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INTERACTION BETWEEN CONCANAVALIN A AND BRAIN LYSOSOMAL ACID HYDROLASES

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SUMMARY

All the five sheep brain lysosomal acid hydrolases tested, namely arylsulphatase A, acid phosphatase, β-*N*-acetylhexosaminidase, β-galactosidase and β-glucuronidase bind with Concanavalin A and form enzymatically active precipitate. This enzyme–Concanavalin A complex can be dissociated by α-methyl-D-glucoside and the dissociation is pH dependent; except for arylsulphatase A which dissociates maximally at pH 9.0, all other enzymes dissociate maximally at pH 4.0. The enzyme–Concanavalin A complex formation is inhibited by α-methyl-D-glucoside. Using the differential dissociation of the enzyme–Concanavalin A complex, these enzymes have been purified to the extent of 30–180-fold over the soluble lysosomal fraction. The dissociation of the enzyme–Concanavalin A complex and the inhibition of its formation by α-methyl-D-glucoside suggest the glycoprotein nature of the enzymes.

INTRODUCTION

Earlier studies from our laboratory have shown that all the six lysosomal acid hydrolases tested, namely the arylsulphatases A and B (arylsulphate sulphohydrolase, EC 3.1.6.1), acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), β-*N*-acetylhexosaminidase (chitobiose acetamide deoxyglucohydrolase, EC 3.2.1.29), β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) and β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) from rat brain bind with Concanavalin A which is linked to an insoluble sepharose matrix [1]. Even at a high salt condition, where electrostatic interaction is a minimum, the addition of Concanavalin A to a crude mixture of soluble lysosomal enzymes results in the precipitation of the enzymes containing 60–90% of the total lysosomal enzymes. These are due to the binding which may take place through specific interaction between saccharides present in enzymes and Concanavalin A, as these enzymes are thought to be glycoprotein in nature. Moreover, protein–protein interaction might contribute significantly in the stabilisation of the lysosomal enzyme-Concanavalin A complex. Thus it is important to know to what extent protein–protein interactions, if any, are involved in stabilising the enzyme–Concanavalin A complex under varying conditions of pH, ionic strength and temperature. Information of this kind would

help one to find out the optimum condition necessary, for the separation of various lysosomal enzymes. These would further elucidate the glycoprotein nature of these lysosomal enzymes and at the same time would help to understand the nature of the chemical specificity that is involved in the stabilisation of the glycoprotein–lectin complex. Recently, several investigators have shown that the glycoprotein–lectin interaction is basic to many processes involving recognition of the cell surface [2].

The present paper describes the binding characteristics in detail of the sheep brain lysosomal acid hydrolases with Concanavalin A under various conditions of ionic strength, pH and in the presence and absence of Concanavalin A-specific sugar namely α-methyl-D-glucoside. A part of this work has been reported earlier [3, 4]. The acid phosphatase–Concanavalin A complex is least stable in the lower pH values in contrast to the arylsulphatase A–Concanavalin A complex and this behaviour has been used for further purification of these enzymes.

MATERIALS AND METHODS

Water-soluble Concanavalin A was prepared by the method described earlier [1].

Preparation of pH 5.0 supernatant enzymes

The crude soluble lysosomal fraction from sheep brain was prepared essentially according to the method reported elsewhere [1]. pH 5.0 supernatant enzymes were prepared by suspending the crude mitochondrial fraction which also contained lysosomes in a volume of 0.02 M Tris–HCl buffer (pH 7.4) equal to half the weight of original fresh tissue. The lysosomes were ruptured by repeated freezing and thawing and then centrifuged at 12 000 × *g* for 30 min. The turbid supernatant solution was adjusted to pH 5.0 with 1 M acetate buffer (final concn 0.05 M) and kept at 37 °C for 15 min and then again centrifuged. The clear supernatant was then dialyzed overnight against 0.02 M Tris–HCl buffer (pH 7.4) and this enzyme preparation was called pH 5.0 supernatant enzyme and was used in this study except where it was indicated.

