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Purification and Properties of α-D- and β-D-Mannosidases from Hen Oviduct†

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ABSTRACT: An α-D- and a β-D-mannosidase (EC 3.2.1.24 and 3.2.1.25) have been purified from hen oviduct approximately 1300- and 10,000-fold, respectively. Molecular weight determinations by the Sephadex gel exclusion procedure revealed α-D-mannosidase to be about 250,000 and β-D-mannosidase, about 100,000. The latter enzyme was stable to high pH and the former to heat, properties that were exploited to remove undesirable contaminating glycosidases from the respective mannosidases. The α-D-mannosidase was more labile to treatment with Ag^+ and guanidine hydrochloride than β-D-mannosidase. Almost complete inhibition of α-D-mannosidase could be effected by Ag^+, with no apparent effect on β-D-mannosidase. However, Hg^{2+} impaired both enzymes to almost the same degree. β-D-Mannosidase was apparently more susceptible to inhibition by mannono(1→5)lactone than α-D-mannosidase. The K_i of this inhibitor was 17 μM for the former enzyme and 110 μM for the latter. Of significance is the finding that Asn-$(GlcNAc)_2(Man)_1$, a widespread constituent of many glycoproteins, is a substrate for β-D-mannosidase, but not for α-D-mannosidase. The latter will, however, convert Asn-$(GlcNAc)_2(Man)_6$ to Asn-$(GlcNAc)_2$-$(Man)_1$. Evidence is presented to support the thesis that a single enzyme hydrolyzes both *p*-nitrophenyl β-D-mannopyranoside and Asn-$(GlcNAc)_2(Man)_1$. Further proof that the mannosyl residues in the latter compound is β linked to *N*-acetylglucosamine was obtained by optical rotatory dispersion and infrared analysis on the Man(→)GlcNAc disaccharide isolated from Asn-$(GlcNAc)_2(Man)_1$.

In the course of studies (Tarentino *et al.*, 1970) on the oligosaccharide sequence of RNase B glycopeptide, it was found that five of the six mannosyl residues associated with the glycopeptide could be removed by jack bean meal α-D-mannosidase. The proposal offered for the resistance of the last mannosyl residue to hydrolysis in the glycosyl-Asn sequence, Asn-$(GlcNAc)_2(Man)_1$,[1] isolated from RNase B, was that the Man(→)GlcNAc bond was possibly in the β configuration or that if α, it was resistant to cleavage for reasons still to be determined. Similar results were obtained with an apparently identical glycosyl-Asn derivative from ovalbumin.

Incubation of Asn-$(GlcNAc)_2(Man)_1$ with extracts from hen oviduct, under conditions that minimized the action of glycosyl asparaginase, revealed the presence of an enzyme that released the terminal mannosyl residue (Sukeno *et al.*,

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[1] Abbreviations used are: Asn-GlcNAc, 2-acetamido-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; Asn-$(GlcNAc)_2(Man)_1$, 2-acetamido-4-*O*-[*O*-mannopyranosyl(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; *p*CMB, *p*-chloromercuribenzoate; DON, 5-diazo-4-oxo-L-norleucine.

1971). Partial purification of this hydrolytic protein revealed that it purified with an enzyme capable of hydrolyzing *p*-nitrophenyl β-D-mannopyranoside. Although β-D-mannosidases have been reported in fungi (Reese and Shibata, 1965), insects (Courtois *et al.*, 1961), and mollusks (Courtois *et al.*, 1962; Levvy *et al.*, 1964; Nagaoka, 1949; Muramatsu and Egami, 1967), none to our knowledge has been characterized in higher phylogenetic forms. As a consequence, the β-D-mannosidase in hen oviduct was purified extensively and found to be enriched similarly in its capacity to hydrolyze the mannose associated with Asn-$(GlcNAc)_2(Man)_1$. This sequence appears to be rather widespread in nature, as terminal mannosyl residues resistant to jack bean α-D-mannosidase have been alluded to in glycoprotein polysaccharide sequence studies in several instances (Li *et al.*, 1968; Lee, 1971; Scocca and Lee, 1969; Huang *et al.*, 1970). Because of the physiologic significance of the β-D-mannosidase and its potential importance in the structural analysis of polysaccharides, a study of the enzyme's properties and its separation from other contaminating hydrolases was undertaken. The results of some of these findings are presented in this report. A somewhat similar purification of the α-D- and β-D-mannosidases from the marine gastropod, *Turbo cortunis*, was reported earlier (Muramatsu and Egami, 1967).

Experimental Section

Materials

The *p*-nitrophenyl glycoside derivatives were purchased from the Pierce Chemical Co., Rockford, Ill., except for *p*-nitrophenyl β-D-mannopyranoside which was obtained from the Sigma Chemical Co., St. Louis, Mo. Asn-GlcNAc (Marks *et al.*, 1963) and D-mannono(1→5)lactone (Levvy *et al.*, 1964) were synthesized chemically. DON was a gift from Dr. R. Handschumacher, while *O*-α-D-mannopyranosyl(1→3)-2-acetamido-2-deoxy-D-glucose (Shaban and Jeanloz, 1971) was kindly provided by Dr. Roger Jeanloz. The 1→4 and 1→6 derivatives of *O*-α-D-mannopyranosyl-2-acetamido-2-deoxy-D-glucose were generously donated by Dr. O. P. Bahl. Ovalbumin glycopeptide, Asn-$(GlcNAc)_2(Man)_6$, was isolated by the procedure of Huang *et al.* (1970) and converted to Asn-$(GlcNAc)_2(Man)_1$, as described earlier (Tarentino *et al.*, 1970). Calcium phosphate gel was prepared by the procedure of Keilin and Hartree (1938).

Cellulose phosphate was obtained from Schleicher and Schuell, Inc., Keene, N. H., and Sephadex G-200 from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Rabbit muscle lactate dehydrogenase was purchased from Worthington Biochemical Corp., Freehold, N. J., and yeast hexokinase (type V), NADH, ATP, and phosphoenolpyruvate from the Sigma Chemical Co.

