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GLYCOSIDASES IN HUMAN BLOOD PLASMA AND URINE. MOLECULAR FORMS AND ACTIVITY LEVELS

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SUMMARY

The isoelectric focusing patterns and pH activity curves of five glycosidase enzymes in blood plasma and urine were studied. This study demonstrated the presence of a single molecular form in α-glucosidase and of two molecular forms of the enzymes N-acetyl-β-D-glucosaminidase, Nacetyl-β-D-galactosaminidase and β-glucuronidase in both plasma and urine. β-Galactosidase existed as a single major molecular form in plasma and at least two molecular forms in urine. Specific activity of each enzyme was determined in plasma and urine The validity of taking the ratio between two enzyme activities as an index for screening of genetic disease is discussed.

INTRODUCTION

For many years the diagnosis of genetic diseases was exclusively based on clinical symptoms and signs. The possibility of determining hereditary enzyme deficiency has changed this pattern. As for many inborn errors of

metabolism the diagnosis of a patient can now in principle be established before clinical symptoms become apparent. (R) A patient can also be classified on the basis of the enzyme deficiency detected independently of the clinical picture. Enzyme assay is generally performed on cells originated from different types of tissue or from fibroblast cell culture and for antenatal diagnosis in cells from amniotic fluid. Only a thorough knowledge of the enzymes in such cells and the use of specially designed and standardized enzymes assay methods can prevent diagnostic mistakes. Assays of some enzymes have also been performed in blood plasma and in a few cases in urine. The usefulness of blood plasma and urine in this respect appears to reflect, to a large degree, the enzyme activity of various tissues and organs from which it originates. In this investigation enzyme activity levels, pH curves and molecular properties of several glycosidase enzymes in blood plasma and urine are reported. These enzymes are N-acetyl-β-D-glucosaminidase(EC 3.2.1. 30), deficient in GM2-gangliosidosis I,II,III,(1),N-acetyl-β-D-galactosaminidase which is identical with N-acetyl-β-D-glucosaminidase, β-galactosidase(EC 3.2.1. 23), deficient in GM1-gangliosidosis (1),α-glucosidase (EC 3.2.1.20), deficient in Pompe's disease(2) and β-glucuronidase(EC 3.2.1.31), the absence of which is responsible for mucopolysaccharidosis type VII (3).

MATERIALS AND METHODS

Blood samples were prepared by adding 1 ml sodium citrate 3.8% to 4 ml blood. Plasma was separated from erythrocytes by centrifugation at 5000 r.p.m. for 15 minutes.

Urine samples were obtained without any prior treatment

and were kept frozen. For removing precipitates the urine was centrifuged at 5000 r.p.m. for 15 minutes.

Enzyme Assays

All enzyme activities were assayed with 4-methyl-umbelliferyl derivatives (Koch-Light Laboratories) as substrates in 0.5 ml of 0.1 M acetate buffer, pH 4.5, and with 0.050 ml of plasma or urine. The substrate concentrations in the incubation mixtures were as follows, 4- methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside 0.4 mM, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside 0.7 mM, 4-methylumbelliferyl-β-D-galactopyranoside anhydrous 0.9 mM,4-methylumbelliferyl-β-D-glucuronide trihydrate 0.6 mM, 4-methylumbelliferyl-α-D-glucopyranoside 0.9 mM, 4-methylumbelliferyl-α-L-arabinoside 1 mM, 4-methylumbelliferyl-α-D-mannopyranoside 0.9 mM, 4-methylumbelliferyl-α-D-galactopyranoside 0.9 mM, 4-methylumbelliferyl-β-D-glucopyranoside 0.9 mM. The incubation tubes containing buffer and enzyme were prewarmed at 37 C for 20 min before the substrate solution was added. The reaction was allowed to take place from 2 to 4 hours at the same temperature. It was terminated by addition of 2 ml of 0.4 M glycine-NaOH buffer, pH 10.7. The amount of liberated 4-methylumbelliferyl was measured in a Turner model 430 Spectrofluorimeter with exciting wave-length 365 nm and emitting wave-length 448 nm. 4-Methylumbelliferone (Koch-Light Laboratories)in glycine buffer was used as standard solution.

Calculation of Enzyme Activity:

1 Unit of enzyme activity equals 1 mμmol of 4- methylumbelliferyl released by 1 ml of urine or plasma at 37 C in 1 hour. The specific activity of each enzyme was ;

determined as mμmoles of 4-methylumbelliferyl released per mg protein of urine per hour and per 9 protein of plasma per hour.