The enzymes have been assayed by the methods described earlier [1]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of product in 1 h under the assay conditions. The specific activity was defined as that unit of enzyme activity per mg of protein, protein being estimated by the method of Lowry et al. [5] using crystalline bovine serum albumin as standard.

The source of substrates for the different enzymes was reported earlier [1]. Sephadex was obtained from Pharmacia Chemicals, Uppsala, Sweden. All other chemicals were of analytical grade.

Titration of lysosomal enzymes with Concanavalin A

Concanavalin A was added to a solution of lysosomal hydrolases in 0.5 M NaCl. The binding between Concanavalin A and lysosomal hydrolases led to the formation of an insoluble precipitate and the amount of various enzymes present in the precipitate and supernatant was assayed for different lysosomal enzyme activity. For the precipitation study, Concanavalin A and lysosomal enzymes under appropriate conditions were incubated for 1 h at 37 °C and then centrifuged at 12 000 × *g* for 30 min and the pellets were washed once with 0.01 M Tris–HCl buffer (pH 7.0) before assaying for various enzymes.

Determination of relative stability of the lysosomal enzyme–Concanavalin A complex

The relative stability of the lysosomal enzyme–Concanavalin A complex formed has been studied either by determining (1) the amount of α-methyl-D-glucoside required for 50% dissociation of the insoluble enzyme–Concanavalin A complex both in the absence and the presence of 0.5 M NaCl (dissociation study), or (2) by the decrease in the amount of insoluble enzyme–Concanavalin A complex formed in presence of varying amounts of α-methyl-D-glucoside (inhibition studies).

In the dissociation study, the enzyme–Concanavalin A complex at the appropriate pH was incubated both in the absence and in the presence of α-methyl-D-glucoside for 1 h at 37 °C and then centrifuged. The enzyme activity in the supernatant was then measured. The amount of enzyme present in the supernatant under these conditions was the measure of degree of dissociation of enzyme–Concanavalin A complex.

The inhibition study was carried out by incubating pH 5.0 supernatant enzymes, Concanavalin A and α-methyl-D-glucoside in presence of 0.5 M NaCl at pH 7.0 for 1 h at 37 °C and then centrifuged. The enzyme activity in the pellet and supernatant was determined.

Purification of lysosomal acid hydrolases from insoluble enzyme–Concanavalin A complex

The enzyme–Concanavalin A suspension (3.75 mg protein/ml) was adjusted to pH 4.0 with 1 M acetate buffer (final concn 0.05 M) and incubated at 37 °C for 1 h in the presence of 0.3 M α-methyl-D-glucoside. It was centrifuged and the pellet so obtained was again suspended in 0.05 M Tris–HCl buffer (pH 8.0) and then incubated again at 37 °C for 1 h in the presence of 0.3 M α-methyl-D-glucoside. It was centrifuged. The supernatants obtained after dissociation at pH 4.0 and 8.0 were separately passed through Sephadex G-50 columns (2.0 cm × 20 cm) equilibrated with 0.05 M acetate buffer (pH 4.0) and Tris–HCl buffer (pH 8.0), respectively, at 0–4 °C with a flow rate of 12 ml/h.

RESULTS AND DISCUSSION

The addition of Concanavalin A to the solution of lysosomal enzymes led to the formation of an insoluble complex in the pH range of 4–8 and the extent of insolubilization was dependant on the pH of the medium.

The total recovery of the enzymes from the solid phase and solution corresponds to 90–95% of the total under all circumstances. Thus the percentage of enzyme activity remaining in solution is a direct measure of the concentration of the enzyme free in the solution, provided, of course, that the complexes that are formed are completely insoluble. All the five lysosomal enzymes studied, namely arylsulphatase A, acid phosphatase, β-*N*-acetylhexosaminidase, β-glucuronidase and β-galactosidase are precipitable under these conditions. Fig. 1 shows the amount of precipitable enzymes at pH 5.0 under a varying concentration of Concanavalin A. In the case of arylsulphatase A, 90% of the enzyme is precipitable at a ratio of pH 5.0 supernatant protein to Concanavalin A of 5:1 whereas in the case of β-*N*-acetylhexosaminidase and acid phosphatase, only 75 and 70% of the enzymes are precipitable, respectively. It is evident from the figures that complete insolubilization of the enzymes is not