Methods

The glycosidases were measured essentially as described by Levvy and Conchie (1966). The assay mixture contained 1.8 mM *p*-nitrophenyl glycoside, 33.3 mM sodium citrate at the desired pH, and enzyme to a volume of 0.3 ml. α- and β-L-fucosidases, α- and β-D-galactosidases, and β-D-glucosidase were assayed at pH 3.5, while β-D-*N*-acetylglucosaminidase and the mannosidases were measured at pH 4.6; α-D-glucosidase was assayed at pH 5.5. After a 15-min incubation period at 37°, the reactions were stopped by the addition of 2.7 ml of 0.1 M Na_2CO_3 and read at 400 nm. The hydrolysis of methyl α-D- and β-D-mannopyranoside and the *O*-α-D-mannopyranosides of *N*-acetylglucosamine were determined by the coupled lactate dehydrogenase–hexokinase assay (Tarentino *et al.*, 1970). Glycosyl asparaginase was measured by the release of *N*-acetylglucosamine (Tarentino and Maley, 1969). A unit of activity represents the amount of enzyme that will hydrolyze 1 μmole of substrate/min under the above reaction conditions. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Fluorometric Assay of β-D-Mannosidase. A 25-μl reaction mixture containing 10 μl of 0.1 M sodium citrate (pH 4.6), 0.01 unit of β-D-mannosidase, and 2–12 nmoles of Asn-$(GlcNAc)_2(Man)_1$ from ovalbumin was incubated at 37°. After 3 min, a 10-μl aliquot was added to a fluorometer cuvet containing 10 mM triethanolamine-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 0.05 μmole of NADH, 1 mM KCl, 2.5 μmoles of phosphoenolpyruvate, 0.3 μmole of ATP, 2.5 units of lactate dehydrogenase, and 0.5 unit of pyruvate kinase in a volume of 3 ml. The reaction was started by the addition of 5 units of yeast hexokinase and the decrease in fluorescence of NADH at 450 nm (excitation at 350 nm) was measured with a Perkin-Elmer Model 204 fluorescence spectrophotometer. The quantity of mannose released was determined by comparison to a mannose standard. The deviation of the latter from linearity was minimal.

Molecular Weight Determination by Sephadex G-200 Chromatography. A Sephadex G-200 column (1.5 × 97 cm, superfine grade gel) was calibrated with proteins of known molecular weight (Siegel and Monty, 1965). The void volume (67.3 ml) was determined with T2-bacteriophage and the elution volume (V_e) was estimated from the midpoint of each peak. Samples of α-D- or β-D-mannosidase, 0.44 and 0.64 unit, respectively, in 0.5 ml were placed on the column and eluted with 0.05 M potassium phosphate (pH 6.2) at a flow rate of 10.5 ml/hr. Fractions of 2.0 ml were collected.

Polyacrylamide Gel Electrophoresis. Disc electrophoresis was performed in 6% polyacrylamide gels as described by Davis (1964) but with the omission of sample and spacer gels. The protein was layered directly on the top of the polymerized gels in 10% sucrose and the electrophoresis was conducted at 4° for 1.5 hr at 2 mA/gel. A 1% solution of Amido Black in 7% acetic acid was used to reveal the location of the protein bands. α-D- or β-D-mannosidase was detected on duplicate gels by incubating the gels first in 0.1 M sodium citrate (pH 4.5) for 10 min at 37° and then in a solution of 0.05 M sodium citrate (pH 5.5) containing either 5 mM *p*-nitrophenyl α-D-mannoside or the corresponding β-D derivative (Gabriel and Wang, 1969). Within 30 min yellow bands appeared in the region of the respective mannosidase.

Purification of β-D-Mannosidase

Crude Extract. Whole hen oviducts (120) stored at −20° were thawed, minced, homogenized in 4.6 l. of 0.01 M potassium phosphate buffer (pH 7.5), and then centrifuged for 15 min at 12,000*g*. The pellet was washed with 2.0 l. of the same buffer. Supernatant fractions were combined and filtered through glass wool. All of the procedures were conducted at 0–4°, unless specified otherwise.

$(NH_4)_2SO_4$. I. The protein concentration of the supernatant fraction was brought to 25 mg/ml with 0.01 M potassium phosphate buffer (pH 7.5) and 19 g of ammonium sulfate/100 ml of solution were added (0.35 saturation). After the resulting suspension was stirred for 20 min, it was centrifuged at 12,000*g* for 20 min. The supernatant fraction was then brought to 0.60 saturation by the addition of 15.1 g of solid ammonium sulfate/100 ml, stirred for 20 min, and centrifuged. The precipitate was dissolved in a minimal volume of 0.01 M potas-

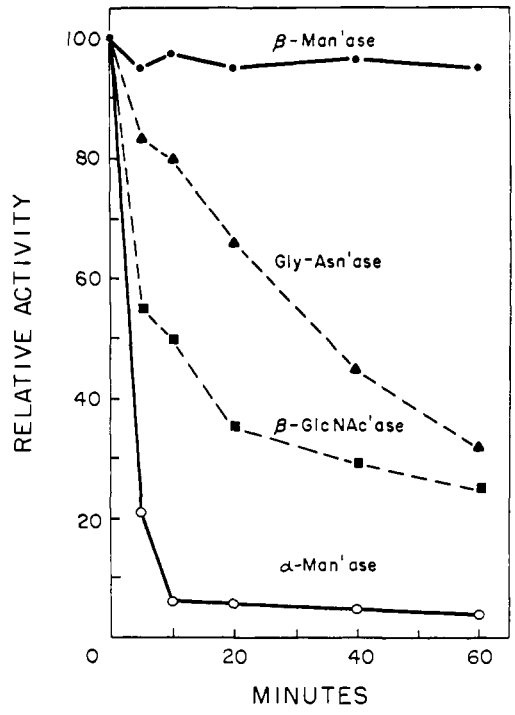

FIGURE 1: Effect of maintaining the pH at 11.0 on enzymes in the phosphocellulose (pH 7.1) eluate. See purification of β-D-mannosidase in Methods for details. Man'ase, mannosidase; Gly-Asn'ase, glycosyl asparaginase; β-GlcNAc'ase, β-*N*-acetylglucosaminidase.

