Biochimica et Biophysica Acta, 391 (1975) 349—360

BBA 67504

CHARACTERIZATION OF HUMAN α-GALACTOSIDASE A AND B BEFORE AND AFTER NEURAMINIDASE TREATMENT

GIOVANNI ROMEO, GIANFRANCO DI MATTEO, MICHELE D'URSO, SU-CHEN LI[1] and YU-TEH LI[1]

International Institute of Genetics and Biophysics, C.N.R., Via Marconi 10, Naples, (Italy) and [1]Delta Regional Primate Research Center, Covington, La. 70633, (U.S.A.)

(Received December 2nd, 1974)

Summary

It has been previously reported that following neuraminidase treatment α-galactosidase A is converted into the B form, as revealed by electrophoresis. By a variety of techniques such as isoelectrofocusing, DEAE-chromatography and by enzyme kinetic parameters, no conversion of α-galactosidase A into B, or the reverse, could be detected after neuraminidase treatment. Only an apparent transformation of α-galactosidase A into B was revealed by Cellogel electrophoresis. In addition, a discrepancy was noticed between the pattern of electrophoretic migration on starch gel and Cellogel and the net electrical charges of the two α-galactosidases as deduced by isoelectrofocusing and DEAE-cellulose. Neuraminidase treatment did not affect the activity of α-galactosidase A towards the natural substrate, ceramidetrihexoside, but the activity of α-galactosidase B decreased by about 30% under the same conditions. The two forms of α-galactosidases A and B used in this study were extensively purified by classical procedures.

Introduction

Several hydrolytic enzymes of lysosomal origin occur in multiple forms that differ in physico-chemical properties such as heat and pH stability, pH optimum and electrophoretic mobility. Analyses carried out on preparations from lysosomes of rat liver and kidney have indicated that many hydrolases are glycoproteins [1], some of which are sialoglycoproteins. Electrophoresis experiments have shown that treatment of these sialoglycoproteins or acid forms

Address to which reprint requests may be sent: Dr Giovanni Romeo, International Institute of Genetics and Biophysics, Via Marconi 10, 80125 Naples, Italy

Abbreviations: Me-Umb-Gal, 4-methyl-umbelliferyl α-galactoside; Nph-Gal, *p*-nitrophenyl-α-galactoside.

of lysosomal hydrolases are converted to the corresponding basic forms after treatment with neuraminidase [1]. This finding suggested that the multiple forms of these enzymes depend on their content in sialic acid [1]. Unfortunately, the acid forms of the hydrolases used in these experiments, as well as in many other similar ones, were only partially purified. Moreover, the crude neuraminidase preparations employed were contaminated by other hydrolytic activities.

Transformation of human α-galactosidase A (α-D-galactoside galactohydrolase EC 3.2.1.22) into B [2–4] following neuraminidase treatment has been shown by electrophoretic techniques, but immunological data contradict this conclusion [5]. The two α-galactosidases can be easily distinguished by their kinetic and structural properties. The B form is more thermostable than the A form, has a higher K_m value and migrates slower towards the anode on starch gel electrophoresis at pH 7.0 [6] or 5.0 [7]. In addition, α-galactosidase A and B possess peculiar antigenic determinants as suggested by the total [5] or partial [8] absence of cross reactivity of antisera made against the two separate enzymes. The pI values of the two enzymes are in accordance with the elution pattern on DEAE-cellulose chromatography, where the A form (pI = 5.1) is eluted first, and the B form (pI 4.9) as a second peak.

Using different techniques such as Cellogel electrophoresis, isoelectrofocusing, DEAE-cellulose chromatography and the kinetic parameters mentioned above, we found that no conversion of one enzyme form into the other takes place following neuraminidase treatment. A discrepancy was noticed between the pattern of electrophoretic migration commonly reported [6,7] and confirmed by us on Cellogel, and the electric charge properties of the two enzymes as deduced by electrofocusing and DEAE-cellulose. In addition, we found that while neuraminidase treatment does not affect the activity of α-galactosidase A towards the natural substrate, ceramidetrihexoside, the activity of α-galactosidase B decreases by about 30% under the same conditions.

Materials and Methods

The two forms of α-galactosidase A and B were purified as described in detail below and already partially reported [9]. A crude preparation of *Clostridium perfringens* neuraminidase was purified by affinity chromatography [10] and its activity was measured by the liberation of sialic acid [11] from 1 mg of bovine submaxillary mucine (Sigma) dissolved in a reaction volume of 0.3 ml at pH 5.5 [10]. An aliquot of 0.1 ml of neuraminidase, which cleaved 50% of the sialic acid bound to mucine in 3 h, was incubated with 0.1 ml of the most pure preparations of α-galactosidase A or B (see Table I) at 37° C for 16 h at pH 5.5 in the same buffer, referred to above. This mixture (0.3 ml) was dialyzed against 1% glycine if gel isoelectrofocusing [12] was performed or against 0.01 M potassium phosphate buffer pH 7.0 containing 0.03 M NaCl before DEAE-cellulose chromatography on small columns [8]. Slab gel isoelectrofocusing was prepared according to the same method used for disc gel isoelectrofocusing [12]. Electrophoresis on Cellogel (Chemetron Italy) was performed at pH 6.0 [13]. The α-galactosidase activity was revealed by incubating the gels with the fluorogenic substrate, as already described [13]. The activities of the

two α-galactosidases towards the 4-methyl-umbelliferyl α-galactoside (Me-Umb-Gal), the *p*-nitrophenyl-α-galactoside (Nph-Gal) or towards ceramidetrihexoside were measured as already reported [14—16]. One milliunit (munit) of α-galactosidase activity was defined as corresponding to 1 nmol of Me-Umb-Gal cleaved per min at 37° C. Specific activity was calculated as munits/mg of protein. Substrates were purchased from Koch-Light, England.

