969), with as standards bovine serum albumin (M_r 68 000), ovalbumin, chymotrypsinogen (M_r 25 000) and chymotrypsin (reduced, M_r 12 000; non-reduced, M_r 25 000).

Determination of M_r by gel filtration (Andrews, 1964) was carried out on a column (2.5 cm $\times$ 89 cm) of Sephadex G-75 equilibrated with 0.05 M-imidazole/HCl buffer, pH 7.3. Standard proteins used were cytochrome *c* (M_r 12 500), ribonuclease A (M_r 13 700), β -lactoglobulin, chymotrypsinogen, ovalbumin and bovine serum albumin. The void volume was determined as the volume at which Blue Dextran or bovine serum albumin dimer was eluted.

Automatic amino acid analysis was carried out with a modified Phoenix P.I. model VG-6200B analyser or a Durrum Instruments model D-500 analyser. Protein samples were hydrolysed under vacuum at 110 °C after being flushed with N_2 , in 6 M-HCl/3 M-toluene-*p*-sulphonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu & Chang, 1971), in 4 M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole (Moore, 1975) or in $Ba(OH)_2$ (Pon *et al.*, 1970). Performic acid oxidation was as described by Hirs (1967). Thiol groups were determined with DTNB (Ellman, 1959) (0.24 mM in 0.1 M-Tris/HCl buffer, pH 8.25), readings being taken against a blank cuvette containing the reagent. Tryptophan was also determined colorimetrically with *p*-dimethylaminobenzaldehyde (Spies & Chambers, 1949) and ninhydrin (Gaitonde & Dovey, 1970), with glycyl-L-tryptophan as standard.

Zinc was determined by atomic absorption spectrometry. Glassware used in diluting the zinc standard and the sample had been stored in 50% (v/v) conc. HNO_3 . In attempting to remove zinc from the enzyme, samples precipitated with $(NH_4)_2SO_4$ were diluted with 0.025 M-piperazine/HCl buffer, pH 5.1; the concentrated buffer and the dialysis flasks had been extracted with diphenylthiocarbazon in chloroform to remove zinc and with chloroform to remove residual diphenylthiocarbazon.

The rate of reaction of the enzyme with DTNB was typically determined by preparing a sample of the enzyme (usually 0.27 μ M) in 0.05 M-Tris/HCl buffer, pH 8.55, total volume 0.108 or 0.109 ml. A 10 μ l portion was removed with an Eppendorf micropipette for

determination of zero-time activity. Then 1 μ l or 2 μ l of DTNB was added (to concentration 0.1, 0.2 or 2 mM, except when [DTNB] was being varied). The mixture was incubated at 30 °C, and at appropriate times after the addition of DTNB samples, usually 10 μ l (a total of five to seven), were removed and added to assay mixture for determination of activity remaining. The rate of inactivation was determined by a linear least-squares treatment of $\ln v$ or $\ln(\%$ of zero-time activity) versus time; rates of inactivation were then fitted to appropriate equations (see the Results section) for dependence on [DTNB] or concentration of competing ligand by non-linear least-squares procedures (Wilkinson, 1961; Fraser & Suzuki, 1973, modified for the equations in this paper by Dr. Peter C. Kahn of this department).

RESULTS

Purification of the enzyme

The progress of a purification is shown in Table 1. The highest specific activity obtained is comparable with that found by Klungsoyr (1966) (516 units/mg, after conversion from his unit). In polyacrylamide-gel electrophoresis, the major band of stained protein corresponded to the single band of activity detected by formazan precipitation; other minor bands, some apparently active by formazan precipitation, were sometimes seen. Material showing only one band of stained protein in gel electrophoresis was used for amino acid analysis and other chemical studies.

Isoelectric focusing in the pH range 3–6 separated the enzyme from minor contaminants; the pH of the three fractions of highest enzyme activity was 4.19, indicating the approximate isoelectric point. However, all ampholyte and sucrose could not be removed, so that amino acid analysis on the combined fractions was not carried out. Recovery of activity was 56% of that applied; the enzyme is at the verge of inactivation at this pH, and indeed some precipitation during the run was generally observed. Attempted preparative gel electrophoresis also resulted in poor recovery (24%) of activity applied, perhaps owing to the high pH (9.4) of the running gel (Jovin, 1973).

Amino acid analysis

The results of amino acid analysis of purified mannitol-1-phosphate dehydrogenase, showing only one

Table 1. Purification *E. coli* mannitol-1-phosphate dehydrogenase

For experimental details see the text.

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Crude extract	174	26.23	4564	129.2	22480	4.93	100
Acid supernatant	172	22.20	3822	129.2	22200	5.82	99
75% -satn.-(NH_4) ₂ SO ₄ precipitate	108	11.90	1285	121.5	13122	10.2	58
49% -satn.-(NH_4) ₂ SO ₄ supernatant	101	4.74	479	120.0	12120	25.3	54
80% -satn.-(NH_4) ₂ SO ₄ precipitate, dialysed	8.0	44.7	358	1263	10100	8.2	45
Sephadex G-75 eluate	35	1.6	56	248	8673	156	39
QAE-Sephadex eluate	14.4	0.67	9.7	338	4865	502	22

Table 2. Amino acid analysis of *E. coli* mannitol-1-phosphate dehydrogenase

Values are based on the assumption that there are 19 aspartic acid residues/molecule, and are given as means $\pm$ S.D.