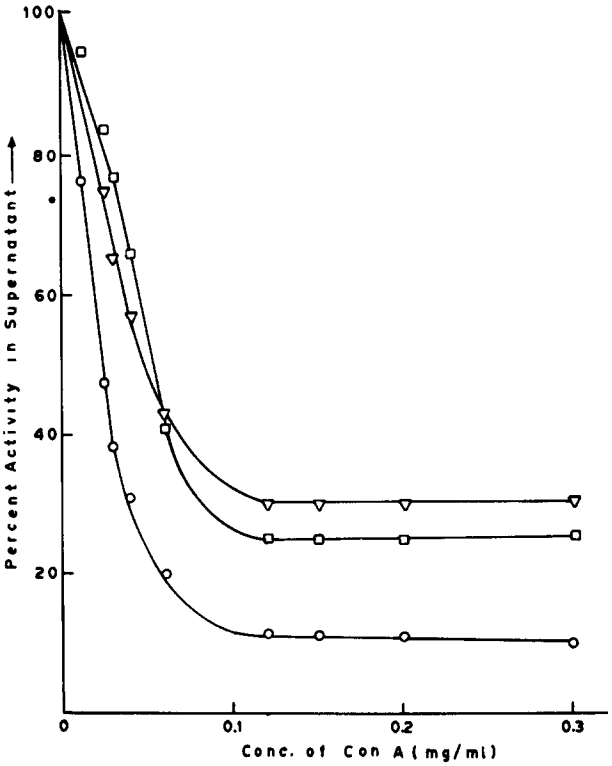

Fig. 1. Effect of Concanavalin A concentration on the formation of the insoluble enzyme–Concanavalin A complex: In a total volume of 1.5 ml, the incubation system contained 0.9 mg of pH-5 supernatant enzyme protein, 0.05 M sodium acetate buffer (pH 5.0), 0.5 M NaCl and different concentration of Concanavalin A. The rest of the conditions were the same as described in the text. Enzymes were assayed both in the pellet and supernatant after centrifugation. The enzyme activity in the supernatant in the absence of Concanavalin A was taken as 100. ○—○, arylsulphatase A; ▽—▽, acid phosphatase; □—□, β-*N*-acetylhexosaminidase.

possible even at a very high concentration of Concanavalin A and this finding could be taken to indicate the microheterogeneity of enzymes, presumably due to the difference in carbohydrate content and the nature of their distribution in various chains.

Fig. 2 shows the arylsulphatase A and acid phosphatase activities in the precipitate for a given mixture of soluble lysosomal enzyme and Concanavalin A at different pH values. It is seen from the figure that maximum precipitation occurs at pH 5.0. Three other enzymes namely β-*N*-acetylhexosaminidase, β-glucuronidase and β-galactosidase also formed a maximum precipitation with Concanavalin A at pH 5.0. The catalytic properties of the insolubilized enzymes tested remained fairly unchanged, although the K_m for acid phosphatase and β-*N*-acetylhexosaminidase bound to Concanavalin A are different from that of the free enzymes (Table I). In both cases the K_m values of the Concanavalin A-bound enzymes were 2.5–3 times more than that of the free enzymes. The addition of substrate under the assay conditions of a particular enzyme to the suspension of the insoluble enzyme–Concanavalin A complex did not lead to any release of enzyme in the supernatant; the enzyme activity in