Purification of α-galactosidase

All the purification steps were performed at 0—4° C. Unless specified otherwise the buffers used were dilutions of 1 M potassium phosphate pH 6.5 or 7.0 as stated. The enzyme showed maximum stability at pH 6.5. Samples of human post-mortem liver, kept frozen at −20° C, were homogenized in a Waring Blender using 2 vol. of 0.05 M buffer pH 6.5. The homogenate was centrifuged at 10 000 × *g* for 30 min and to each ml of the supernatant 176 mg of solid ammonium sulfate were added. The precipitate was discarded and another 198 mg of ammonium sulfate were added for each ml of the original solution. The precipitate was dissolved in 0.05 M buffer pH 6.5 and the enzymatic activity recovered after this purification step was precipitated again by adding dropwise 1.8 ml of 95% cold ethanol for each ml of solution. The mixture was kept in an ice bath and its temperature was lowered by adding NaCl to the ice mixture until it reached −12° C. The material precipitated at 1200 × *g* for 20 min was resuspended by stirring in 0.05 M buffer pH 6.5 and centrifuged again at 16 000 × *g* for 30 min. This washing was repeated two more times and to the pooled supernatant were added 4 ml of 1 M $ZnSO_4$ per 100 ml. The precipitate was discarded, the supernatant concentrated by precipitation with ammonium sulfate (390 mg/ml) and dialyzed extensively against 0.01 M buffer pH 7.0 containing 0.04 M NaCl. The dialyzed enzyme was chromatographed on a DEAE-50 cellulose column (2.5 × 100 cm), pre-equilibrated with the same buffer used for the dialysis. To elute separately the two forms of α-galactosidase a linear gradient of NaCl was used, from 0.04 M to 0.1 M for 24 h, followed by a second one from 0.1 to 0.4 M for 24 h. The fractions containing enzymatic activity were pooled in two batches (A and B) and precipitated with ammonium sulfate (390 mg per ml). The pellet was resuspended in the smallest possible volume of 0.1 M buffer pH 6.5 and passed through a column (2.5 × 100 cm) of Sephadex G-150 in the same buffer. Optimal results of this step were obtained by adding to the column no more than 75 mg of protein per run. After gel filtration the two batches of α-galactosidase A and B, containing less than 2 mg of protein/ml, were dialyzed against 1 mM sodium phosphate buffer pH 7.0 and loaded on a small column (1.4 × 2 cm) of hydroxylapatite (Clarkson Chemical Co.) previously equilibrated with the same buffer. The maximum load for such a column was 50 mg of protein. After washing the column with 1 mM sodium phosphate pH 7.0 the enzyme was eluted with 5 mM and 10 mM buffer, stepwise. The B form came out with the 5 mM buffer while the A form required the 10 mM buffer to complete the elution. The active fractions were concentrated to a small volume by countercurrent dialysis against dry Sephadex G-150. Used hydroxylapatite could be regenerated with 0.2 M buffer, pH 8.0, and then reequilibrated with

TABLE I

PURIFICATION OF α-GALACTOSIDASE

	Vol. (ml)	Protein (mg/ml)	Activity (munits/ml)	Spec. act.	Total Act.	Purif. (-fold)	Yield (%)
Crude extract (10 000 × *g* supernatant from 600 g of liver)	1500	21.9	24	1.1	36091	—	—
After $(NH_4)_2SO_4$ fractionation	180	43.1	146	3.4	26347	3.1	73
After ethanol precipitation	143	9.0	126	14.0	18048	12.7	50
After $ZnSO_4$ precipitation	146	6.7	119	17.7	17326	16.0	48
After DEAE-cellulose chromatography							
A	304	0.5	30	60.0	9070	58.4	25
B	180	1.3	23	17.7	4100	16.0	11
α-galactosidase A							
After Sephadex G-150 chromatography	53	0.4	102	255	5400	232	15
After Hydroxylapatite chromatography (concentrated)	12	0.24	232	967	2786	878	8
After Ecteola-cellulose chromatography*	40	0.015	43	2867	1722	2606	5
α-galactosidase B							
After Sephadex G-150 chromatography	41	1.4	66	47	2700	43	8
After Hydroxylapatite chromatography (concentrated)	8	0.7	172	246	1372	223	4

* This step was performed in only one batch of α-galactosidase A, following a published procedure [19].

the starting buffer. The same column was not used for more than three times. An outline for a typical purification procedure is shown in Table I.