Amino acid	Amino acid composition (residues/molecule)	
	Found	Integral value
Cysteine*	1.25 $\pm$ 0.14	1
Aspartic acid	(19)	19
Threonine	8.43 $\pm$ 0.57†	9
Serine	8.51 $\pm$ 0.81‡	9
Glutamic acid	20.77 $\pm$ 1.44	21
Proline	8.76 $\pm$ 1.32	9
Glycine	16.03 $\pm$ 1.53	16
Alanine	22.35 $\pm$ 1.12	22
Valine	16.81 $\pm$ 1.00	17
Methionine	3.85 $\pm$ 0.70	4
Isoleucine	10.85 $\pm$ 0.38	11
Leucine	17.69 $\pm$ 1.20	18
Phenylalanine	5.52 $\pm$ 0.42	6
Tyrosine	3.11 $\pm$ 0.43	3
Histidine	3.99 $\pm$ 0.82	4
Lysine	10.74 $\pm$ 0.38	11
Arginine†	8.85 $\pm$ 0.24‡	9
Tryptophan	4.02 $\pm$ 0.45	4
By <i>p</i> -dimethylamino- benzaldehyde	2.62 $\pm$ 0.11	
By ninhydrin	3.35 $\pm$ 0.05	
		Total 193

* As cysteic acid (not corrected for incomplete recovery; Hirs, 1967).

† Two peaks in some analyses (see the text). Includes determination as ornithine after Ba(OH)₂ hydrolysis.

‡ Extrapolated to zero time.

protein band in gel electrophoresis, are shown in Table 2. Included are data from analysis after: four standard 6 M-HCl hydrolyses (two 24 h, one 48 h, one 72 h); three hydrolyses in 3 M-toluene-*p*-sulphonic acid and one in 4 M-methanesulphonic acid, for tryptophan recovery; one Ba(OH)₂ hydrolysis, and some data from another; three 6 M-HCl hydrolyses (20 h) after performic acid oxidation, to convert cysteine into cysteic acid. All have been normalized on an assumption of 19 aspartic acid/asparagine residues per molecule, which gives good approaches to integral values for most of the relatively uncommon amino acids, and corresponds to a minimum M_r (21 040) close to that found by gel electrophoresis in the presence of SDS. Values for serine and threonine have been extrapolated to zero time of hydrolysis; arginine also seemed unstable, giving in some cases two peaks, corresponding to six and three residues, and recoveries have also been extrapolated to zero time. After Ba(OH)₂ hydrolysis, 8.5 residues of ornithine were found in a single peak. Values for valine, isoleucine and leucine are based on 48 h and 72 h hydrolyses. Tryptophan determination by reaction with ninhydrin (Gaitonde & Dovey, 1970) agreed reasonably well with determination by amino acid analysis, although a somewhat lower value was found with *p*-dimethylaminobenzaldehyde.

The most notable observation is that the enzyme contains only a single cysteine residue. Tyrosine and histidine contents are also low, facilitating investigations of the possible role of these residues in dehydrogenase catalysis.

Polyacrylamide-gel electrophoresis in the presence of SDS

A sample of purified mannitol-1-phosphate dehydrogenase was subjected to gel electrophoresis in the presence of 0.2% SDS. The mobility of the enzyme was unaffected by inclusion of 2-mercaptoethanol in the preincubation mixture, indicating that it does not contain chains held together by a disulphide bond (cf. chymotrypsin). The logarithm of the M_r values of the standard proteins was plotted versus distance (cm) migrated; the location of the mannitol-1-phosphate dehydrogenase band on this line indicated a protomer M_r of about 22 500, in reasonable agreement with the minimum M_r suggested by amino acid analysis and the value (25 000) reported by Klungsøyr (1966).

Determination of M_r of the native protein

The first method used was gel-filtration chromatography (Andrews, 1964), on a calibrated Sephadex G-75 column equilibrated with 0.03 M-imidazole/HCl buffer, pH 7.3. The M_r values found in three experiments were 48 300, 45 100 and 44 300, clearly consistent with a dimer of monomer M_r about 22 500, rather than a monomer. Essentially the same M_r was found if the buffer was 0.05 M-Tris/HCl buffer, pH 7.3, containing 0.05 M-NaCl and 1 mM-2-mercaptoethanol, as used by Klungsøyr (1966) in ultracentrifugation, or 0.05 M-sodium acetate buffer, pH 5.3, containing 0.05 M-NaCl.

Similarly, gel electrophoresis of the enzyme, along with standard proteins, at several polyacrylamide concentrations (Hedrick & Smith, 1968) gave an M_r value of 45 500.