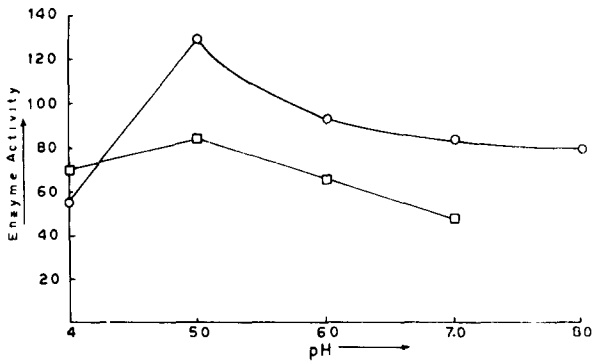

Fig. 2. The pH optima of the precipitation of acid hydrolases with Concanavalin A: In a total volume of 1.0 ml the incubation system contained 0.05 M Tris–acetate buffer of different pH values 1 4 mg of soluble lysosomal protein 0.7 mg of Concanavalin A and 0.5 M NaCl. The rest of the conditions were the same as described in Fig. 1. Enzyme activities in the pellet were assayed after suspending it in 0.01 M Tris–HCl buffer (pH 7.0). ○—○ arylsulphatase A; □—□, acid phosphatase.

the supernatant was assayed after dialysis. Thus pH 5.0 was chosen to prepare the insoluble enzyme–Concanavalin A complex for dissociation studies. For this, the insoluble enzyme–Concanavalin A complex was, however, resuspended in buffers of a different pH containing different amounts of α-methyl-D-glucoside. The amount of enzyme (%) released into the supernatant (which is the measure of the degree of dis-

TABLE I

K_m OF FREE ENZYMES AND CONCANAVALIN A BOUND ENZYMES

Standard assay conditions were maintained in measuring the enzyme activities. *p*-Nitrophenyl-phosphate and *p*-nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside were used as substrates for acid phosphatase and β-*N*-acetylhexosaminidase, respectively. K_m values were determined by plotting S/v against S. pH 5.0 supernatant enzyme was termed as free enzyme. Enzyme–Concanavalin A complex was prepared by the methods described in the text.

	Acid phosphatase (mM)	β-*N*-Acetylhexosaminidase (mM)
Free enzyme	0.2	0.8
Enzyme–Concanavalin A complex	0.6	2.0

sociation) at different pH values in the absence and presence of a fixed amount of α-methyl-D-glucoside (0.1 M) is shown in Fig. 3. It is seen from the figure that the degree of dissociation of acid phosphatase decreased with the increase in pH and was maximum at pH 4.0. At this pH 4.0, the extent of self dissociation (dissociation in the absence of α-methyl-D-glucoside) was about 16% and at a higher pH, no self dissociation occurs. In contrast to the acid phosphatase, the degree of dissociation of arylsulphatase A increased with the increase in pH and dissociated maximally at pH 9.0. In the absence of α-methyl-D-glucoside, arylsulphatase A dissociated from the insoluble complex in the alkaline range and it was 50% at pH 9.0. The other three enzymes, like acid phosphatase were also maximally dissociated at pH 4.0. The