Enzyme Fractionation

The precipitation of all enzymes in blood plasma and urine was performed with equal amounts of saturated ammonium sulfate solution. The ammonium sulfate precipitate was dissolved in distilled water and dialysed overnight against 0.02 M phosphate buffer, pH 7.0. The dialysed solution was acidified with 1 M acetic acid until its pH reached 4.5. The precipitate was finally removed by centrifugation.

Preparative Isoelectric Focusing:

Soluble enzyme preparations obtained by the above procedures were subjected to isoelectric focusing in a pH gradient ranging between 4 and 8. Carrier ampholytes(40%) of two types (LKB 8152.pH 4-6; LKB 8154, pH 6-8) were mixed (1:1) to yield the desired pH range. For the pH gradient 3.5 to 10 carrier ampholyte (40%) of the type LKB 1809-101 pH 3.5-10 was used. An LKB column for the separations was used (110 ml,LKB 8100-1). The anode solution at the bottom contained 0.15 ml sulfuric acid and 12 g of sucrose in 14 ml of distilled water and the cathode solution at the top contained 0.15 ml of ethanolamine in 10 ml of distilled water. The separation took place at 300 Volts for 20 hours, after which time the voltage was increased to 800 Volts for 24 hours. To improve resolution, the isoelectric focusing procedure was repeated once more with the same preparation. Fractions of 1 ml were collected after each separation.

Protein Determination:

Protein concentration was determined by the method

described by Lowry et al. (4) using bovine albumin as protein standard.

Results

pH curve

Five glycosidase activities in plasma and urine were determined at different pH in 0.1 M acetate buffer. The enzyme activities with respect to pH are shown in Figure 1. N-Acetyl-β-D-glucosaminidase from both plasma and urine had a pH optimum around 5.5-6.0. N-Acetyl-β-D-galactosaminidase enzyme also exhibited a pH curve with optimum between 5.5-6.0 for both plasma and urine. β-Galactosidase exhibited pH curve with optimum at 4.5 for urine and 3.6 for plasma. β-Glucuronidase in urine showed a pH optimum at 4.5 while the same enzyme in plasma had its highest level of activity at pH 3.6. α-D-Glucosidase had a broad pH optimum around pH 4-5 for urine and 5-6 for plasma.

Enzyme Activity

The specific activity of nine glycosidase enzymes was assayed in plasma and urine (table 1,2.).Specific activity of each enzyme was determined as mμmols 4-methylumbelliferyl released per mg or g protein per hour at 37 C. In table 1 and 2 the activity ratio of N-acetyl-β-D-glucosaminidase to other enzymes is given. This ratio was subjected to the same variable factors and fluctuations as specific activities of some of the enzymes.

Molecular Forms

Isoelectric focusing was used for detecting multiple molecular forms of several glycosidases in blood plasma and urine. N-Acetyl-β-D-glucosaminidase was fractionated by isoelectric focusing into two isoenzymes having pI values of 4.8 and 6.1 for plasma and pI values of 5.4

Table 1

Enzymes in plasma		Number of specimens	Units of activity*		Specific acitivity**		Ratio of two enzymes***	
			Range	Average	Range	Average	Range	Average
N-Acetyl-β-D Glucosaminidase	(A)	19	17-67	43	196-906	570	———	
N-Acetyl-β-D Galactosaminidase	(B)	19	23-45	35	250-809	466	(A/B) 0.7-1.8	1.2
β-Galactosidase	(C)	19	0.12-8.7	1.4	1.4-111	19	(A/C) 5.4-389	85
β-Glucuronidase	(D)	19	16-62	28	202-810	362	(A/D) 0.6-3	1.6
α-Glucosidase	(E)	19	1.2-13	2.7	13-161	36	(A/E) 1.2-38	22
α-L-Arabinosidase	(F)	19	0	0	0	0	(A/F) 0	0
α-D-Mannosidase	(G)	11	8.2-52	21	112-638	274	(A/G) 0.9-3.7	2.4
α-D-Galactosidase	(H)	12	0.1-0.47	0.2	1.1-6.4	2.4	(A/H) 47/605	316
β-D-Glucosidase	(I)	14	0.1-3.2	0.6	0.9-44	8.4	(A/I) 7-448	222

* 1 Unit equals 1mμmol of 4-methylumbelliferyl released by 1 ml of plasma at 37C. in 1 hour.