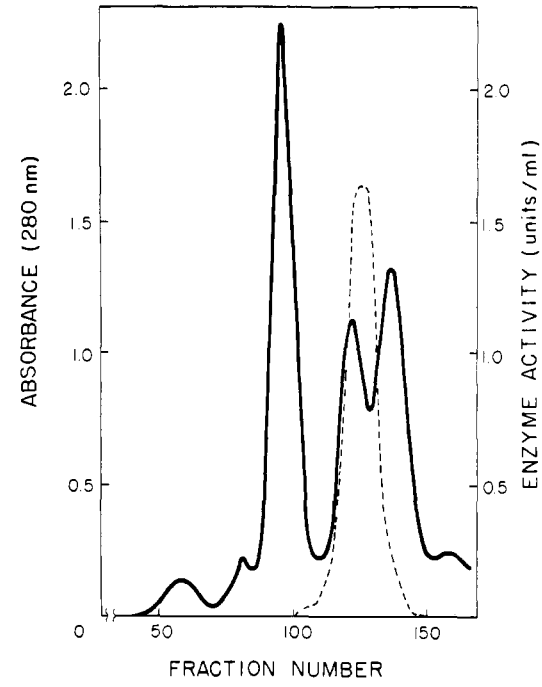

FIGURE 2: Sephadex G-200 chromatography of β-D-mannosidase. Absorbance (——); enzyme activity (- - - - - -). For details, see purification procedures in Methods.

sium phosphate buffer (pH 7.5) and dialyzed against 20 l. of the same buffer with three changes.

pH 5.5. The dialyzed extract was adjusted to pH 5.5 with 1 N acetic acid and the resulting turbidity was removed by centrifugation.

$(NH_4)_2SO_4$. II. The supernatant fraction was brought to a 0.70 saturation by adding 43.6 g of solid ammonium sulfate/100 ml, stirred for 20 min, and centrifuged. The resulting pellet was dissolved in a minimal volume of 0.01 M potassium phosphate buffer (pH 7.1) and dialyzed for 24 hr against three 6-l. changes of the same buffer.

Phosphocellulose (pH 7.1). The dialyzed extract (1600 ml), containing 34.1 g of protein, was applied to a phosphocellulose column (7.6 × 32 cm), which had been equilibrated previously with 0.01 M potassium phosphate buffer (pH 7.1). Elution of the column with the same buffer yielded both α- and β-D-mannosidases as a nonretarded fraction (2300 ml), which could be stored at −10° without loss in activity. For the purification of α-D-mannosidase, 700 ml of this fraction was brought to pH 6.1 by dialysis against 0.01 M potassium phosphate and applied to a phosphocellulose (pH 6.1) column, as described in the section on α-D-mannosidase purification.

pH 11.0. The pH of the phosphocellulose eluate (1600 ml) was adjusted carefully with 0.1 N NaOH (about 260 ml) until it reached 11.0. The solution was maintained at this pH for 40 min with occasional addition of 0.1 N NaOH, and then brought to pH 7.0 with about 30 ml of 0.5 M sodium citrate buffer (pH 4.5). The resulting turbidity was removed by centrifugation and the supernatant fraction (1800 ml) was dialyzed against 0.01 M sodium phosphate buffer (pH 6.1). With this treatment, 95% of the α-D-mannosidase was inactivated (Figure 1).

Phosphocellulose (pH 6.1). The dialyzed extract was placed on a phosphocellulose column (7.6 × 40 cm) equilibrated previously with 0.01 M sodium phosphate (pH 6.1) and washed with the same buffer until the unadsorbed protein was eluted. β-D-Mannosidase was eluted with a solution of 0.01 M sodium phosphate (pH 6.1)–0.2 M sodium chloride. Fractions of 25 ml were collected and the enzyme-containing fractions (50–120) were pooled (total volume, 1800 ml).

Acetone. The pH 6.1 phosphocellulose eluate was concentrated to 1200 ml (2.5 mg of protein/ml) in an Amicon Model 50 ultrafilter containing a PM-10 membrane. Acetone at 4° (514 ml) was added dropwise to 0.30 saturation and the temperature was maintained at 4°. The resultant suspension was stirred for 30 min, and then centrifuged at 0° for 15 min at 16,000*g*. The supernatant fraction was decanted, brought to 0.60 saturation with 1280 ml of acetone, stirred, and centrifuged as described above. The precipitate was extracted four times with 50-ml volumes of a solution containing 0.05 M sodium citrate buffer (pH 4.6), 0.1 mM zinc acetate, and 0.05 M sodium chloride.

Sephadex G-200. The β-D-mannosidase solution (885 mg of protein) was concentrated to 13 ml and applied to a Sephadex G-200 column (3.7 × 173 cm) equilibrated with 0.05 M sodium citrate (pH 4.6), 0.1 mM zinc acetate, and 0.05 M sodium chloride. Fractions of 8.8 ml were collected. The enzyme-containing fractions, 112–142 (Figure 2), were pooled and concentrated to 10 ml in the Amicon ultrafilter. About 96% of the β-D-*N*-acetylglucosaminidase from the preceding step was removed by this procedure.

$Ca_3(PO_4)_2$ Gel. The concentrated β-D-mannosidase solution (96 mg of protein in 10 ml) was dialyzed against three changes of 100 volumes each of 0.01 M potassium phosphate buffer (pH 7.1) and applied to a calcium phosphate gel column (2.0 × 8.0 cm) equilibrated with the same buffer. The support for the column was prepared by slowly pouring 500 ml of gel (30 mg/ml) into 1 l. of a 10% suspension of Whatman cellulose powder. Stepwise elutions were carried out with 0.05 and 0.10

TABLE I: Purification of Hen Oviduct β-D-Mannosidase.

Step	Vol (ml)	Protein (mg)	Total Act. (Units)	Sp Act. (Units/mg)	Yield (%)
Crude extract	6600	262,000	550	0.0021	100
$(NH_4)_2SO_4$ I	2800	72,000	420	0.0058	76
pH 5.5	3000	39,000	378	0.0092	69
$(NH_4)_2SO_4$ II	1600	34,100	407	0.0119	74
Phosphocellulose (pH 7.1)	2300[a]	28,000	369	0.0125	67
pH 11.0	1800	17,400	202	0.0116	53
Phosphocellulose (pH 6.1)	1800	3,240	164	0.0506	43
Acetone	200	885	162	0.183	42
Sephadex G-200	10	96	150	1.56	39
$Ca_3(PO_4)_2$ gel	1.2	3.2	65	20.6	17

[a] 700 ml were taken at this stage for the subsequent purification of α-D-mannosidase, as discussed in Methods. To compensate for this removal, the % yield from the pH 11 step on was obtained by multiplying total activity by 23/16 and dividing by 550.