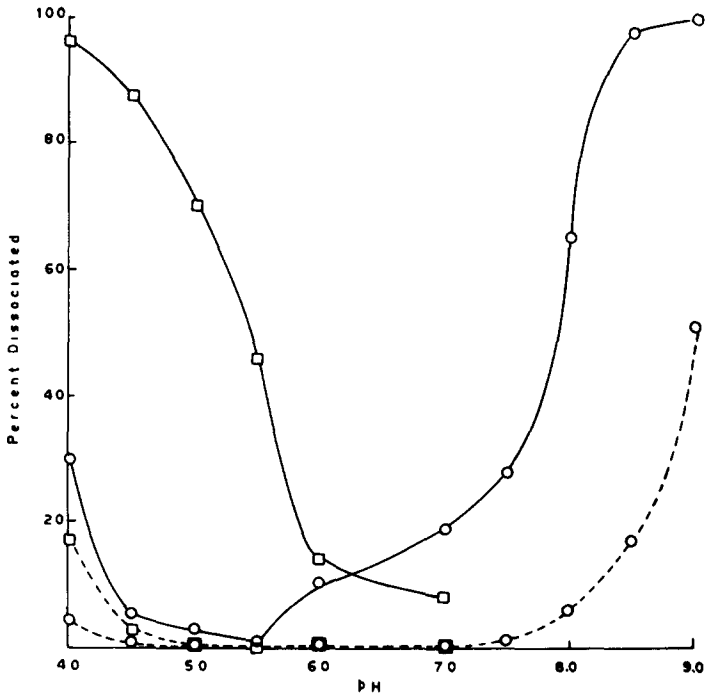

Fig. 3. Effect of pH on the α-methyl-D-glucoside-dependent dissociation of the enzyme–Concanavalin A complex: In a total volume of 0.5 ml, the incubation system contained 0 6 mg protein of enzyme–Concanavalin A complex, 0.05 M sodium acetate buffer (for pH 4.0–6.0) or Tris–HCl buffer (for pH 7.0–9.0) and 0.1 M α-methyl-D-glucoside Control tubes did not contain any α-methyl-D-glucoside. The rest of the conditions were the same as indicated in Fig. 1. Acid phosphatase in the presence (□—□), and absence (□---□) of α-methyl-D-glucoside. Arylsulphatase in the presence (○—○) and absence (○---○) of α-methyl-D-glucoside.

pH-dependent dissociation of the enzymes has been fruitfully utilized in their purification (see latter).

Fig. 4 shows the percent dissociation of acid phosphatase and arylsulphatase A in the presence of varying amounts of α-methyl-D-glucoside at different pH values. The amount of α-methyl-D-glucoside required for 50% dissociation of the enzyme–Concanavalin A complex at different pH values can be read directly from Fig. 4c. At pH 5.0 and in the presence of 0.5 M NaCl, the dissociation profile of the arylsulphatase A–Concanavalin A complex is surprisingly different from that of its absence and is shown in Fig. 5. But NaCl does not have any influence on the dissociation of the acid phosphatase–Concanavalin A complex.

When the pH 5.0 supernatant enzyme was incubated with Concanavalin A at pH 7.0 in the presence of a varying concentration of α-methyl-D-glucoside, the formation of the insoluble enzyme–Concanavalin A complex was inhibited and this is shown in Fig. 6. Since the inhibition and dissociation studies were not done at the same total concentration of lysosomal enzyme and Concanavalin A, the values of α-methyl-D-glucoside for 50% dissociation and 50% inhibition are not comparable. Nevertheless, it showed that the stabilities of the enzyme–Concanavalin A complex