**Specific activity was measured as mμmol 4-methylumbelliferyl released per g protein per hour at 37C.

***Ratio of two enzymes is a unit of activity of certain enzyme divided by N-acetyl-β-D-glucosaminidase.

Table 2

Enzymes in urine		Number of specimens	Units of activity*		Specific activity**		Ratio of two enzymes***	
			Range	Average	Range	Average	Range	Average
N-Acetyl-β-D Glucosaminidase	(A)	20	1.4-29	13	0.4-3.8	2.3	——	——
N-Acetyl-β-D Galactosaminidase	(B)	20	1.4-41	16	0.8-7.5	2.9	(A/B) 0.06-3.9	1.02
β-Galactosidase	(C)	20	3-330	75	1.7-47	13.5	(A/C) 0.01-2.1	0.4
β-Glucuronidase	(D)	20	6-83	25	0.6-17	5	(A/D) 0.04-4.4	0.8
α-Glucosidase	(E)	20	7-146	48	1.3-33	8.7	(A/E) 0.03-1.1	0.4
α-L-Arabinosidase	(F)	20	0.1-20	5	0.02-4.4	0.9	(A/F) 0.1-156	13.5
α-D-Mannosidase	(G)	20	0.07-44	2.8	0.03-5.4	0.4	(A/G) 0.7-79	30
α-D-Galactosidase	(H)	20	0.3-29	4.5	0.09-3.5	0.7	(A/H) 0.2-44	11
β-D-Glucosidase	(I)	20	0.1-1.9	0.4	0.02-0.24	0.07	(A/I) 5.5-142	46

* 1 Unit equals 1 mμmol of 4-methylumbelliferyl released by 1 ml of urine at 37C, in hour.