Results

Following neuraminidase treatment each sample of α-galactosidase was analyzed by the different procedures described below, together with appropriate controls. No α-galactosidase activity was contributed by the neuraminidase preparation, as shown by the absence of enzymatic reaction incubating neuraminidase with Me-Umb-Gal for 4 h under the conditions described [14].

Cellogel electrophoresis

The two forms of α-galactosidase could be distinguished on Cellogel electrophoresis at pH 6.0 [13], and, as shown in Fig. 1, the A form migrated faster than the B. This difference in migration disappeared when a higher pH (0.05 M potassium phosphate buffer pH 7.5) was used.

The α-galactosidase A after treatment with neuraminidase migrated as α-galactosidase B on Cellogel at pH 6.0, while no change in mobility was

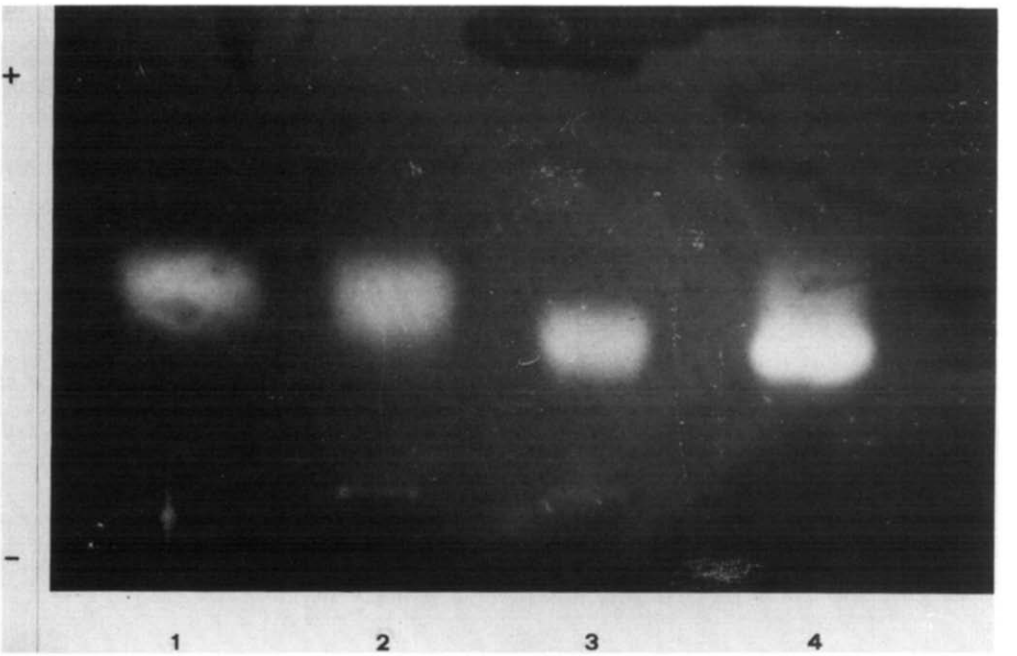


Fig. 1. Effect of neuraminidase treatment on α-galactosidase A as revealed by Cellogel electrophoresis. (1) α galactosidase A; (2), α-galactosidase A plus neuraminidase added at the end of the incubation; (3), α-galactosidase A incubated with neuraminidase; (4), α-galactosidase B. The conditions for the neuraminidase treatment and for the staining of α-galactosidase activity are indicated under Materials and Methods.

noticed for the control to which neuraminidase was added at the end of the incubation (Fig. 1) or for the α-galactosidase B treated in the same way.

DEAE-cellulose chromatography

Purified preparations of α-galactosidase A and B did not show any degree of interconversion after neuraminidase treatment as shown by the elution profiles on DEAE-cellulose columns. The results of two different experiments are plotted in Figs 2 and 3. Since the two forms of α-galactosidase are easily distinguishable on the basis of their different K_m values and different thermolability rates, each peak of α-galactosidase activity from these experiments was tested for the above parameters. No significant change was found in the K_m values of α-galactosidase A or B before and after treatment with neuraminidase (Fig. 4). It is likely that the peak 2 of α-galactosidase A (Fig. 2) was contaminated by α-galactosidase B, as suggested by its K_m value (Fig. 4). The kinetics of thermal inactivation for the two enzymes were not altered by the neuraminidase treatment, the A form remaining thermolabile and the B form thermostable at 51° C for 45 min.