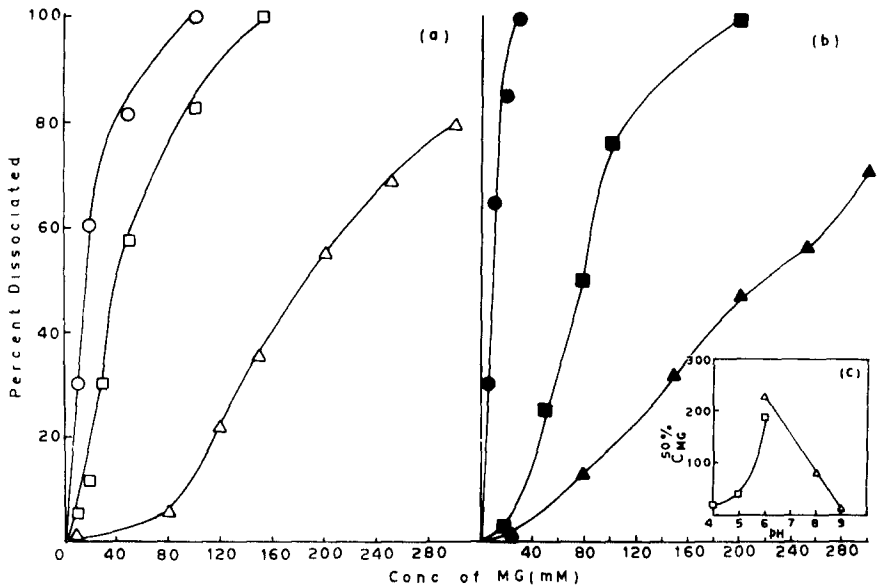

Fig. 4. Dissociation of the enzyme–Concanavalin A complex by different concentrations of α-methyl-D-glucoside (MG) at various pH values: In a total volume of 0.5 ml, the incubation system contained 0.56 mg protein of enzyme–Concanavalin A, 0.05 M Tris–acetate buffer of various pH values and different concentrations of α-methyl-D-glucoside. The rest of the conditions were the same as described in Fig. 1. (a). Acid phosphatase at pH 4.0 (○—○), 5.0 (□—□) and 6.0 (△—△). (b). Arylsulphatase A at pH 6.0 (▲—▲), 8.0 (■—■) and 9.0 (●—●). (c). Concentration of α-methyl-D-glucoside (mM) required for 50% dissociation ($c^{50\%}_{MG}$) of acid phosphatase (□—□) and arylsulphatase A (△—△) at different pH values. Corrections were made for self-dissociation in determining the percentage of dissociation in the presence of α-methyl-D-glucoside.

as measured by the amount of enzyme released in the supernatant is very much dependent on the pH and α-methyl-D-glucoside.

Arylsulphatase A, acid phosphatase, β-*N*-acetylhexosaminidase and β-glucuronidase were purified from the enzyme–Concanavalin A complex by differential dissociation of all these enzymes and both the specific activities and total activities of these enzymes are shown in Table II. It is seen from the table that acid phosphatase, β-*N*-acetylhexosaminidase and β-glucuronidase and arylsulphatase A were purified to the extent of 52-, 31-, 75- and 180-fold, respectively, over the soluble lysosomal fraction.

Concanavalin A, a phytohaemagglutinin, has the property of binding with carbohydrates and glycoproteins [6–8]. The fact that the insoluble enzyme–Concanavalin A complex can be dissociated by α-methyl-D-glucoside strongly suggests that binding between Concanavalin A and the enzymes takes place through the carbohydrate recognition site of Concanavalin A. This is further substantiated by the following observations: (i) increase in specific activity of enzymes in the enzyme–Concanavalin A complex over the pH 5.0 supernatant enzyme (Table II), (ii) increase in the neutral sugar content per mg of total protein of enzyme–Concanavalin A complex over the pH 5.0 supernatant enzymes (data not presented).