M potassium phosphate buffer (pH 7.1); fractions of 8 ml were collected (Figure 3). Fractions 32–38 were pooled and concentrated to 1.2 ml in the Amicon ultrafilter. The final purification of β-D-mannosidase was 10,000-fold over the initial extract with a recovery of 17%. The purified β-D-mannosidase from this step was devoid of detectable α-D-mannosidase, α-D- and β-D-galactosidases, α-L- and β-L-fucosidases, lysozyme (less than 0.1%), but contained 0.2% glycosyl asparaginase and 0.13% β-D-*N*-acetylglucosaminidase. Glycosyl asparaginase was inactivated completely by 2×10^{-3} M DON without any loss of β-D-mannosidase activity. The purification of β-D-mannosidase is summarized in Table I.

Purification of α-D-Mannosidase

Phosphocellulose (pH 6.1). A portion (700 ml) of the phosphocellulose (pH 7.1) elution step was used for the purification of α-D-mannosidase. This solution, containing a total of 8.5 g of protein, was dialyzed against three changes of 15 l. each of 0.01 M sodium phosphate buffer (pH 6.1) and applied to a phosphocellulose column (3.7 × 39 cm) equilibrated with the same buffer. The column was washed with the 0.01 M phosphate buffer until most of the unadsorbed protein was removed and the enzyme was eluted with a solution of 0.01 M sodium phosphate (pH 6.1)–0.2 M NaCl. Fractions of 20 ml were collected and the enzyme in fractions 27–40 was combined (340 ml).

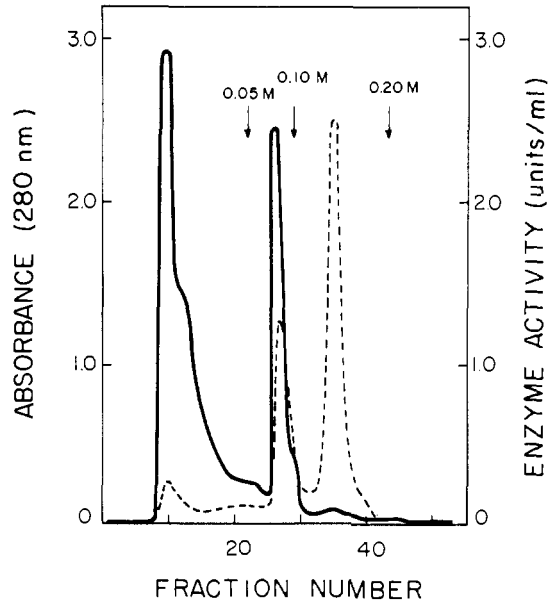

FIGURE 3: Calcium phosphate gel column chromatography of β-D-mannosidase. Absorbance (——); enzyme activity (- - - -). The potassium phosphate (pH 7.1) buffer changes are indicated by the arrows. For further details, see Methods.

Heat Treatment. To the enzyme solution, 0.34 ml of 0.1 mM zinc acetate was added. The solution was placed in a water bath at 85° until the temperature of the solution reached 65° and was maintained at this temperature for 1 hr. The turbid solution was chilled with ice and centrifuged. The precipitate was washed once with a small volume of 0.01 M sodium phosphate buffer (pH 6.1) and the clear supernatant fractions were combined. By this treatment, β-D-mannosidase, β-D-*N*-acetylglucosaminidase, and glycosyl asparaginase were decreased in activity by 94, 97, and 91%, respectively, while α-D-mannosidase was unaffected (Figure 4).

Acetone. The solution (360 ml) from the above heat step, containing 2.2 mg of protein/ml, was brought to a 0.30 saturation with 154 ml of cold acetone, stirred for 30 min, and centrifuged. The conditions were identical with those reported above for β-D-mannosidase. Acetone (380 ml) was then added to the supernatant fraction to 0.60 saturation. The precipitate was collected by centrifugation and extracted four times with approximately 50-ml volumes of a solution containing 0.05 M sodium citrate buffer (pH 4.6), 0.1 mM zinc acetate, and 0.05 M sodium chloride. The extracts were pooled and concentrated to 6 ml in the Amicon ultrafilter.

Sephadex G-200. The concentrate was placed on a Sephadex G-200 column (2.5 × 173 cm) equilibrated with a solution of 0.05 M sodium citrate buffer (pH 4.6), 0.1 mM zinc acetate, and 0.05 M sodium chloride. Fractions of 10 ml were collected. The enzyme-containing fractions 35–45 were combined and concentrated to 5 ml in the Amicon ultrafilter.

The overall purification of the enzyme from the initial extract was 1290-fold with a recovery of 27%. None of the following enzymes was detectable: β-D-mannosidase, α-D- or β-D-*N*-acetylglucosaminidase, α-D- or β-D-galactosidase, α-L- or β-L-fucosidase, α-D- or β-D-glucosidase, lysozyme (less than 0.1%), but about 1% glycosyl asparaginase was present.

TABLE II: Purification of Hen Oviduct α-D-Mannosidase.[a]

Step	Vol (ml)	Protein (mg)	Total Act. (Units)	Sp Act. (Units/mg)	Yield (%)
Phosphocellulose (pH 6.1)	340	1,840	94	0.0511	43
Heat (65° for 1 hr)	360	775	86	0.111	39
Acetone	190	241	76	0.314	35
Sephadex G-200	5	19	58	3.02	27

[a] The purification for α-D-mannosidase follows exactly that described for β-D-mannosidase in Table I through the phosphocellulose (pH 7.1) step. The initial total activity of α-D-mannosidase in the crude extract of Table I was 718 units.

Contamination of the latter enzyme was completely inhibited by 2×10^{-3} DON without any loss of α-D-mannosidase activity. The purification of α-D-mannosidase is summarized in Table II.

Results and Discussion

Purification of α-D- and β-D-Mannosidases. To facilitate the separation of the mannosidases from one another, and from undesirable contaminating glycosidases, conditions of differential inactivation were explored. The apparent stability of β-D-mannosidase to high pH suggested a potentially useful procedure for its purification or at least a separation from other glycosidases. Support for the latter contention is presented in Figure 1 where it is evident that maintaining the pH at 11.0 for 1 hr resulted in a considerable loss in glycosyl asparaginase, β-D-*N*-acetylglucosaminidase, and α-D-mannosidase, without any effect on β-D-mannosidase.