Gel isoelectrofocusing

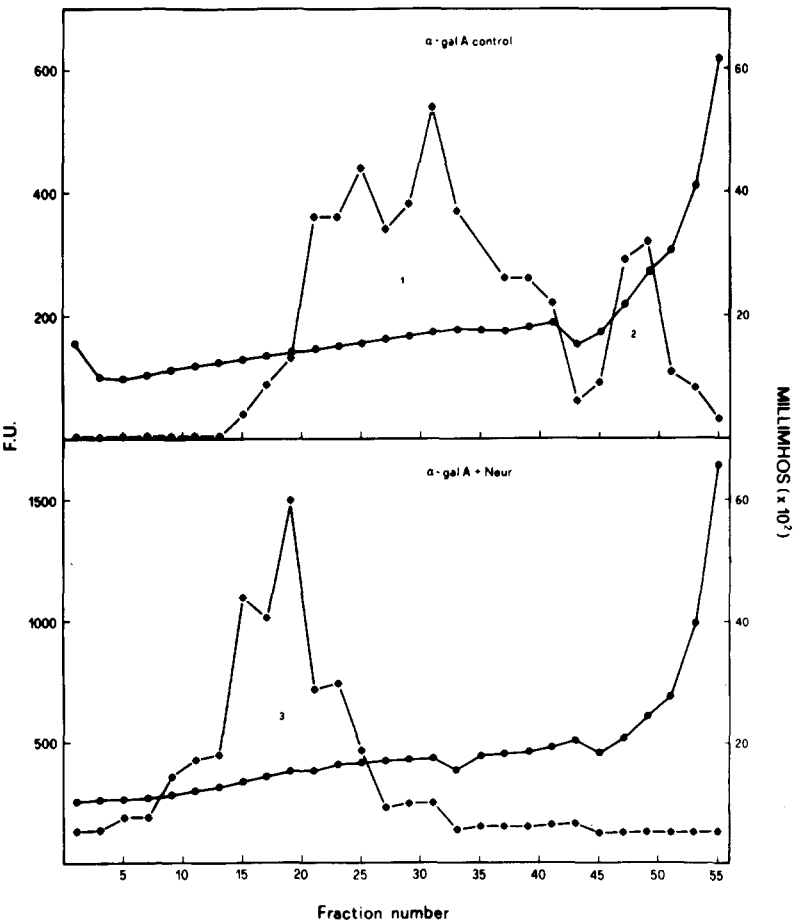
Isoelectrofocusing on acrylamide slab gel, performed as described under Materials and Methods, yielded a clear separation of α-galactosidase A and B as

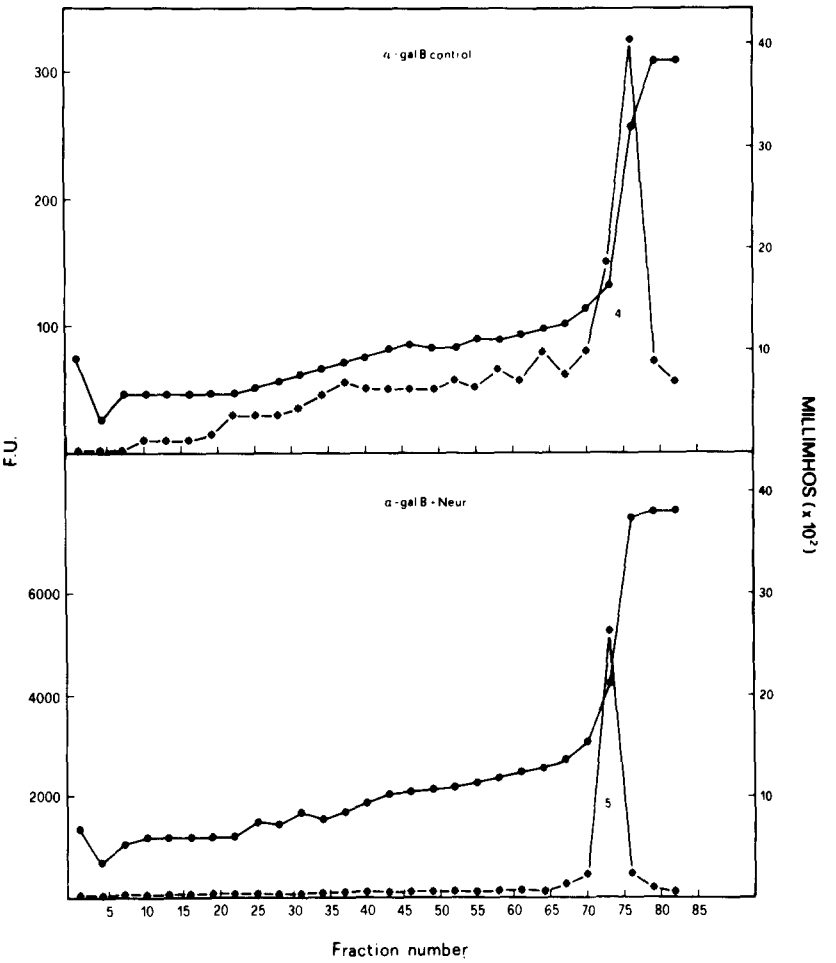
shown in Fig. 5, but no evidence for conversion of these two enzymes following neuraminidase treatment was obtained. The pI of α-galactosidase B was more acidic (4.9) than that of the A form (5.1), as confirmed by disc isoelectrofocusing in the same pH range (Fig. 6) and assaying with both Me-Umb-Gal and Nph-Gal.

Following neuraminidase treatment the pI values of the two enzymes did not change with respect to controls, as shown in Fig. 7. Although the enzyme units used in these experiments were the same for the sample treated with neuraminidase and for its untreated control, a stimulation of α-galactosidase activity seemed to take place in the neuraminidase treated samples. Since, as indicated above, no detectable α-galactosidase activity was contributed to any of the two enzymes by the neuraminidase preparation, the stimulatory effect of a common protein like albumine was tested. The results reported in Fig. 8 showed a more marked stimulatory effect towards α-galactosidase B than A at the concentrations of albumine used.