**Specific activity was measured as mμmol 4-methylumbelliferyl released per mg protein per hour at 37C.

***Ratio of two enzymes is a unit of activitv of certain enzyme divided by N-acetyl-β-D-glucosaminidase.

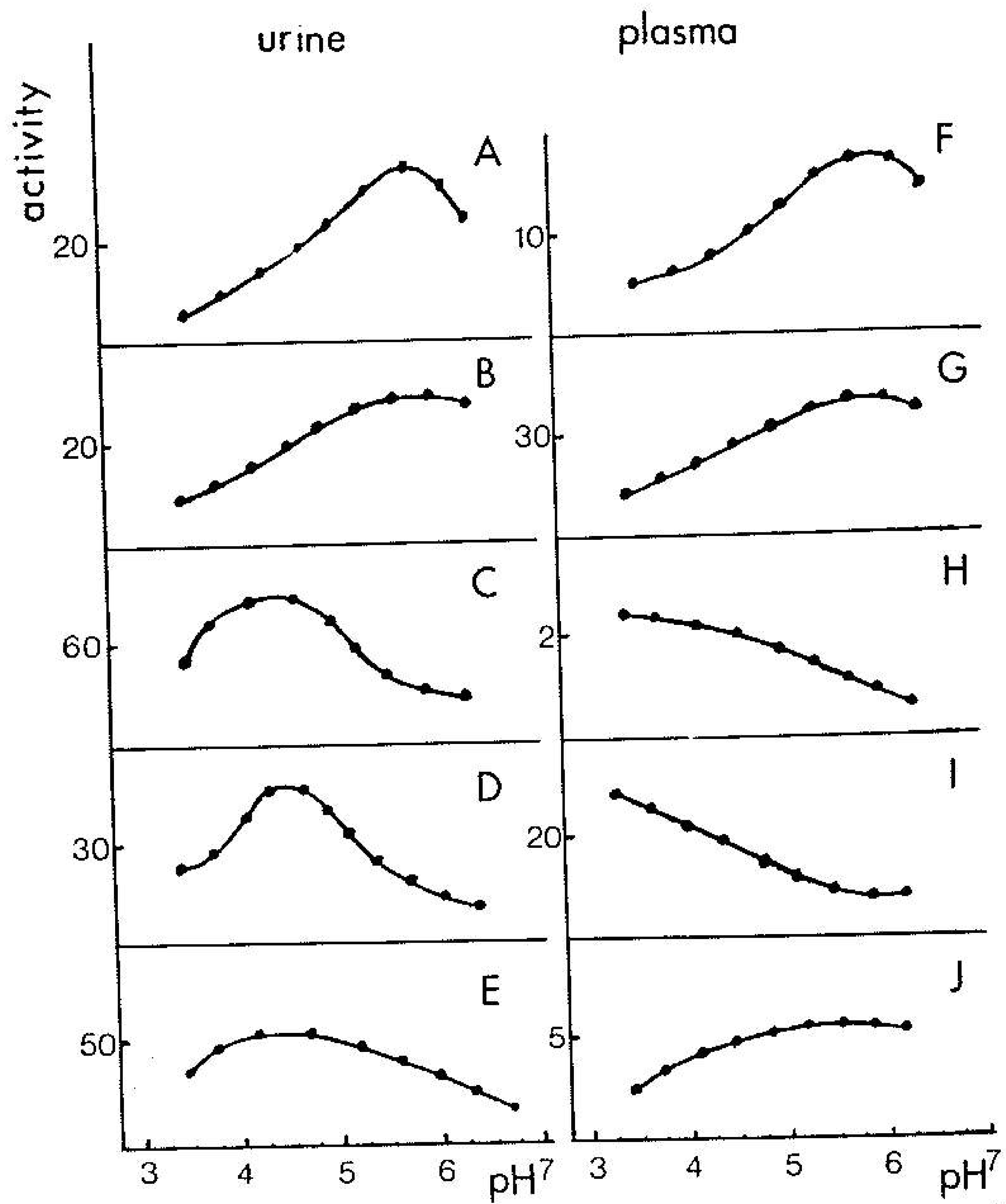

Fig.1. PH dependence of the enzyme activities. (A,F) N-acetyl-β-D-glucosaminidase, (B,G) N-acetyl-β-D-galactosaminidase, (C,H) β-glucuronidase, (D,I) β-galactosidase, (E,J) α-D-glucosidase.