Zinc content

Initial zinc assays of purified mannitol-1-phosphate dehydrogenase found 1.14 Zn atoms/monomer, suggesting the presence of catalytically important zinc, as in yeast and liver alcohol dehydrogenases (Theorell *et al.*, 1955; Drum *et al.*, 1967). However, dialysis against phosphate buffers, which inactivates liver alcohol dehydrogenase by removal of zinc at pH 5.0 (Drum *et al.*, 1967), did not inactivate mannitol-1-phosphate dehydrogenase significantly at pH values above 4.2, nor was zinc consistently removed even when the enzyme was inactivated. Incubation in 0.1 M-diethyldithiocarbamate, pH 7.0, which inactivates liver alcohol dehydrogenase completely in 24 h (Drum *et al.*, 1967), did inactivate the enzyme, by 95% in 100 h, but the inactivation was prevented by the presence of 0.14 M-2-mercaptoethanol. This suggests that diethyldithiocarbamate reacts with an enzyme thiol group rather than with Zn²⁺, 2-mercaptoethanol preventing inactivation by reducing any mixed disulphide formed with the reagent. $\alpha\alpha'$ -Bipyridyl and 1,10-phenanthroline, which bind tightly to the catalytic zinc of liver alcohol dehydrogenase (K_i values 0.4 mM and 0.008 mM; Sigman, 1967), are only very weak inhibitors of mannitol-1-phosphate dehydrogenase [K_i values, by the method of Dixon (1953), 11 $\pm$ 4 mM and 8 $\pm$ 4 mM respectively]. The non-chelating isomer 4,7-phenanthroline was almost equally inhibitory (K_i 12 $\pm$ 6 mM). Finally, zinc determinations on other

Fig. 1. Reaction of *E. coli* mannitol-1-phosphate dehydrogenase with DTNB

Enzyme (0.473 mg, by amino acid analysis) was incubated at 25 °C in 1.0 ml of 0.1 M-Tris/HCl buffer, pH 8.25. After addition of DTNB (0.24 mM), A_{412} was monitored, and at the indicated times samples (1 μ l) were removed and assayed for activity. A control incubation without DTNB showed no loss of activity over this period of time. ●, Activity remaining at time t , as percentage of initial activity; ○, the comparable value for A_{412} , $100 \times [1 - (A_{412} \text{ at time } t / \text{final } A_{412})]$; the 'final A_{412} ' is a theoretical value chosen to linearize the plot over the first 17 min (92% of inactivation), since actual A_{412} decreased after that time. ■, Percentage activity remaining after time t , under the same conditions (0.1 ml of incubation mixture) + 5.0 mM-NAD⁺; □, percentage activity remaining after time t , under the same conditions (0.1 ml of incubation mixture) + 1.0 mM-mannitol 1-phosphate.

preparations of mannitol-1-phosphate dehydrogenase gave values well below 1 Zn atom/subunit: typically 0.20–0.25 Zn atom/subunit, and as low as 0.10 Zn atom/subunit.

Reaction of the thiol group with DTNB

This reagent reacts relatively slowly with mannitol-1-phosphate dehydrogenase at pH 8.25; the rates of appearance of A_{412} (formation of 2-nitro-5-mercaptobenzoate dianion) and disappearance of enzymic activity could readily be monitored (Fig. 1), and are essentially identical ($0.231 \pm 0.006 \text{ min}^{-1}$ and $0.230 \pm 0.005 \text{ min}^{-1}$ respectively). In another experiment, with enzyme reduced with 1,4-dithiothreitol under N₂ and filtered through Sephadex G-25 to remove the reductant, the molarity of 2-nitro-5-mercaptobenzoate produced was

Fig. 2. Dependence on [DTNB] of rate of inactivation of *E. coli* mannitol-1-phosphate dehydrogenase

The reaction as carried out in 0.05 M-Tris/HCl buffer, pH 8.6, as described in the text, at various values of [DTNB].

81% of the subunit concentration indicated by protein determination, showing that the two active sites react independently with DTNB. A similar result was obtained with the less bulky 2-nitro-5-thiocyanatobenzoate (Degani & Patchornik, 1974). If only DTNB-reactive subunits are enzymically active, their catalytic-centre activity is 265 s^{-1} , or the specific activity is $702 \text{ } \mu\text{mol/min per mg}$.

The rate of inactivation depends hyperbolically on the DTNB concentration (Fig. 2), indicating that a non-covalent E·DTNB complex is formed before the covalent modification reaction. The observed rates were fitted to a version of the Michaelis–Menten equation:

$$b = \frac{k[\text{DTNB}]}{K_d + [\text{DTNB}]} \quad (1)$$

where b is the observed rate of inactivation, k is the limiting rate of inactivation at this pH, and K_d is the dissociation constant of the E·DTNB non-covalent complex. The fit gave $k = 0.723 \pm 0.034 \text{ min}^{-1}$ and $K_d = 0.0551 \pm 0.00765 \text{ mM}$.