Activity towards ceramidetrihexoside

A typical experiment regarding the effect of neuraminidase treatment on





Figs 2 and 3. Elution profiles of α-galactosidase A and B from DEAE-cellulose chromatography. Samples of the two enzymes incubated with neuraminidase for 16 h (lower half) or additioned with neuraminidase at the end of the incubation (upper half) were dialyzed as indicated in the text and loaded on small DEAE-cellulose columns (1 X 16 cm). A reproducible gradient of NaCl concentration, measured in units of conductivity (millimho), was obtained mixing in an LKB gradient maker (Ultrograd 11300) two solutions of NaCl (respectively 0.03 M and 0.4 M) in 1 mM phosphate buffer pH 7.0. F.U., Fluorescent Units from the α-galactosidase assay, performed as indicated in the text. Each peak from the two experiments is numbered and the correspondent K_m values are reported in Fig. 5. ●———●: Conductivity.

the activity of the two α-galactosidases towards ceramidetrihexoside is reported in Table II. This treatment decreased the catalytic activity of α-galactosidase B, but not A, towards ceramidetrihexoside, while no effect was noticed towards Me-Umb-Gal.

Discussion

The evidence presented in this study contradicts previous reports concerning the conversion of α-galactoside A and B by neuraminidase treatment [2—4] and confirms that only an apparent transformation of α-galactosidase A into B

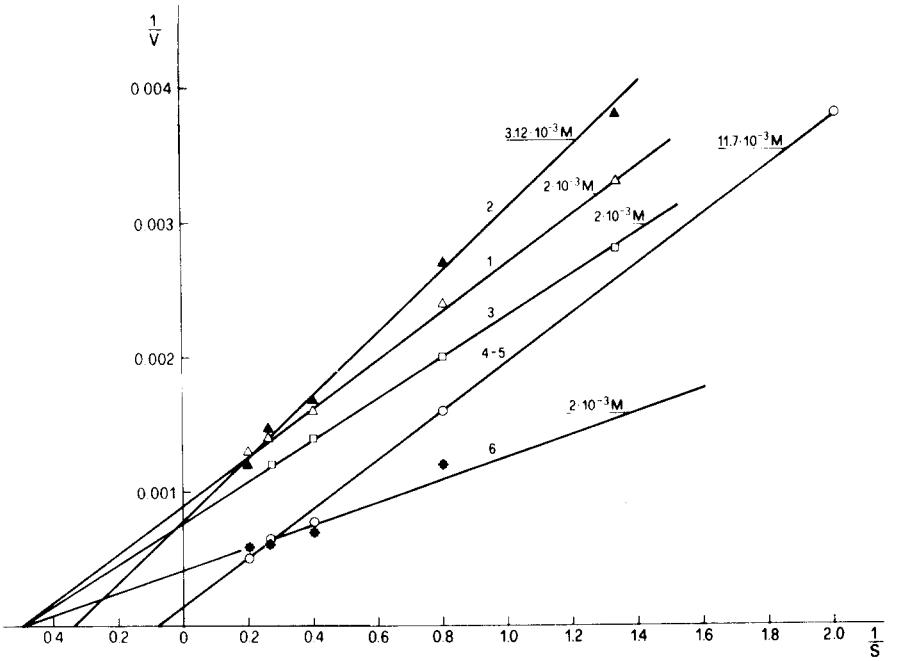


Fig. 4. Apparent K_m values towards Me-Umb-Gal of the different peaks of α-galactosidase activity from the experiments represented in Figs 2 and 3. The K_m values calculated by the Lineweaver and Burk method are indicated for each peak (1 to 5) and for α-galactosidase A (6).

is manifested if one uses electrophoresis as the discriminating condition [5]. This particular phenomenon was caused only by neuraminidase treatment since in the control experiments in which neuraminidase was added at the end of the incubation the electrophoretic migration of the A enzyme remained unchanged as shown in Fig. 1. Up to now little attention has been paid, however, to the