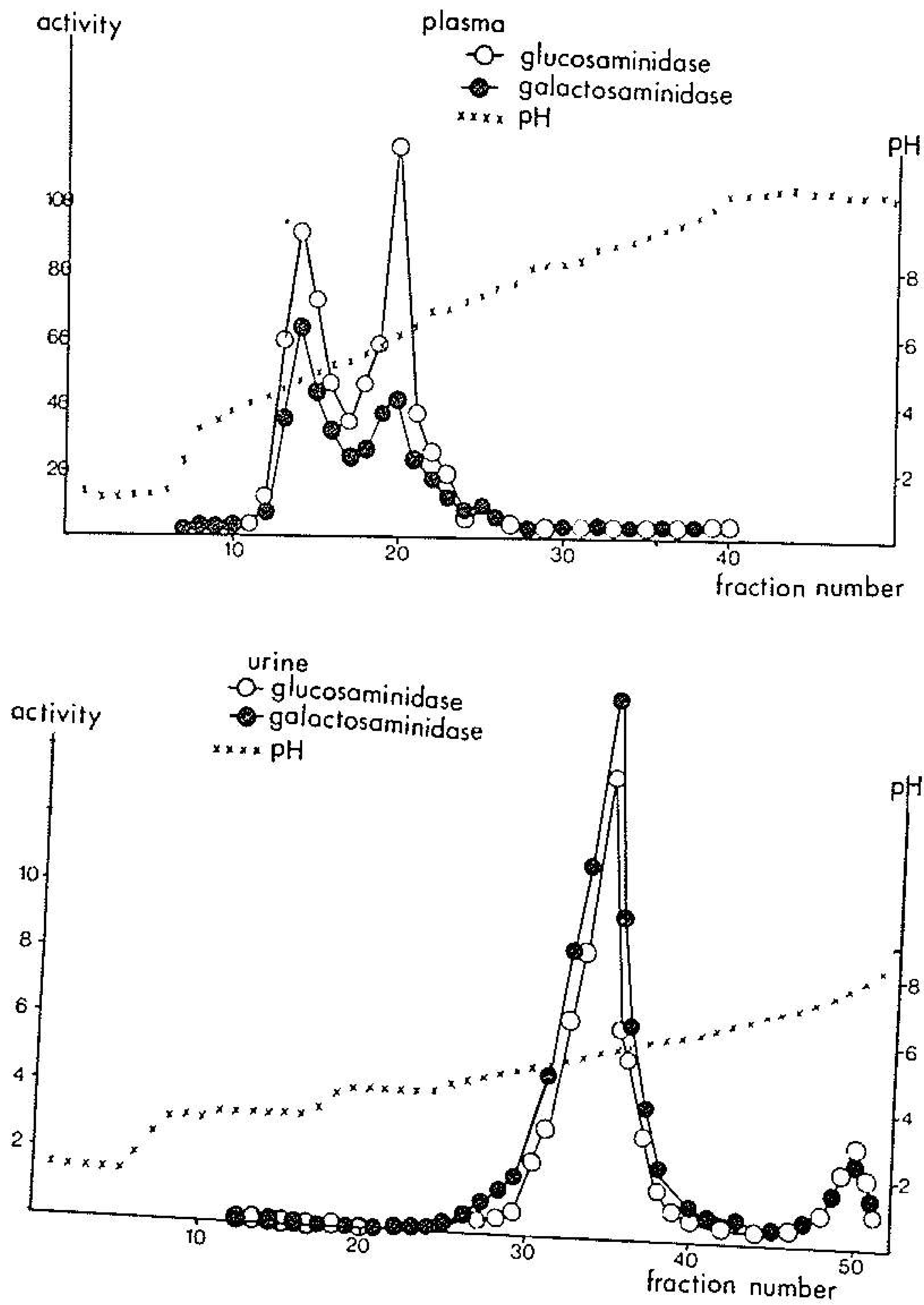

Fig. 2. Distribution of N-acetyl-β-D-glucosaminidase and N-acetyl-β-D-galactosaminidase activity of plasma and urine after isoelectric focusing (pH.3-10).