In contrast, the marked stability of α-D-mannosidase to heat provided a means of obtaining this enzyme practically devoid of other glycosidases (Figure 4). The heat stability of the hen oviduct enzyme is thus similar to that described for the oyster (Courtois *et al.*, 1962), snail (Nagaoka, 1949), gastropod (Muramatsu and Egami, 1967), and hog kidney (Okumura and Yamashina, 1970) α-D-mannosidases.

With this information, the overall purification scheme for the two mannosidases was devised (Tables I and II). Some of the steps, although not providing an improvement in the specific activity of the desired enzyme, were designed to remove an undesired activity. Thus, as seen from the list of glycosidases present in hen oviduct (Table III), the predominant enzyme activity by far was β-D-*N*-acetylglucosaminidase. Most of this enzyme (*ca.* 80%) was eliminated with the phosphocellulose (pH 7.1) step. The pH 11.0 and heat steps exploited the information presented in Figures 1 and 4 and enabled the removal of most of the enzymes presented in Table III. Undoubtedly, a much greater specific activity for α-D-mannosidase could have been obtained, but in view of the numerous purifications described for this enzyme and because the enzyme offered no advantage over the jack bean α-D-mannosidase, it was not purified further.

Sephadex G-200 chromatography was found useful in achieving a 10-fold purification for both enzymes, and from the variation in elution volumes, a distinct difference in their molecular weights was indicated. For the final step in the β-D-mannosidase purification, $Ca_3(PO_4)_2$ gel chromatography yielded an additional 10-fold purification with the resultant enzyme enriched about 10,000-fold over that in the initial crude extract. As indicated in Figure 3, two major peaks of β-D-mannosidase were obtained, but only the second high specific activity component was retained. The reason for the two peaks of activity is not known, but glycosidases (Robinson and Stirling, 1968; Johnson and DeBusk, 1970; Wiederschain and Rosenfeld, 1971; Robinson *et al.*, 1967) have been shown to possess multiple components.

Despite the extensive purification of the β-D-mannosidase, acrylamide gel electrophoresis revealed the enzyme protein to migrate slightly ahead of the major protein band. Since most of the unwanted glycosidases were removed or reduced to acceptable levels for structural studies, further purification of the β-D-mannosidase was not attempted.

Molecular Weights of α-D- and β-D-Mannosidases. The disparity in molecular weights between the α-D- and β-D-mannosidases was readily apparent on elution of the enzymes from calibrated Sephadex G-200 columns. An estimated molecular weight of 100,000 was obtained for β-D-mannosidase and 250,000 for α-D-mannosidase.

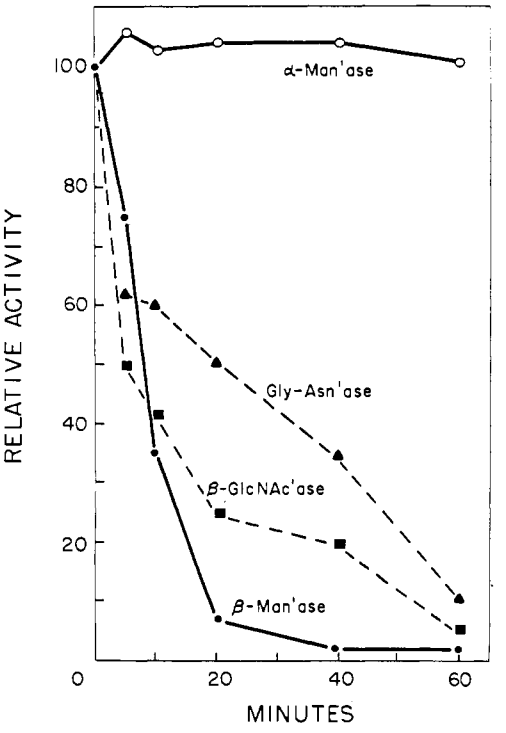

FIGURE 4: Effect of heating at 65° on enzymes in phosphocellulose (pH 6.1) eluate. See purification of α-D-mannosidase in Methods for details. Same abbreviations as in Figure 1.

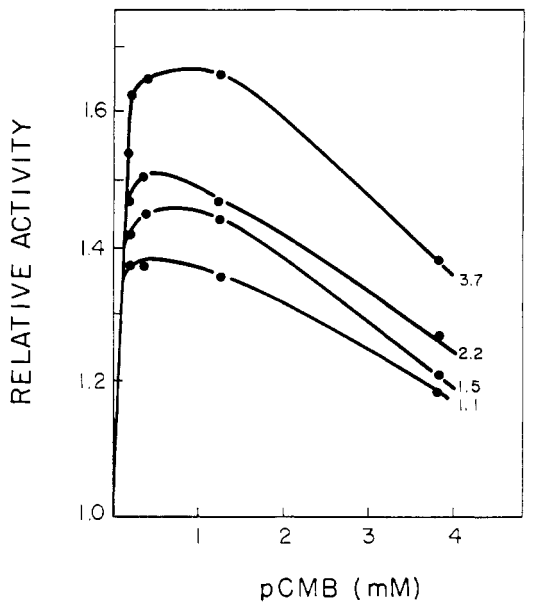

FIGURE 5: The activation of β-D-mannosidase by *p*CMB. The enzyme (0.06 unit of highest purity) was incubated at 37° in 1.2 ml of 50 mM sodium citrate (pH 4.6) and the indicated concentration of *p*CMB. After 20 min, 0.2 ml of the mixture was added to 0.1 ml of substrate and the final solution was incubated for an additional 15 min at 37°. The final substrate concentrations are indicated to the right of each curve. The reaction was stopped and assayed as in Methods. Controls were run without *p*CMB.

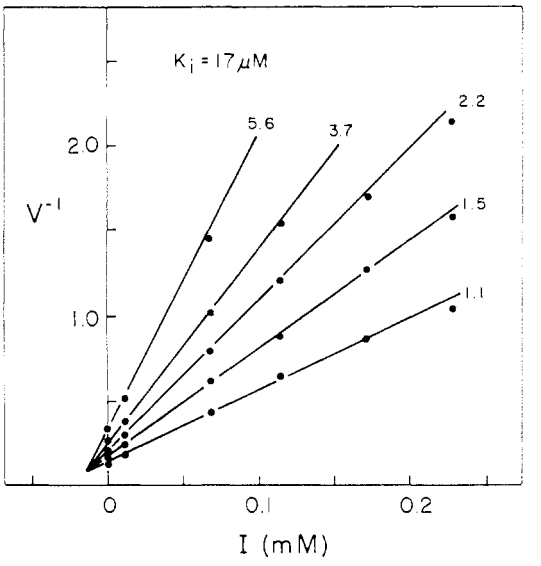

FIGURE 6: Dixon plot of β-D-mannosidase inhibition by D-mannono-(1→5)lactone. The assay mixture was as described in Methods, except that the mM substrate concentration was varied as indicated by the numbers to the right of the lines. The inhibitor (I) concentration was varied also as indicated (●). The reaction mixture containing 0.006 unit of highest purity enzyme was incubated at 37° for 15 min and assayed as in Methods.