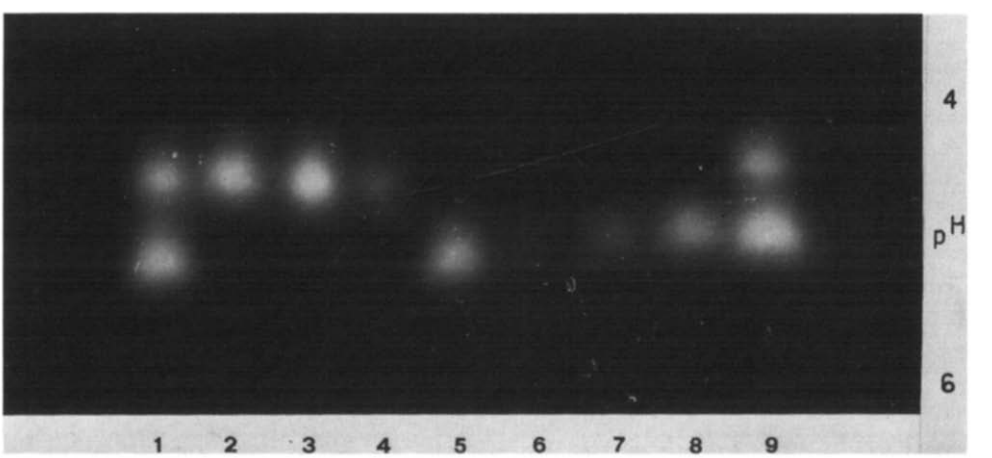


Fig. 5. Isoelectrofocusing on acrylamide slab gel. 1 and 9, mixtures of α-galactosidase A and B; 2—4 α-galactosidase B incubated at 37°C without (2) or with (3) neuraminidase or kept at 4°C (4); (7), α-galactosidase A kept at 4°C or incubated with (5) or without (8) neuraminidase at 37°C; Slot 6 was empty. The conditions of this experiment were the same as those indicated for the experiment of Fig. 1.

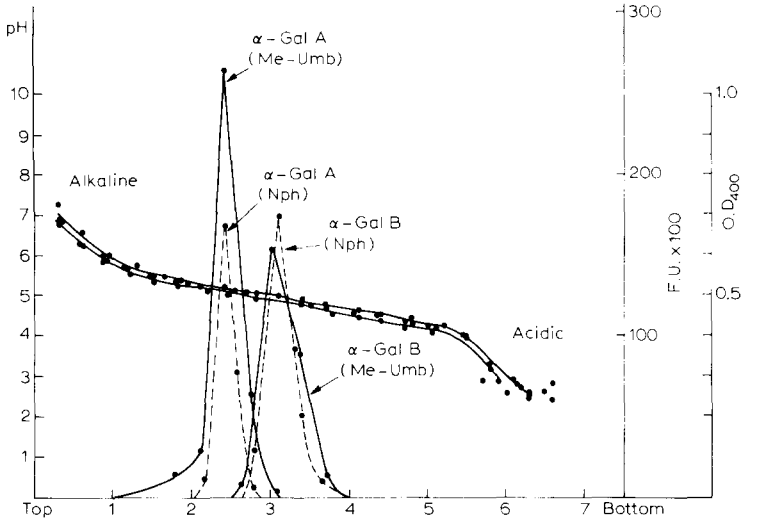


Fig. 6. Values of pI of α-galactosidase A and B determined by isoelectrofocusing on disc-gel prepared as described in the text. The gels were cut in half longitudinally and slices of 3 or 4 mm were equilibrated with 0.3 ml of 0.1 M acetate buffer pH 4.6 or with 0.5 ml H_2O overnight at 4° C. These mixtures were assayed for enzymatic activity and pH, respectively. F.U. fluorescent units from the Me-Umb-Gal assay (30 m) and $O.D._{400}$, readings from the Nph-Gal (16 h).

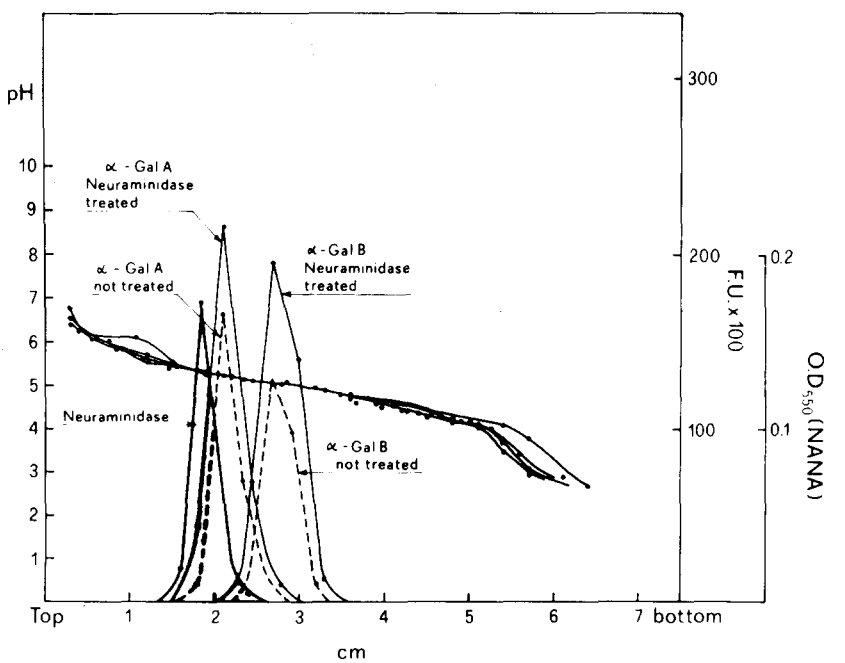


Fig. 7. Isoelectrofocusing on disc-gel of α-galactosidase A and B before and after treatment wit[1]. neuraminidase. The pI of neuraminidase, assayed by the liberation of *N*-acetyl-neuraminic acid (NANA from mucin as described in the text, is shown to differ from those of either α-galactosidase. F.U fluorescent units. The conditions for the assay of activity and pH were those described under Fig. 6.