The variation of the rate of inactivation with pH was studied over the range 7–11 (Fig. 3). The logarithm of the rate of inactivation increased linearly with pH up to about pH 10, then levelled off. The data from pH 8.75 to 11.0 were fitted to the equation:

$$b = \frac{k[\text{DTNB}]}{K_d \left(1 + \frac{[\text{L}]}{K_L} \right) + [\text{DTNB}]} \quad (2)$$

[the hyperbolic form of eqn. (7) of Chase & Shaw (1969)]; [L] is the concentration of some protecting ligand, and K_L is its dissociation constant from the enzyme. In this case L is H⁺, K_d is 0.0551 mM, and K_L is the dissociation constant of the reacting thiol group. In two experiments values of K_L found were $27.1 \pm 13.5 \text{ pM}$ and $13.1 \pm 4.5 \text{ pM}$ ($\text{p}K_L = 10.57$ and 10.88); but, considering the limited data in this pH region, it can only be asserted that the $\text{p}K_a$ is greater than pH 10.5. The constancy of K_d in the pH range 7–10.5 is indicated by the fit of the data to a single straight line (Fig. 3); a change in K_d , at a non-saturating [DTNB], would shift the line up or down as the rate changed with degree of saturation.

Fig. 3. Dependence on pH of rate of inactivation of *E. coli* mannitol-1-phosphate dehydrogenase

Rates were determined in ○, 0.05 M-Tris/HCl buffer, [DTNB] = 0.1 mM; ▲, 0.05 M-ethanolamine/HCl buffer, [DTNB] = 0.01 mM; □, 0.05 M-Na₂CO₃ buffer, [DTNB] = 0.002 mM. Rates at 0.01 mM- and 0.002 mM-DTNB were adjusted to those expected at 0.1 mM by using eqn. (1) (see the text). Rates at pH above 9.0 are corrected for DTNB-independent rate of loss of activity (0.36 min⁻¹ at pH 11.0).

Fig. 1 also shows that the presence of either NAD⁺ or mannitol 1-phosphate further lowered the rate of reaction with DTNB, indicating that both bind, independently, to free enzyme and protect the thiol group. NADH and fructose 6-phosphate act similarly.

The possibilities for reaction of DTNB with the enzyme in the presence of a ligand L are shown in the following scheme:

$$b = \frac{kK_L[\text{DTNB}]}{K_dK_L + K_d[\text{L}] + K_L[\text{DTNB}] + (K_d/K'_d)[\text{L}][\text{DTNB}]} + \frac{k'[\text{L}][\text{DTNB}]}{K'_dK_L + K'_d[\text{L}] + K_L(K'_d/K_d)[\text{DTNB}] + [\text{L}][\text{DTNB}]} \quad (4)$$

or the equivalent:

$$b = \frac{kK_L[\text{DTNB}]}{K_dK_L + K_d[\text{L}] + K_L[\text{DTNB}] + (K_L/K'_L)[\text{L}][\text{DTNB}]} + \frac{k'[\text{L}][\text{DTNB}]}{K_dK'_L + K_d(K'_L/K_L)[\text{L}] + K'_L[\text{DTNB}] + [\text{L}][\text{DTNB}]} \quad (5)$$

Fig. 4. Protection by mannitol 1-phosphate (□) and fructose 6-phosphate (○) against DTNB inactivation of *E. coli* mannitol-1-phosphate dehydrogenase

The points show experimentally determined rates of inactivation by DTNB (0.1 mM, in 0.05 M-Tris/HCl buffer, pH 8.5, at 30 °C), the curves the best fits to eqn. (4).

where TNB represents 2-nitro-5-mercaptobenzoate. Reaction may occur either from the binary complex E·DTNB, at rate k , or from the ternary complex E·L·DTNB, at rate k' . The observed rate of reaction b is a function of [DTNB], [L], k , k' and the dissociation constants; $K_dK'_L = K_d'K_L$, so that only three of the dissociation constants need be determined independently.

The observed rates of reaction (b) at various concentrations of the substrates were initially fitted to the equation:

$$b = \frac{kK_L + k'[\text{L}]}{K_L + [\text{L}]} \quad (3)$$

which is the equation for direct reaction of DTNB (at a fixed concentration) with the enzyme without a non-covalent intermediate E·DTNB complex. For L = NAD⁺, NADH (and H⁺) this gave values of $k' \leq 0$, indicating that the E·L·DTNB complex is not formed or does not react. The appropriate equation is then eqn. (2), and fits of the data for L = NAD⁺ gave values of $K_L = 0.18 \pm 0.03$ mM with two different enzyme preparations. Two experiments at enzyme concentrations 0.27 μM and 0.029 μM gave K_L for NADH = 0.605 ± 0.12 μM and 0.60 ± 0.06 μM respectively. This indicates that the enzyme does not, as has been suggested for bovine heart cytoplasmic malate dehydrogenase (Koren & Hammes, 1975), dissociate with a change of K_L in this concentration range.