The marked effect of pH and salt on the α-methyl-D-glucoside induced disso-

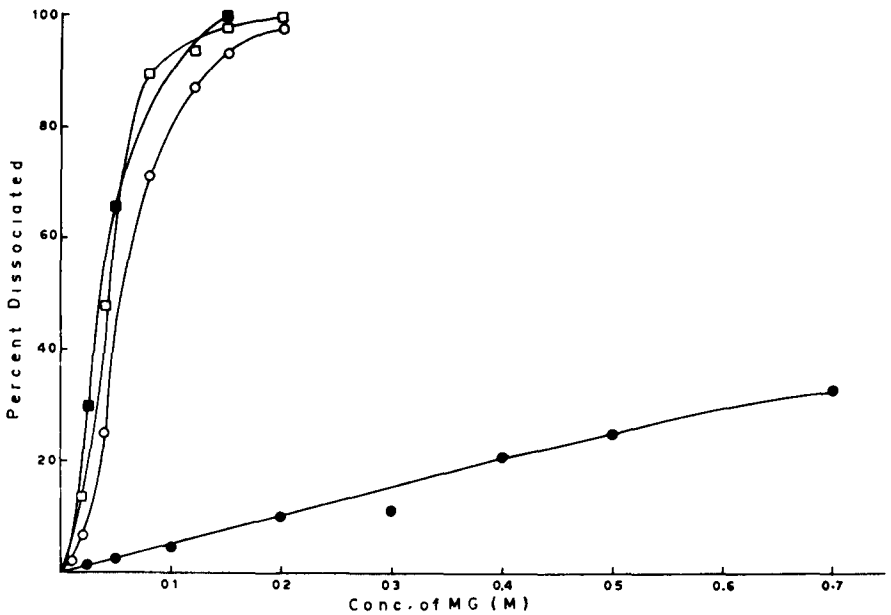

Fig. 5. Effect of NaCl on the α-methyl-D-glucoside-dependent dissociation of enzyme–Concanavalin A at pH 5.0: Conditions of the experiment were the same as described in Fig. 4 except that 0.5 M NaCl was included when indicated. Acid phosphatase in the absence (■—■) and presence (□—□) of 0.5 M NaCl. Arylsulphatase A in the absence (●—●) and presence (○—○) of 0.5 M NaCl.

ciation of the enzyme–Concanavalin A complex suggests that the protein–protein interactions are also involved in the stabilization of the enzyme–Concanavalin A complex. The electrostatic contribution towards the total stability of the enzyme–Concanavalin A complex will decrease with increasing salt concentration, if both enzyme and Concanavalin A carry charges opposite to each other. The concentration of α-methyl-D-glucoside required for a 20% dissociation of the arylsulphatase A–Concanavalin A complex at pH 5.0 in the absence of NaCl was higher than that in the presence of 0.5 M NaCl by a factor of 10 (Fig. 5).

In contrast, NaCl does not have any appreciable effect on the c α-methyl-D-glucoside (c_{MG}) for the dissociation of acid phosphatase. Furthermore, the pH for maximal binding of the enzymes with Concanavalin A (pH 5.0) is different from that reported for polysaccharides [9] (pH 6.2–7.1). This difference can be ascribed to the additional contribution of the protein–protein interaction. The data presented here are, however, not sufficient to evaluate the contribution due to carbohydrate–Concanavalin A and protein–Concanavalin A separately but our data on the rate of formation and the α-methyl-D-glucoside-induced dissociation of arylsulphatase A–Concanavalin A and acid phosphatase–Concanavalin A complexes show that the stability of the complex is of the order of 10^7–10^8 M^{-1} at 29 °C and is higher by 3–4 orders of magnitude than that of the Concanavalin A–carbohydrate complex (Bishayee, S., Bachhawat, B. K. and Podder, S. K., manuscript in preparation).

Though the stability of the enzyme–Concanavalin A complex is influenced by the pH and the salt concentration in the suspension, the dissociation of the enzyme–