Differential Responses of α-D- and β-D-Mannosidases to Various Inhibitors. Hen oviduct α-D-mannosidase was inhibited 50% by 50 μM Ag^+, an effect similar to that described for the almond emulsin (Schwartz *et al.*, 1970) and jack bean meal enzymes (Li, 1967). In contrast to α-D-mannosidase, β-D-mannosidase was barely affected by 0.7 mM Ag^+. However, both enzymes were impaired approximately 50% by 50 μM Hg^{2+}. The effect of 0.6 M guanidine hydrochloride was similar to that of Ag^+ in that α-D-mannosidase was impaired much more than β-D-mannosidase (70% inhibition as compared to 30%).

Since Hg^{2+} affected both mannosidases in a detrimental manner, other sulfhydryl inhibitors were tested, including iodoacetate, *N*-ethylmaleimide, and *p*CMB. All were ineffective, but in the case of the latter compound, a 65% increase in β-D-mannosidase activity was effected by 0.5 mM *p*CMB (Figure 5). As indicated, the extent of the increase in activity was dependent also on substrate concentration. The apparent anomaly of activation by *p*CMB is not unique as an even more profound effect has been described with dihydrofolate reductase (Perkins and Bertino, 1964).

As shown by Levvy *et al.* (1964), D-mannono(1→5)lactone is an effective competitive inhibitor of limpet α-D- and β-D-mannosidase. The Dixon plot of Figure 6 reveals the lactone to inhibit hen oviduct β-D-mannosidase competitively with a K_i of 17 μM. The α-D-mannosidase was also inhibited competitively by the lactone, but with a K_i of 110 μM. Similar values were obtained by Levvy *et al.* (1964) for the limpet β-mannosidase.

EDTA was found to enhance the lability of the α-D-mannosidase much more so than that of the β-D-mannosidase, an effect that could be reversed by Zn^{2+}. Although an extensive study of stabilizing metal ions, such as described by Snaith and Levvy (1968) with the jack bean α-D-mannosidase, was not conducted, findings similar to theirs on the stabilizing influence of Zn^{2+} were obtained with the hen oviduct enzyme. Thus, incubation of the α-D-mannosidase for several hours at 37° in the presence of Zn^{2+} had a profound stabilizing effect on the enzyme as compared to that in its absence. Similar results were obtained with the hog kidney α-D-mannosidase (Okumura and Yamashina, 1970). β-D-Mannosidase was much more stable over the course of such an incubation and was little affected by Zn^{2+} or EDTA.

Substrate Specificity. The relative rates of hydrolysis of several potential substrates for the mannosidases were compared and as shown in Table IV, the *p*-nitrophenyl derivatives

TABLE III: Glycosidases in Hen Oviduct before and after Partial Purification.

Enzyme	Activity (Units/mg × 10^{-3}) Extract[a]	Partially Purified[b]
β-D-*N*-Acetylglucosaminidase	165	29
α-L-Fucosidase	0.33	0
β-L-Fucosidase	0.24	0
α-D-Galactosidase	0.93	2.0
β-D-Galactosidase	4.2	2.0
α-D-Glucosidase	1.2	0.74
β-D-Glucosidase	0.29	0.38
α-D-Mannosidase	2.34	14.2
β-D-Mannosidase	2.07	12.5
Glycosyl asparaginase	0.29	0.94
Lysozyme	61.0	2.5

[a] Crude extract of Table I. [b] Phosphocellulose (pH 7.1) stage of purification in Table I.

were the best substrates, a finding in general accord with the hydrolysis of glycosides by glycosidases. No hydrolysis of Asn-$(GlcNAc)_2(Man)_1$ could be detected on treatment with hen oviduct α-D-mannosidase, although 4.2 mole equiv of mannose were released from Asn-$(GlcNAc)_2(Man)_5$ by this enzyme. However, treatment of Asn-$(GlcNAc)_2(Man)_5$ with β-D-mannosidase resulted in the release of only marginal amounts of mannose,[2] suggesting that the β-D-mannosidic linkage is either proximally located in the polymannose chain or that the $(Man)_4$ chain sterically restricts the hydrolysis of the β-D-mannosidic bond to *N*-acetylglucosamine.

It appears from the data in Table IV that α-D-mannosidase hydrolyzes α-D-Man(1→4)GlcNAc and α-D-Man(1→6)GlcNAc at approximately two orders of magnitude slower than β-D-mannosidase hydrolyzes the trisaccharide, β-D-Man-(1→4)-β-D-GlcNAc(1→4)GlcNAc. Whether β-D-Man(1→4)-GlcNAc is hydrolyzed at rates comparable to the latter trisaccharide has not been accurately determined yet, but recent studies indicate that this disaccharide is as good, if not a better, substrate than the trisaccharide.

A kinetic analysis of the data through the use of double-reciprocal plots revealed the K_m's of α-D- and β-D-mannosidase for their respective *p*-nitrophenyl substrates to be 4.5 and 2.9 mM. The K_m of β-D-mannosidase for Asn-$(GlcNAc)_2(Man)_1$, the presumed natural substrate, was 16.9 mM. However, the V_{max} for *p*-nitrophenyl β-D-mannopyranoside and Asn-$(GlcNAc)_2(Man)_1$ when calculated on the basis of micromoles per minute per milligram of protein was approximately the same, indicating that the latter could be as good a substrate as the former, if present at a high enough concentration. Preliminary studies with $(GlcNAc)_2(Man)_1$ indicate that it is as good a substrate as its corresponding asparagine derivative. It is possible that the above trisaccharide, when linked to a polypeptide or protein, would be an even better substrate for β-D-mannosidase, but no evidence is available on this subject as yet.