Fits of the data with mannitol 1-phosphate and fructose 6-phosphate to eqn. (3) indicated appreciable values of k' , i.e. these ligands do not completely protect against inactivation by DTNB even at saturating concentrations (see Fig. 4). The proper equation for the above scheme with $k' > 0$ is:

Table 3. Substrate dissociation constants from binary and abortive ternary complexes

Ligand	Binding to	Eqn. no.	[DTNB] (mM)	K_L found (μM)
DTNB	E	(1)	0.01–0.5	55.1 ± 7.65
	E·Fru-6-P	(1) } (4) }	0.01–2.0	$\left\{ \begin{array}{l} 876 \pm 113^* \\ 890 \pm 68^* \end{array} \right.$
	E·Man-1-P	(1) } (4) }	0.05–4.0	$\left\{ \begin{array}{l} 805 \pm 91^\dagger \\ 807 \pm 153^\dagger \end{array} \right.$
NAD ⁺	E	(2)	0.1	184 ± 36 177 ± 23
	E·Fru-6-P	(2)	0.1	$16.25 \pm 0.22^*$
NADH	E	(2)	0.1	$0.597 \pm 0.064^\ddagger$ $0.605 \pm 0.121^\S$
	E·Man-1-P	(2)	0.1	$15.98 \pm 2.27^\dagger$
			2.0	$16.52 \pm 3.0^\dagger$
Fructose 6-phosphate	E	(4)	0.1	598 ± 33
	E·NAD ⁺			$53.8 \pm 9.3\parallel$
Mannitol 1-phosphate	E	(4)	0.1	9.86 ± 1.11
			0.2	8.15 ± 4.46
	E·NADH			$267 \pm 68\parallel$

* Determined at 30 mM-fructose 6-phosphate, corrected to infinite [fructose 6-phosphate].

† Determined at 15 mM-mannitol 1-phosphate, corrected to infinite [mannitol 1-phosphate].

‡ Determined at 0.028 μM -enzyme.

§ Determined at 0.269 μM -enzyme.

|| Calculated from the other dissociation constants (see the text).

To obtain K_L , estimates were first obtained by fitting to eqn. (3). Values of K_d' were then obtained by varying [DTNB] at very high concentrations of fructose 6-phosphate and mannitol 1-phosphate. These values were then used as fixed parameters, along with [DTNB], K_d and k (obtained from determinations of b at $[L] = 0$ and eqn. 1), to fit values of b at various values of $[L]$ to eqn. (4). Finally, the values of K_d' were adjusted slightly by using the obtained values of k' and K_L as fixed parameters in eqn. (4). The results are shown in Table 3. The ratios k'/k , representing the degree of protection by substrate, were 0.44 ± 0.044 for fructose 6-phosphate, and 0.50 ± 0.09 and 0.51 ± 0.16 for two determinations with mannitol 1-phosphate.

Since protection is not complete at saturating concentrations of the sugar phosphate ligand, the dissociation constants of coenzymes from the abortive ternary complexes enzyme·NAD⁺·fructose 6-phosphate and enzyme·NADH·mannitol 1-phosphate can be determined similarly, by varying [coenzyme] at high sugar phosphate concentration (with NADH this was performed at two concentrations of DTNB) and fitting to eqn. (2). The results are shown in Table 3. The dissociation constants for sugar phosphates from these complexes (K_s') can then be calculated from the dissociation constant for the coenzyme (K_c') and the dissociation constants of the binary complexes (K_s and K_c), since $K_s'K_c = K_sK_c'$.

DISCUSSION

The present results differ from those reported by Klungsøyr (1966) in two respects. The level of activity in crude extracts, 2.5–4 units/mg of protein, is one-fifth to one-third that observed by Klungsøyr (1966). Other strains of *E. coli* surveyed in this laboratory (P.

Krevetski & T. Chase, Jr., unpublished work), including A.T.C.C. 8739 as used by Klungsøyr (1966), had activity levels comparable with strain ΔAC9 used in this study, as did *E. coli* B surveyed by Liss *et al.* (1962) and strains surveyed by Solomon & Lin (1972).

More importantly, two methods dependent on the hydrodynamic radius of the native protein molecule indicated an M_r of about 45000, in contrast with Klungsøyr's (1966) value of 25000. Liss *et al.* (1962) reported an M_r of 40000 for the *Aero. aerogenes* enzyme, Brown & Banks (1977) reported an M_r of 45000 for the *S. mutans* enzyme, and Niehaus & Kiser (1981) reported an M_r of 40000 for the *Asp. niger* enzyme. Gel electrophoresis in the presence of SDS indicated an M_r of 22500, suggesting that the native enzyme is a dimer. No reason for the discrepancy is readily apparent. Strain difference is not responsible, since the enzyme from A.T.C.C. 8739 also emerged from a Sephadex G-75 column at a position indicating an M_r of 45000 (P. Krevetski & T. Chase, Jr., unpublished work). Presence of a large amount of non-amino-acid material attached to the peptide chain, with resultant increase in both hydrodynamic radius and partial specific volume, could reconcile the results from ultracentrifugation, Sephadex chromatography and gel electrophoresis of the native enzyme, but not the behaviour in gel electrophoresis in the presence of SDS. Monomeric dehydrogenases do exist [e.g. dihydrofolate reductase (Gundersen *et al.*, 1972; Nagelschmidt & Jaenicke, 1972), octopine dehydrogenase (Olomucki *et al.*, 1972) and aldehyde reductase (Turner & Tipton, 1972)], but are not common, and typically use NADP⁺ as coenzyme rather than NAD⁺.