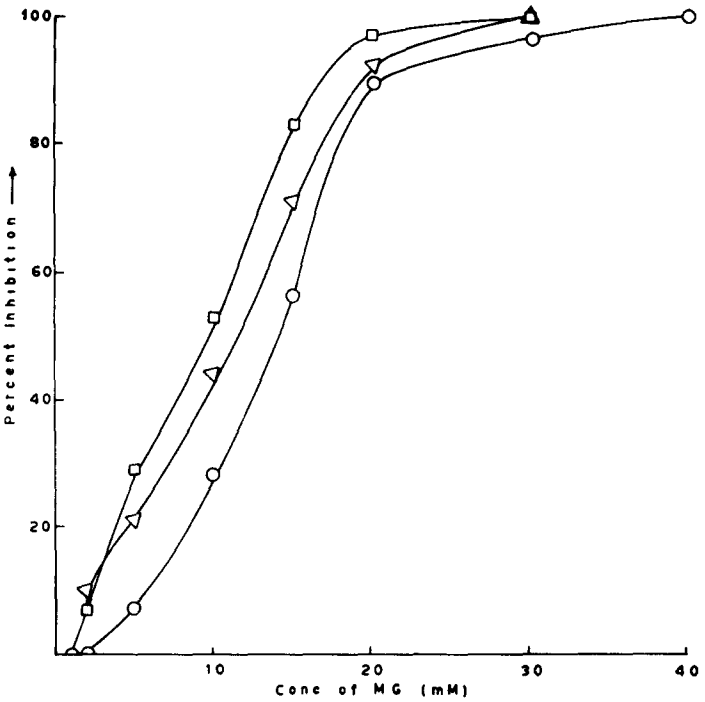

Fig. 6. Inhibition of enzyme–Concanavalin A complex formation by α-methyl-D-glucoside (MG) at pH 7 0 In a total volume of 1 5 ml, the incubation system contained 0.9 mg of pH 5.0 supernatant enzyme protein, 0.05 M Tris–HCl buffer (pH 7.0), 0.5 M NaCl and different concentrations of α-methyl-D-glucoside. The time of incubation was 1 h at 37 °C. The total enzyme activity in the pellet in the absence of α-methyl-D-glucoside was taken as 100%. The rest of the conditions were the same as indicated in Fig. 1. (○—○), arylsulphatase A; (▽—▽), β-*N*-acetylhexosaminidase; (□—□), acid phosphatase.

Concanavalin A complex by Concanavalin A-specific sugar, α-methyl-D-glucoside (Figs. 3 and 4) suggests that α-methyl-D-glucoside can displace the lysosomal enzymes from bound Concanavalin A and indicates thereby that the binding of Concanavalin A is through the carbohydrate moiety of the lysosomal enzymes. This is further proved by the inhibition of the enzyme–Concanavalin A complex by α-methyl-D-glucoside (Fig. 6). Both the dissociation of the enzyme–Concanavalin A complex and its inhibition of the formation by α-methyl-D-glucoside indicate that all the five enzymes isolated from rat kidney and liver can form a precipitate with Concanavalin A and that this precipitation is inhibited by α-methyl-D-glucoside (Bishayee, S. and Bachhawat, B. K., unpublished observation).

The enzyme–Concanavalin A complex in the solid phase is active and even under the assay conditions of the enzymes, these enzymes are not released from the complex. This also suggests that the Concanavalin A binding site and substrate binding site of the enzymes are different. However, the 2.5–3.0-fold increase in the K_m values of β-*N*-acetylhexosaminidase and acid phosphatase in enzyme–Concanavalin A complex over the free enzymes may be because of the particulate nature of the complex or it may be due to the fact that the binding of Concanavalin A to the enzymes somehow re-

TABLE II

PURIFICATION OF LYSOSOMAL ACID HYDROLASES BY INTERACTION WITH CONCANAVALIN A

Conditions of the experiments were the same as described in the text except that the soluble lysosomal fraction was prepared according to the method described earlier [1]. It was adjusted to pH 5.0 with 1 0 M sodium acetate buffer (pH 5.0) (final concn 0.05 M) and incubated at 37 °C for 15 min. It was centrifuged and the supernatant was used for preparing the enzyme–Concanavalin A suspension.

Steps	Total volume (ml)	Protein (mg/ml)	Arylsulphatase A		Acid phosphatase		β-*N*-Acetylhexosaminidase		β-Glucuronidase	
			Spec. act.	Recovery (%)	Spec. act.	Recovery (%)	Spec. act.	Recovery (%)	Spec. act.	Recovery (%)
Soluble lysosomal fraction	95.0	3.0	0.70	100	0.90	100	0.45	100	0.004	100
pH-5.0 Supernatant	100.0	1.3	1 6	95	2 0	100	0.82	85	0.008	90
Enzyme–Concanavalin A suspension	3.5	3.75	11.2	75	14.3	70	6.60	64	0.060	71
Supernatant after dissociation at pH 4.0	3.9	—	—	28	—	65	—	60	—	70
Sephadex G-50 at pH 4.0	11.7	0 16	15.0	14	46.6	30	15 0	21	0.30	50
Supernatant after dissociation at pH 8.0	2.2	—	—	42	—	—	—	—	—	—
Sephadex G-50 at pH 8.0	6.6	0.06	130.0	26	—	—	—	—	—	—

duces the affinity of the enzyme for the substrate. This type of phenomenon has also been observed with other solid supported enzymes, the effect being either to increase or decrease the substrate binding capacity [11, 12].

The method of enzyme fractionation using Concanavalin A gives a purification of about 30–180-fold over the soluble lysosomal fraction. This procedure can be used also for glycoprotein enzymes other than those mentioned here.

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