The pH optimum for both β-D-mannosidase substrates was essentially the same, 4.6, a value identical with that obtained for α-D-mannosidase. This pH is similar to that reported for other mannosidases and within the range of enzymes believed to be of lysosomal origin. Additional evidence that the same enzyme hydrolyzes both *p*-nitrophenyl β-D-mannopyranoside and Asn-$(GlcNAc)_2(Man)_1$ was provided by the finding that the ratio of hydrolysis rates of both compounds by 20-fold purified enzyme and 10,000-fold purified enzyme was the same. In addition, polyacrylamide disc gel electrophoresis of the most purified β-D-mannosidase preparation separated most of the contaminating proteins from the β-D-mannosidase region, but did not separate the *p*-nitrophenyl β-D-mannopyranoside and Asn-$(GlcNAc)_2(Man)_1$ hydrolyzing activities. The finding that *p*-nitrophenyl β-D-mannoside inhibited the hydrolysis of Asn-$(GlcNAc)_2(Man)_1$, but that the corresponding α derivative did not (Sukeno *et al.*, 1971) supports the β-anomeric structure of the mannosyl unit in Asn-$(GlcNAc)_2(Man)_1$.

Further Proof on the Nature of the Mannosidic Linkage in Asn-$(GlcNAc)_2(Man)_1$. Although the evidence presented above supports the belief that the mannosidic linkage in Asn-$(GlcNAc)_2(Man)_1$ from ovalbumin is β, it does not constitute unequivocal proof. More satisfactory proof would be provided by chemical evidence and recent studies have presented such proof. With the use of an enzyme from *Streptomyces griseus* (A. L. Tarentino, F. Maley, and T. H. Plummer, unpublished data) which was found to hydrolyze Asn-$(GlcNAc)_2(Man)_1$ to Asn-GlcNAc and Man(→)GlcNAc, the latter compound could be isolated in sufficient yield for optical rotatory dispersion (ORD) and infrared measurements. ORD analysis revealed a negative Cotton effect with a trough at 220 nm, characteristic of the asymmetric interaction of a 2-acetamido group with a dominant β anomer on C_1 of *N*-acetyl-D-glucosamine (Listowsky *et al.*, 1968). The $[m]_{218}-[m]_{300}$ (where m = molecular rotation) was −4440°, which is in the range for β-galactosidic linkages to *N*-acetylglucosamine (Beychok and Kabat, 1965). While $[m]$ for the latter compound may not be the same for different β-glycosidic linkages to *N*-acetyl-D-glucosamine, recent ORD studies with α-D-Man(1→4)GlcNAc revealed $[m]$ to be positive from 210 to 300 nm, with an $[m]_{218}-[m]_{300}$ of −970°. In addition, gas chromatographic analysis of the trimethylsilyl derivatives of the natural and chemically prepared disaccharides revealed them to migrate differently (F. Maley, unpublished data). Infrared analysis of the natural disaccharide yielded an absorption band at 880 cm^{-1} which is in the type 2B absorption region reported for β-glycosidic bonds (Barker *et al.*, 1954). An absorption band in the α-glycosidic region of 844 cm^{-1} could not be detected.

The products of the hydrolysis were identified tentatively as Asn-$(GlcNAc)_2$ and mannose (Sukeno *et al.*, 1971). The latter has been characterized more definitively since then by the carbohydrate analysis procedure of Walborg *et al.* (1969).

TABLE IV: Substrate Specificity of Hen Oviduct α-D- and β-D-Mannosidases.

Substrate	Relative Rate: α-D-Mannosidase (%)	Relative Rate: β-D-Mannosidase (%)
p-Nitrophenyl α-D-mannopyranoside[a]	100	0
p-Nitrophenyl β-D-mannopyranoside[b]	0	100
Methyl α-D-mannopyranoside	0.25	0
Methyl β-D-mannopyranoside	0	0.73
α-D-Man(1→4)GlcNAc	0.48	0
α-D-Man(1→6)GlcNAc	0.27	0
Asn-$(GlcNAc)_2(Man)_1$[c]	0	25
$(GlcNAc)_2(Man)_1$[d]	0	33

[a] Hydrolyzed at a rate of 4.4 μmoles/min per ml of enzyme. [b] Hydrolyzed at a rate of 6.4 μmoles/min per ml of enzyme. [c] From ovalbumin. [d] Prepared by glycosyl asparaginase treatment of Asn-$(GlcNAc)_2(Man)_1$.

Conclusions

Lysosomal enzymes capable of degrading polysaccharide chains of glycoproteins and gangliosides to their individual monosaccharides have been found in most tissues. Their importance is emphasized by the finding that a genetic deficiency in one of these enzymes, as in the case of the hexosaminidase A of Tay-Sachs disease (O'Brien *et al.*, 1970), can have fatal consequences. Thus, it was not surprising to find

[2] Incubation of Asn-$(GlcNAc)_2(Man)_5$ in a 0.5-ml reaction mixture with 7 units of β-D-mannosidase for 3 days hydrolyzed only 0.31 mole equiv of mannose. This amount of enzyme is sufficient to completely release mannose from Asn-$(GlcNAc)_2(Man)_1$ in a few minutes.

that another mannosidase was present in animal tissues capable of hydrolyzing the mannosyl residue in the glycosylasparagine moiety of glycoproteins that were resistant to hydrolysis by α-D-mannosidase. This enzyme, as amply demonstrated, is specific for β anomers of mannose, thus providing an explanation for the resistance of Asn-$(GlcNAc)_2$-$(Man)_1$ to α-D-mannosidases from jack bean meal and hen oviduct.

In addition to RNase B and ovalbumin (Sukeno *et al.*, 1971), such glycoproteins as pineapple stem bromelein (Lee, 1971), *Aspergillus oryze* α-amylase (Lee, 1971), and porcine thyroglobulin (Fukuda and Egami, 1971) appear to contain mannose residues resistant to hydrolysis by jack bean α-D-mannosidase. In other cases, such as taka-amylase A (Yamaguchi *et al.*, 1970), IgG (Kornfeld *et al.*, 1971), IgA (Dawson and Clamp, 1968), IgM (Johnson and Clamp, 1971), bovine fibrinogen (Mester, 1968), α_1-acid glycoprotein (Sato *et al.*, 1967), silk fibroin (Sinohara *et al.*, 1971), DNase (Salnikow *et al.*, 1970), fetuin (Spiro, 1964), and probably many others where a proximal mannose is associated with di-*N*-acetylchitobiose or *N*-acetylglucosamine, the mannose may be linked in the β configuration.[3] This possibility should at least be considered in view of the findings presented in this paper. Until the present time, most, if not all, of the mannose residues in glycoproteins were believed to be in the α configuration on the basis of α-D-mannosidase digestion studies. Most of these studies employed extensive incubation periods, some for as much as several days. During this time, minute quantities of contaminating glycosidases could obscure the nature of the monosaccharides hydrolyzed and as a consequence lead to erroneous interpretations. It is for this reason that an α-D-mannosidic linkage was suggested for Asn-$(GlcNAc)_2(Man)_1$ (Lee, 1971), and for glycopeptides from IgG (Kornfeld *et al.*, 1971), and IgM (Johnson and Clamp, 1971). What significance can be attached to a β-D-mannose residue and the apparent specificity of its location is still to be uncovered. In any event, the isolation of a β-D-mannosidase free of contaminating glycosidases should be useful in structural studies on oligosaccharides.