Although not as simple as was hoped, mannitol-1-phosphate dehydrogenase of *E. coli* remains a useful example for investigation of the mechanism of action of nicotinamide nucleotide-dependent dehydrogenases. The

low content of cysteine, tyrosine, histidine and tryptophan (one, three, four and four residues/subunit respectively) should facilitate examination of their possible roles in catalysis and conformational change, either by chemical modification or by biosynthetic incorporation of ^{13}C -labelled amino acids or fluorinated analogues for n.m.r. studies (Browne *et al.*, 1973; Hull & Sykes, 1974).

Although totally zinc-free preparations of the enzyme have not been obtained, no experiment indicated zinc to be an essential component of it. It is difficult to rule out the presence of zinc-less inactive enzyme in even a highly purified preparation, but a zinc content of 0.10 mol/mol of subunit, for a preparation of specific activity 284 units/mg (55% of the highest observed), seems too low for a specific role. No combination of chelation and dialysis completely removed zinc from an enzyme preparation, and only prolonged incubation in diethyl-dithiocarbamate destroyed activity; this was prevented by the presence of 2-mercaptoethanol, suggesting a thiol reaction to be responsible for inactivation. Weak inhibition by 1,10-phenanthroline and $\alpha\alpha'$ -bipyridyl was matched by non-chelating 4,7-phenanthroline, and probably is the result of non-specific binding at the coenzyme-binding site. It is concluded that the residual zinc content is without significance, and results from non-specific tight binding.

The inactivation of the enzyme by DTNB, and the protection against inactivation by substrates as well as coenzymes, suggests that the thiol group lies very near the active site, although a chemical role in catalysis is not thereby suggested. Either the protection by coenzymes or (more probably) that by substrates could result from an enzyme conformational change rather than direct steric interference, but it is unlikely that both protect merely by a conformational change. We have reported elsewhere (Cerione & Chase, 1983) evidence for different conformational changes caused by binding of NAD^+ and NADH to the enzyme, and yet other, smaller, changes on binding of mannitol 1-phosphate and fructose 6-phosphate to pyridoxal phosphate-modified enzyme. However, DTNB might react with and stabilize a different, inactive, conformation of the enzyme whose formation is prevented by binding of either substrates or coenzymes. Presence or a thiol group near the catalytic site is common in dehydrogenases; in one case the chemical non-essentiality of the thiol group was demonstrated by re-activation of DTNB-inactivated enzyme (isocitrate dehydrogenase from *Azotobacter*) by conversion into the thiocyanato-enzyme with CN^- (Chung *et al.*, 1970). In the present case the location of the thiol group, and potentially of other reactive residues such as histidine, may be further mapped by observing the protective effect of substrate and coenzyme analogues.

The sugar phosphate substrates protect principally by increasing the K_a of DTNB about 15-fold (Table 3). The rate of reaction at saturating [DTNB] is decreased only about 2-fold, suggesting DTNB to be slightly less well placed for reaction in the complex. Complete protection by coenzymes suggests that DTNB binds in the coenzyme site, possibly the nicotinamide portion (near the substrate site) rather than the adenine portion.

The slowness of the reaction with DTNB is due to the unusually high $\text{p}K_a$ of the cysteine residue, which could result from proximity of a negatively charged group. Dividing the limiting rate of reaction at saturating

[DTNB] and complete dissociation of the thiol group, $65 \text{ min}^{-1} = 1.1 \text{ s}^{-1}$, by the K_a , $55 \mu\text{M}$ (which is equivalent to substituting $[\text{E}][\text{DTNB}]$ for $[\text{E}\cdot\text{DTNB}]$ in the first-order rate equation, observed rate $= k[\text{E}\cdot\text{DTNB}]$) yields an apparent second-order rate constant $20000 \text{ s}^{-1}\cdot\text{M}^{-1}$, in good agreement with the second-order rate constant for the reaction of DTNB with GSH, $21700 \text{ s}^{-1}\cdot\text{M}^{-1}$ at complete dissociation ($149 \text{ s}^{-1}\cdot\text{M}^{-1}$ at pH 6.5; Degani & Patchornik, 1974). Thus the reaction rate does not appear to be affected by any factors other than those causing the high $\text{p}K_a$ of the cysteine residue.