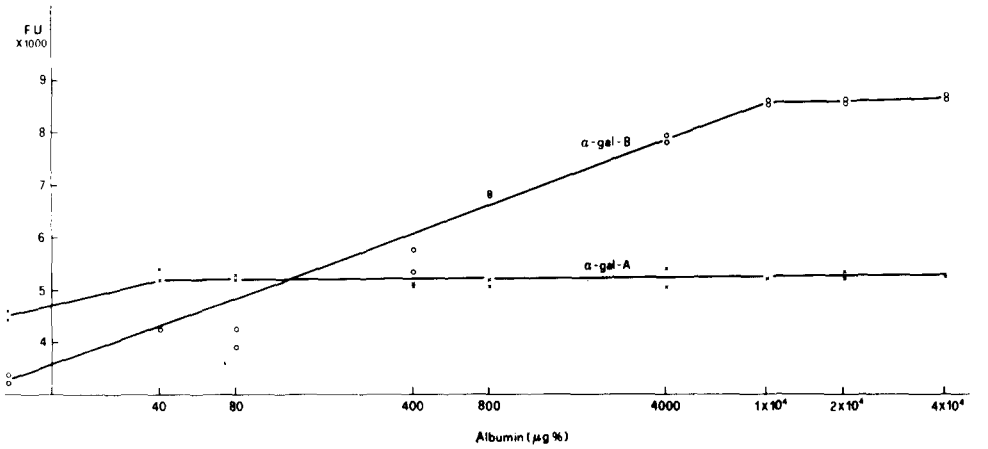


Fig. 8. Effect of increasing concentrations of albumine on the activity of the most pure preparations of α-galactosidase A and B (see Table I) assayed with Me-Umb-Gal.

discrepancy between the observed electrophoretic migration and that expected on the basis of the pI values of these two enzymes. At a pH higher than the isoelectric points of these two enzymes the B form should move more anodically than the A, as its pI value is slightly more acidic than that of the A form. This situation is exemplified by another lysosomal hydrolase, β-hexosaminidase whose A form (pI = 5.0) and B form (pI = 7.3) are respectively fast- and slow-moving on starch-gel electrophoresis [17], and are eluted from DEAE-cellulose chromatography as peak I (B form) and II (A form) [18]. The small difference in pI values between α-galactosidase A (5.1) and B (4.9), found in this study and in accordance with previous reports [19,20], agrees with the separation of the two enzymes on DEAE-cellulose chromatography where A is eluted as the first peak and B as the second [19,9]. The denomination of α-galactosidase A, on the other hand, was first related to its fast electrophoretic migration on starch gel [6,7] with respect to the slow-moving B form. This

TABLE II

Effect of neuraminidase treatment on the catalytic activity of α-galactosidase A and B towards Me-Umb-Gal and ceramidetrihexoside. An aliquot of 3 μl was taken from the reaction mixture during the incubation with neuraminidase and assayed at the times indicated using Me-Umb-Gal as substrate. Ceramidetrihexoside assay was performed according to a procedure already described [16] after a 24 h incubation with neuraminidase.

Enzyme	Me-Umb-Gal				Ceramidetrihexoside assay
	Fluorescent Units				(cpm)
	0	4 h	8 h	23 h	24 h
α-Galactosidase A	8800	11300	11500	10600	761
+ neuraminidase	9400	11000	12000	10800	738
α-galactosidase B	11100	11600	11700	11500	244
+ neuraminidase	11800	12300	12000	11600	174

denomination is now commonly used to define the thermolabile A form, which is deficient in Fabry's disease [6,7,14] and inhibited by *myo*-inositol [21] as different from the thermostable B form, characterized by its higher K_m value [19], and by its lack of inhibition by *myo*-inositol [21,22]. One should be aware, however, that it is the B enzyme that is the more acidic form and not the A, as previously believed [2]. The anomalous electrophoretic migration cannot be explained on the basis of molecular sieving, since both forms have molecular weights of 150 000 [19], and could be dependent on the supporting media such as the starch gel [6,7] or the Cellogel used in this and in another study [20]. A possible explanation of this discrepancy could be that these two enzymes, although similar with respect to their molecular weights, interact differently with the supporting media (Cellogel and starch gel) because of their dissimilar conformation (e.g. ovoidal versus spherical) whose effect is not felt in a system like electrofocusing. In turn, the treatment with neuraminidase could affect only this interaction with the supporting media and not the pI on electrofocusing. A more detailed study on the structural properties of these two enzymes is required to substantiate this hypothesis.

An additional effect of neuraminidase treatment on α-galactosidase A is the one shown in Fig. 2. A considerable degree of molecular heterogeneity of α-galactosidase A on DEAE-cellulose chromatography had already been noticed [8] and the present experiments suggest that this heterogeneity can be attributed to the different number of sialic acid residues, since it is significantly reduced by neuraminidase treatment. A similar hypothesis has been proposed to account for the differences in mobility of α-galactosidase A from different human tissues [23].

Finally, the observation that, after neuraminidase treatment, only α-galactosidase B loses some of its activity towards ceramidetrihexoside, and the different stimulatory effect by albumine on the two enzymes, is suggestive again of the different kinetics of these two α-galactosidases. On the other hand, the substrate specificities of the two forms of α-galactosidase towards ceramidetrihexoside and melibiose previously reported [19,24] have now been disproved [9,16,20], since both enzymes can cleave these two substrates, although with different kinetics [9,16].