Acknowledgments

We express our appreciation to Dr. Angelo Lobo of our Division for his aid with the infrared analysis and to Dr. Yash Myer of the Department of Chemistry, State University of New York at Albany, for the optical rotatory dispersion measurements.

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[3] We were recently informed by Dr. C. H. W. Hirs that two β-mannosyl residues linked to the Asn-$(GlcNAc)_2$ moiety in porcine ribonuclease were found in his laboratory (Kabasawa and Hirs, 1972). However, recent findings by Yamaguchi *et al.* (1971) indicate that all of the mannosyl residues in taka-amylase A glycopeptide, Ser-Asn-$(GlcNAc)_2(Man)_6$ are α linked. While this may be the case, the 12-day incubation used by these workers with an α-D-mannosidase preparation containing several contaminating glycosidases, including β-D-mannosidase, tends to cloud their results.

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Diphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase from Pig Heart. Characterization of the Active Substrate and Modes of Regulation†

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ABSTRACT: This paper explores the hypothesis that only one of the ionic forms of isocitrate participates in the metal-dependent reaction catalyzed by DPN-specific isocitrate dehydrogenase and suggests that activators can function indirectly by modifying the distribution of the various forms of isocitrate. Determinations of the Michaelis constants for DPN and total isocitrate at 1.25 mM $MnSO_4$ showed that the K_m for DPN was relatively constant, but that the K_m for total isocitrate increased nearly 100-fold as the pH was raised from 6.0 to 8.0. Higher concentrations of $MnSO_4$ increased the K_m for total isocitrate at each pH. An analysis of the distribution of the various forms of isocitrate, facilitated by a computer program, revealed that the K_m for dibasic isocitrate (approximately 30.0 μM) was independent of both pH and manganese concentration. The observed variation in the K_m for total isocitrate can therefore be explained as a reflection of differential modifications of the equilibria between the ionic forms of isocitrate caused by changes in the concentrations of hydrogen and manganous ions. Free dibasic isocitrate is the actual substrate of the enzyme. Substrate inhibition, noted at pH 6, but not at 7 and 8, could be attributed to the binding of the manganous complex of dibasic isocitrate. The change of V_{max} with pH indicates the requirement for a basic form of an amino acid in the enzyme–substrate complex with a pK of 6.25. The DPN-dependent isocitrate dehydrogenase is activated by both citrate and nucleotides. "Apparent activation" results when chelating agents such as citrate, GDP, and UDP lower the K_m for total isocitrate by raising the relative proportion of the active species of isocitrate; the K_m calculated for dibasic isocitrate is unchanged. In contrast, ADP lowers the K_m for the active form of isocitrate, as well as for total isocitrate, suggesting that this nucleotide functions both as a chelating agent and as a specific allosteric modifier of enzyme activity. The demonstration that modifiers of enzyme activity can function indirectly, *via* their chelating ability, provides an alternate mechanism to that of allosteric regulation which should be considered when evaluating the kinetics of a metal-dependent enzyme.

The kinetics of the DPN-dependent isocitrate dehydrogenases [*threo*-D_s-isocitrate:NAD-oxidoreductase (decarboxylating), EC 1.1.1.41] have been extensively studied (Plaut and Aogaichi, 1968; Atkinson *et al.*, 1965; Sanwal and Cook, 1965). These enzymes, however, require divalent metal ions for activity and in the past the role of metal chelation of substrates has not generally been considered. Under physiological conditions, the substrate isocitrate exists in equilibrium with both hydrogen and metal ion and is present in both its dibasic and tribasic forms and their corresponding metal chelates (Grzybowski *et al.*, 1970). It seemed possible that only one of these four ionic species would specifically react with the enzyme. Any variation, therefore, in the concentrations of hydrogen and metal ions might alter the affinity of the enzyme for total isocitrate, but not for the active species of substrate. The active substrate for the pig heart TPN-dependent isocitrate dehydrogenase has previously been shown to be the metal chelate of the tribasic form of isocitrate (Colman, 1972c). A distinction in the form of isocitrate utilized by the two isocitrate dehydrogenases from the same organ and species might suggest a complementary role for the two enzymes *in vivo*.

The DPN-specific isocitrate dehydrogenases from several species have been shown to be activated by citrate and/or adenine nucleotides. In general, citrate modifies those dehydrogenases extracted from plants (Cox and Davies, 1967; Coultate and Dennis, 1969), while the adenine nucleotides modify the mammalian enzymes (Chen *et al.*, 1964; Stein *et al.*, 1967). Both citrate and adenine nucleotides, however, activate the enzymes from yeast and microorganisms (Hathaway and Atkinson, 1963; Sanwal and Cook, 1965; LéJohn *et al.*, 1969). Despite the fact that these positive effectors are all chelating agents, the possibility that they function indirectly by altering the distribution of the ionic forms of isocitrate in solution has not previously been explored. This paper reports kinetic data for the DPN-dependent isocitrate dehydrogenase from pig heart which not only allows the identification of the actual substrate of this reaction, but also analyzes the roles of different activators in terms of their effect on the Michaelis

† From the Biochemical Research Laboratory, Massachusetts General Hospital and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts. *Received October 14, 1971.* This work was supported by U. S. Public Health Service Grants 1-R01-AM14635 and RR 05486, The Medical Foundation, Boston, Mass., and U. S. Public Health Service Career Development Award 7-K4-GM-9576 to R. F. C. A preliminary report has been presented (Cohen and Colman, 1971a).