Protection against DTNB inactivation by substrates as well as coenzymes indicates that, unlike the substrates of lactate dehydrogenase, malate dehydrogenase and alcohol dehydrogenase, they bind to the enzyme in the absence of coenzyme. The slightly tighter binding of mannitol 1-phosphate, compared with NAD^+ , supports the earlier suggestion (Chase & Lucek, 1974) that the binding order may be random for this direction. A random, but not rapid-equilibrium, mechanism could explain the concave-downward plots of $1/v$ versus $1/[\text{mannitol 1-phosphate}]$ that have been observed (Klungsoyr, 1967). However, the dramatic increase of K_L for NADH in the presence of mannitol 1-phosphate supports Klungsoyr's (1967) suggestion that this apparent substrate activation results from more rapid dissociation of NADH from the abortive enzyme $\cdot\text{NADH}\cdot\text{mannitol 1-phosphate}$ complex than from the normal enzyme $\cdot\text{NADH}$ complex, though such a kinetic argument cannot be proven by determination of a dissociation constant, the ratio of 'off' and 'on' rate constants. Dissociation of NADH might also be favoured in the enzyme $\cdot\text{NADH}\cdot\text{fructose 6-phosphate}$ complex, as compared with enzyme $\cdot\text{NADH}$; but this cannot be investigated by the present technique.

The higher K_L for fructose 6-phosphate (as compared with that for mannitol 1-phosphate) is expected, since at most 4.1% is in the open chain (keto) form that presumably is the substrate (Midelfort *et al.*, 1976). The true K_L for this form is thus about $24 \mu\text{M}$, similar to that for mannitol 1-phosphate. However, the very much lower K_L for NADH, as well as the high practical K_L for fructose 6-phosphate, ensures that the reaction from this side will be essentially ordered (this does not necessarily hold for release of nascent fructose 6-phosphate product, since it is already in the open-chain form). The abortive ternary complex resulting from the mutual tightening of NAD^+ and fructose 6-phosphate binding presumably is responsible for the substrate inhibition seen at high fructose 6-phosphate concentrations (Klungsoyr, 1966).

The different effects on dissociation constants in the ternary complexes, namely increased in enzyme $\cdot\text{NADH}\cdot\text{mannitol 1-phosphate}$ but decreased in enzyme $\cdot\text{NAD}^+\cdot\text{fructose 6-phosphate}$, presumably result from different conformational changes caused by coenzyme binding, rather than by sugar phosphate binding (since the two sugar phosphates have virtually the same effects on DTNB binding). Such different conformational changes have also been observed by the different effects of the two coenzymes on protein fluorescence (Cerione & Chase, 1983), though small differences in enzyme $\cdot\text{mannitol 1-phosphate}$ and enzyme $\cdot\text{fructose 6-phosphate}$ complexes are also visible in the fluorescence spectra of pyridoxal phosphate-modified enzyme. Mannitol-1-phosphate dehydrogenase remains a useful case for the study of the role of coenzyme- and

substrate-directed conformational changes in dehydrogenase catalysis.

I thank Mrs. Greta Drahovsky, Mrs. Karen Brooks and Mrs. Pamela Enslin for general technical assistance, Mr. Bob Johnson for gel electrophoresis in the presence of SDS, Dr. David Strumeyer, Mr. Bradford Fisher and Mr. Rudolph Lucek for amino acid analysis, and Mr. Lucek also for preliminary experiments on DTNB inactivation, Dr. Robert Cousins and Dr. Katherine Squibb for atomic absorption spectrometry, Dr. Peter Kahn for computer fitting of inactivation-rate data, and Mr. Paul Krevetski for studies on other strains of *E. coli*. This paper is New Jersey Agricultural Experiment Station Publication no. D-01110-2-85, and the work was supported by State funds and by Hatch Act funds, the Rutgers University Research Council, a Biomedical Sciences Support Grant to Rutgers University and the Merck Foundation.