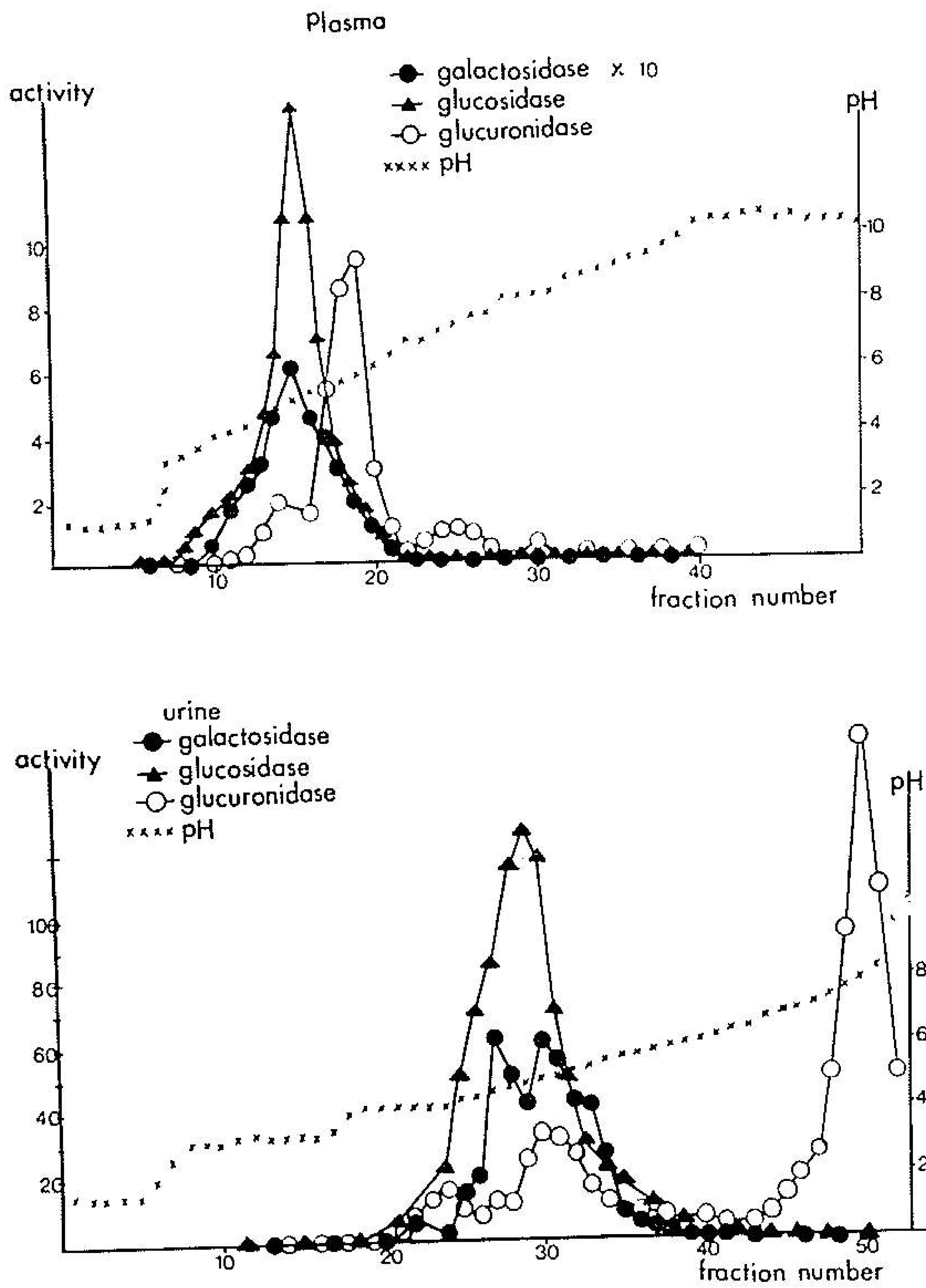

Fig.3. Distribution of β-galactosidase, α-glucosidase and β-glucuronidase activity of plasma and urine after isoelectric focusing (pH.3-10).

and 7.8 for urine enzymes (Fig 2). N-Acetyl-β-D-glucosaminidase and N-acetyl-β-D-galactosaminidase activities of each isoenzyme were associated with a single protein, this was supported by isoelectric focusing profiles and a parallel ratio obtained throughout this fractionation procedure for these two enzymes (Fig.2).The results of isoelectric focusing for β-glucuronidase from plasma and urine show that this enzyme exists as two molecular forms. The two molecular forms of β-glucuronidase in plasma having pI values of 4.9 and 5.7 and in urine having pI values of 4.9 and 7.7 (Fig 3). Isoelectric focusing of β-galactosidase in plasma suggests that this enzyme exists as a single major molecular form having a pI value of 4.9. The same enzyme in urine exists as at least two molecular forms with pI values of 4.6 and 4.9 (Fig.3). The result of isoelectric focusing of α-glucosidase shows that this activity is associated with a single major molecular form with an isoelectric point at pH 5.0 in both plasma and urine (Fig. 3).

DISCUSSION

A considerable number of enzyme activities are expressed in urine and plasma which appears to reflect, to a large degree, the enzyme activities of various tissues and organs from which they arise. Using these sources of enzymes it should be possible to introduce a reliable test to screen for patients with different genetic diseases. It should be pointed out that variations in factors, such as age, dilution and collection methods of urine and plasma, might on occasions result in low enzyme activities in urine and plasma samples from normal individuals. While it is evident that repeated assays on several urine and plasma samples from a single individual

can correct for false negatives introduced by these factors. It has been found that the simultaneous assay of a second glycosidase enzyme, subject to the same variable factors, serves also as a good control. By relating the two enzyme activities to each other as a ratio,it should be possible to separate individuals with an enzyme deficiency from the control group. In the method given here, N-acetyl-β-D-glucosaminidase is used as the control enzyme. The results are then given as a ratio of N-acetyl-β-D-glucosaminidase to another enzyme.

The use of isoelectric focusing in this study revealed that N-acetyl-β-D-glucosaminidase from both plasma and urine exists as two molecular forms. This enzyme also exists as two molecular forms (A and B) with some common subunit structure in many mammalian tissues (5,6).

Evidence for the identity of N-acetyl-β-D-glucosaminidase and N-acetyl-β-D-galactosaminidase enzymes was obtained by examination of the ratio of these two enzyme activities in plasma and urine samples.

The elution profiles of two enzymes after isoelectric focusing and similarity in pH activity curves also support the conclusion that activities reside in the same protein molecule. Several different genetic diseases are related to N-acetyl-β-D-hexosaminidase deficiency in man. In Sandhoff disease, or GM(2)gangliosidosis type II,there is a deficiency of hexosaminidase A and B forms (7,1). Scrivastava and Beutler (8) suggested that hexosaminidase A and B share a common subunit which is lacking in Sandhoff disease, whereas a subunit unique to hexosaminidase A is deficient in Tay-Sachs disease. Chern et al.(9) demonstrated that hexosaminidase A is composed of alpha

15 and 5 respectively. Hexosaminidase B is a homopolymer of beta chains. Tay Sachs disease results from a deficiency of alpha chains and Sandhoff disease from a deficiency of beta chains. Mac Leod et al. (10) described a form of hex•A and B deficiency with juvenile onset. This may be an allelic form of Sandhoff disease comparable to the juvenile form of Tay-Sachs disease.