The hydrolytic activity of both α-galactosidase A and B towards ceramidetrihexoside was manifest only in the presence of a proper activator [25] which was always added to the reaction mixture as described in detail elsewhere [16]. In the absence of this activator and in the presence of a detergent like sodium cholate, the activity towards ceramidetrihexoside seems to be much higher for the thermolabile α-galactosidase A purified from placenta than for the corresponding thermostable B form [26].

On the basis of this study it can be excluded that the difference between α-galactosidase A and B is due to the differences in the content of their sialic acid residues. The same conclusion has been reached recently for β-hexosaminidase A [27] which had been previously reported to be transformed into B form by neuraminidase treatment [28]. Therefore, the general hypothesis stating that the correlation between acid and basic forms of lysosomal hydrolases consists in the number of sialic acid residues attached to these enzymes is not confirmed at least for two different glycosidases.

Acknowledgments

We thank Dr E. Macchia of the University of Naples for a generous gift of a crude *Cl. perfrigens* neuraminidase preparation and Dr P. Cuatrecasas for a gift of *N*-(*p*-aminophenyl)-oxamic acid linked to agarose beads, and Mr A. de Falco for his skilful technical assistance. Supported by grants from the Italian National Research Council (CNR), National Institutes of Health NS 09626 and the National Foundation March of Dimes. Part of this work was performed while Drs S.C. Li and Y.T. Li were visiting scientists in Naples under a cooperative project sponsored by the U.S. National Science Foundation and the Italian C.N.R.

References

1 Goldstone, A., Konecny, P. and Koenig, H. (1971) FEBS Lett. 13, 68–72
2 Kint, J A. and Huys, A. (1971) Arch. Int. Phys. Biochem. 79–83
3 Ho, M.W., Beutler, S., Tennant, L. and O'Brien, J.S. (1972) Amer. J. Hum. Gen. 24, 256–266
4 Mapes, C.A. and Sweeley, C.C. (1973) Arch. Biochem. Biophys. 158, 297–804
5 Beutler, E. and Kuhl, W. (1972) Nature New Biol. 239, 207–208
6 Beutler, E. and Kuhl, W. (1972) Amer. J. Hum. Gen. 24, 237–249
7 Wood, S. and Nadler, H.L. (1972) Amer. J. Hum. Gen. 24, 250–255
8 Romeo, G., D'Urso, M., Pisacane, A., Blum, E., De Falco, A. and Ruffilli, A. (1975) submitted for publication
9 Romeo, G., D'Urso, M., Lee, Y.C., Wan, C.C., Li, S.C. and Li, Y.T. (1974) Fed. Proc. 33, 1299
10 Cuatrecasas, P. and Illiano, G. (1971) Biochem. Biophys. Res. Commun. 44, 178–184
11 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
12 Wrigley, C.W. (1972) Methods. Enzymol., Vol. 23, pp. 559–578
13 Grzeschik, K.H., Grzeschik, A.M., Benoff, S., Romeo, G. Siniscalco, M. van Someren, H., Meera Khan, P., Westerveld, A. and Bootsma, D. (1972) Nature New Biol. 240, 48–50
14 Romeo, G., Childs, B. and Migeon, B.R. (1972) FEBS Lett. 27, 161–166
15 Romeo, G. and Migeon, B.R. (1970) Science 170, 180–181
16 Romeo, G., D'Urso, M., Di Matteo, G., Wan, C.C., Li, S.C. and Li, Y.T. (1975) Biochem. Exp. Biol., in press
17 Okada, S. and O'Brien, J.S. (1969) Science 165, 698–700
18 Sandhoff, K. and Wassle, W. (1971) Hoppe-Seyler's Z. Phys. Chem. 352, 1119–1133
19 Beutler, E. and Kuhl, W. (1972) J. Biol. Chem. 247, 7195–7200
20 Kano, I. and Yamakava, T. (1974) J. Biochem. 75, 347–354
21 Crawhall, J.C. and Banfalvi, M. (1972) Science 177, 527–528
22 Rietra, P.J.G.M., Tager, J.M. and Borst, P. (1972) Biochim. Biophys. Acta 279, 436–445
23 Beutler, E., Guinto, E. and Kuhl, W. (1973) Amer. J. Hum. Gen. 25, 42–46
24 Ho, M.W. (1973) Biochem. J. 133, 1–15
25 Li, S.C., Wan, C.C., Mazzotta, M.Y. and Li, Y.T. (1974) Carbohydrate Res. 34, 189–193
26 Johnson, W.G. and Brady, R.O. (1972) in Methods in Enzymology (Colowick and Kaplan, eds) Vol. 28, pp. 849–856, Academic Press
27 Tallman, J.F., Brady, R.O., Quirk, J.M., Villalba, M. and Gal, A.E. (1974) J. Biol. Chem. 249, 3489–3499
28 Robinson, D. and Stirling, J.L. (1968) Biochem. J. 107, 321–327