REFERENCES

- Alizalde, M. A., Gaede, K. & Brendel, K. (1976) Hoppe-Seyler's Z. Physiol. Chem. **357**, 1163–1169
- Andrews, P. (1964) Biochem. J. **91**, 222–223
- Bernhard, S. A., Dunn, M. F., Luisi, P. L. & Schack, P. (1970) Biochemistry **9**, 185–192
- Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
- Brown, A. T. & Banks, R. D. (1977) Infect. Immun. **16**, 163–173
- Browne, D. T., Kenyon, G. L. & Hegeman, G. D. (1970) Biochem. Biophys. Res. Commun. **39**, 13–19
- Browne, D. T., Kenyon, G. L., Packer, E. L., Sternlicht, H. & Wilson, D. M. (1973) J. Am. Chem. Soc. **95**, 1316–1323
- Cerione, R. A. & Chase, T., Jr. (1983) J. Protein Chem. **2**, 371–381
- Chase, T., Jr. (1972) Abstr. Annu. Meet. Am. Soc. Microbiol. **72nd** 72
- Chase, T., Jr. & Lucek, R. F. (1974) Fed. Proc. Fed. Am. Soc. Exp. Biol. **33**, 1237
- Chase, T., Jr. & Shaw, E. (1969) Biochemistry **8**, 2212–2224
- Chung, A. E., Franzen, J. S. & Braginski, J. E. (1970) Biochemistry **10**, 2872–2876
- Davis, B. J. (1964) Ann. N.Y. Acad. Sci. **121**, 404–427
- Degani, Y. & Patchornik, A. (1974) Biochemistry **13**, 1–11
- Dixon, M. (1953) Biochem. J. **55**, 170–171
- do Nascimento, K. H. & Davies, D. D. (1975) Biochem. J. **149**, 553–557
- Drum, D. H., Harrison, J. H., Li, T.-K., Bethune, J. L. & Vallee, B. L. (1967) Proc. Natl. Acad. Sci. U.S.A. **57**, 1434–1440
- Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 70–77
- Fraser, R. D. B. & Suzuki, E. (1973) in Physical Principles and Techniques of Protein Chemistry (Leach, S. J., ed.), pp. 301–355, Academic Press, New York
- Gaitonde, M. V. & Dovey, T. (1970) Biochem. J. **117**, 907–911
- Gundersen, L. E., Dunlap, R. B., Harding, N. G. C., Freisheim, J. H., Otting, F. & Huennekens, F. M. (1972) Biochemistry **11**, 1018–1023
- Harada, K. & Wolfe, R. G. (1968) J. Biol. Chem. **243**, 4131–4137
- Hedrick, J. L. & Smith, A. J. (1968) Arch. Biochem. Biophys. **126**, 155–164
- Hirs, C. H. W. (1967) Methods Enzymol. **11**, 197–199
- Horwitz, S. B. & Kaplan, N. O. (1964) J. Biol. Chem. **239**, 830–838
- Hull, W. E. & Sykes, B. D. (1974) Biochemistry **13**, 3431–3437
- Hult, V., Veide, A. & Gatenbeck, S. (1980) Arch. Microbiol. **128**, 253–255
- Jovin, T. M. (1973) Biochemistry **12**, 890–898
- Klungsoyr, L. (1966) Biochim. Biophys. Acta **128**, 55–62
- Klungsoyr, L. (1967) Biochim. Biophys. Acta **146**, 10–17
- Klungsoyr, L. (1969) Anal. Biochem. **27**, 91–98
- Koren, R. & Hammes, G. G. (1975) Biochemistry **14**, 1021–1025
- Liss, M., Horwitz, S. B. & Kaplan, N. O. (1962) J. Biol. Chem. **237**, 1342–1350
- Liu, T.-Y. & Chang, Y. H. (1971) J. Biol. Chem. **246**, 2842–2848
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
- Mayers, V. L. & Spizizen, J. (1954) J. Biol. Chem. **210**, 877–884
- Midelfort, C. F., Gupta, R. K. & Rose, I. A. (1976) Biochemistry **15**, 2178–2185
- Moore, S. (1975) in Chemistry and Biology of Peptides (Meienhofer, J., ed.), pp. 629–653, Ann Arbor Publications, Ann Arbor
- Munkres, K. D. (1965) Biochemistry **4**, 2186–2196
- Nagelschmidt, M. & Jaenicke, L. (1972) Hoppe-Seyler's Z. Physiol. Chem. **353**, 773–781
- Niehaus, W. G., Jr. & Kiser, R. C. (1981) Arch. Biochem. Biophys. **211**, 613–621
- Olomucki, A., Huc, C., Lefebure, F. & Thoai, N. V. (1972) Eur. J. Biochem. **28**, 261–268
- Pon, N. G., Schnackherz, K. D., Blackburn, M. N., Chatterjee, G. C. & Noltmann, E. A. (1970) Biochemistry **9**, 1506–1514
- Schachter, H., Sarney, J., McGuire, E. J. & Roseman, S. (1969) J. Biol. Chem. **244**, 4785–4792
- Sigman, D. S. (1967) J. Biol. Chem. **242**, 3814–3824
- Solomon, E. & Lin, E. C. C. (1972) J. Bacteriol. **111**, 566–574
- Spies, J. R. & Chambers, D. C. (1949) Anal. Chem. **21**, 1249–1266
- Theorell, H., Nygaard, A. P. & Bonnichsen, R. K. (1955) Acta Chem. Scand. **9**, 1148–1165
- Turner, A. J. & Tipton, K. F. (1972) Biochem. J. **130**, 765–772
- Ueng, S. T.-H., Hartonowicz, P., Lewandoski, C., Keller, J., Holick, M. & McGuinness, E. T. (1976) Biochemistry **15**, 1743–1749
- Weber, K. & Osborn, M. (1969) J. Biol. Chem. **244**, 4406–4412
- Wilkinson, G. N. (1961) Biochem. J. **80**, 324–332
- Wolff, J. B. & Kaplan, N. O. (1956a) J. Bacteriol. **71**, 557–564
- Wolff, J. B. & Kaplan, N. O. (1956b) J. Biol. Chem. **218**, 849–869

Received 14 May 1986; accepted 26 June 1986