The β-glucuronidase enzyme from plasma and urine also occurs in two molecular forms, which differ by their iso electric points. Sly et al.(3) found that deficiency of β-glucuronidase is associated with mucopolysaccharidosis type VII. Fibroblast cultures from the patient with this syndrom showed a β-glucuronidase deficiency. Chern and Croce (11) revealed the tetrameric association of subunits of the β-glucuronidase enzyme molecule. Knowles et al (12) concluded that the beta-glucuronidase locus is on the long arm of chromosome 7.

Beta-galactosidase in plasma exists as a single molecular form while the same enzyme in urine exists as at least two molecular forms.

Okada and O'Brien(13)demonstrated that β-galactosidase deficiency is the fundamental error in generalized gangliosidosis. O'Brien(14,15) found three different isoenzymes of acid betagalactosidase A,B and C, these three isoenzymes were grossly deficient in all tissues examined from the patient with generalized gangliosidosis. He also discoverd a second type of Gm(1)-gangliosidosis or juvenile type which was clinically and chemically different from type I. Only the B and C isoenzymes of beta-galactosidase were deficient in the juvenile type. A third type of Gm(1) gangliosidosis or adult type with

less severe abnormality than type I and II has also been described (16,17). Galjaard et al. (18) studied complementation in cell hybrids between 4 types of Gm(1)-gangliosidosis and concluded that type I and II involve the same locus, whereas types 3 and 4 result from mutation at a second and separate locus.

The enzyme α-glucosidase investigated here is present as a single major molecular form with the same isoelectric point in both plasma and urine. This enzyme degrades glycogen intra-lysosomally into glucose. Deficiency of acid alpha-glucosidase has also been detected in the urine of patients with Pompe's disease(19).

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REFERENCES

1- J.S. O'Brien, S. Okada, M.W. Ho, D.L. Killerup, M.L. Veath, K. Adams, Fed. Proc, 30 (1971) 956.

2- B. Hultberg, P.A. Okerman, Clin. Chem. Acta.28 (1970) 169.

3- W.S. Sly, B.A. Ouinton, W.H. McAlister, D.L. Rimoin, J. Pediat, 82(1973) 249.

4- O.H. Lowry, N.J. Rosebrough, A.L. Farr, A.L. Randall, J. Biol. Chem, 193(1951) 265.

5- J.F. Tallman, R.O. Brady, R. Navon, B. Padeh, Nature, 252(1974) 254.

6- S.K. Srivastava, Y.C. Awasthi, A. Yoshida, E. Beutler, J. Biol. Chem, 249(1974) 2043.

7- K. Sandhoff, U. Andreae and H. Jatzkewitz, Life Science, 7(1968) 283.

8- S.K. Srivastava, E. Beutler, Nature, 241 (1973) 463.

9- C.J. Chern, E. Beutler, W. Kuhl,F. Gilbert; W.J.Mellman, C.M. Croce, Proc. Nat. Acad. Sci, 73(1976) 3637.

10-P.M. Mac Leod, S. Wood, J.E. Jan, D.A. Applegarth and C.L. Dolman, Neurology, 27 (1977) 571.

11-C.J. Chern, C.M. Croce, Am. J. Hum. Gent, 28(1976) 350.

12-B.B. Knowles, D. Solter, G. Trinchieri, K.M.Maloney, S.R. Ford and F.P. Aden, J. Exp. Med, 145 (1977)314.

13-S.Okada and J.S. O'Brien, Science, 160 (1968) 1002

14-J.S. O'Brien, (Letter) Lancet II, (1969) 805.

15-J.S. O'Brien, J. Pediat, 75 (1969) 167.

16-D.A. Wenger,S.I. Goodman and G.B. Myers, Lancet II, (1974) 1319.

17- J.S. O Brien, E. Gugler, A. Giedion, U. Weissmann,N. Herschkowitz, C. Neier, and J. Leroy, Clin. Genet, 9 (1976) 495.

18- H. Galjaard, A. Hoogeveen, W. Keijzer, H.A.Dewit-Verbeek and A.J.J. Cytogenetics and Cell genetics,14 (1975) 320.

19- I.S. Salafsky and H. L. Nadler J. Pediat, 82 (1973) 294.