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NEUTRAL GLYCOSIDASE IN HUMAN LEUKOCYTES; THE PRESENCE OF NEUTRAL α-MANNOSIDASE AND THE EFFECT OF ACTH ON THE ACID AND NEUTRAL GLYCOSIDASES

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The specific high activity of neutral α-mannosidase (EC 3.2.1.24) was present in the cytosol fraction of human leukocytes. It is not bound by concanavalin A-Sepharose, has a pH optimum of 6.0, more neutral than that of the acid lysosomal α-mannosidase, and protected and activated by divalent cations of Mn^{2+}. Cupric and zinc ions were strongly inhibitory. The neutral α-mannosidase was unaffected by ACTH therapy in infants with infantile spasms, while the acid α-mannosidase and acid β-galactosidase were suppressed by ACTH.

Introduction

α-D-mannosidase (EC 3.2.1.24) is widely distributed in nature. Three types of α-mannosidase with different subcellular localization have been identified in mammalian tissues. One is the acid α-mannosidase which is localized in the lysosomome and has an acidic pH optimum of 4.3-4.6. Its genetic deficiency causes mannosidoses in humans[1)2)] and in Angus cattle[3)4)]. A cytosol-localized hepatic α-mannosidase has a pH optimum of 6.0-6.5[5)6)] and has been purified to homogeneity[7)]. The third type of α-mannosidase has been purified from a hepatic Golgi membrane fraction[8)9)]. It has an intermediate pH optimum of 5.6. A similar enzyme has also been described in human serum or plasma[10)11)]. Of the three, the cytosolic α-mannosidase with the highest pH optimum was not bound by Concanavalin A-Sepharose 4B column, while the other two types were bound[12)].

The presence of the three types of α-mannosidase can be a source of confusion in terminology of the "neutral" α-mannosidase unless clearly defined. In this report we will define the term, "neutral α-mannosidase" to mean any α-mannosidase which is not the lysosomal acid α-mannosidase and not bound by Concanavalin A-Sepharose 4B column. Very little is known about "neutral" α-mannosidase in leukocytes at present, and the above definition may be useful in minimizing a confusion at least until more detailed characterization is achieved.

We have started a systematic exploration of neutral glycosidases and have published some characterizations of the neutral β-N-acetylhexosaminidases in rat brain[13)14)]. A variety of hormones including thyroxine[15~18)] and cortisone[16)18)] have been found to influence on lysosomal enzyme activity in rat liver. Little is known of the sensitivity of these enzymes to hormonal effects during infancy, and of the difference between acid and neutral glycosidases. The present communi-

Abbreviations used; ConA: Concanavallin A, ACTH: adrenocorticotrophin, T_4: thyroxine, 4 MU: 4 methylumbelliferone

cation describes evidence for the presence of the neutral α-mannosidase in human leukocytes and the differential effects of ACTH on acid and neutral glycosidases.

Materials and Methods

Materials: For basic characterization of enzymes, blood of normal adult volunteers of both sexes were used. When the effects of ACTH were examined, infants with infantile spasms who were treated with ACTH were used. Blood of patients was taken with the permission of informed parents. Blood samples were collected between 0:30 p.m. and 3:00 p.m.

Preparation of enzyme sources: Isolation of leukocytes from peripheral blood was prepared in principle according to Snyder et al. with 6% dextran in 0.9% saline[19]. For evaluation of the effects of ACTH, leukocytes were homogenized in appropriate volume (2 ml/6-8 ml blood) of 10 mM phosphate buffer, pH 8.0, containing 15 mM 2-mercaptoethanol, in a hand operated Teflon-Piston homogenizer (Tokyo Garasu Kikai Co, Tokyo). The homogenate was sonicated mildly with Sonifier B-12 (Branson Sonic Power Co, Dangury, CT, USA) before enzyme assay. The whole procedure was carried out at 4°C unless described.

Concanavalin A-Sepharose 4B chromatography: The total bed volume of Concanavalin A-Sepharose 4B was adjusted to approximately 1 ml for 6-8 ml of peripheral blood. The column size was usually 1.0 × 1.3 cm. Leukocytes were homogenized in appropriate volume of 10 mM tris-HCl buffer, pH 8.0, containing 15 mM 2-mercaptoethanol, 0.5 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. This buffer was used only in this column study to facilitate the binding by Concanavalin A-Sepharose 4B. The column was prewashed with 3 bed volumes of the buffer. The 11,000 g for 20 minutes supernate was applied to the column. The unbound fraction was collected together with 2 bed volumes of washings applied after the sample. The neutral glycosidases were recovered in this fraction while the acid glycosidases were bound to the column.

Subcellular fractionation: When subcellular distribution of the enzymes was tested, leukocytic subcellular fractions were prepared in principle according to DeRobertis et al[20]. Leukocytes were homogenized in appropriate volume (2.5 ml/50 ml blood) of ice-cold of 0.32 M sucrose in a Teflon-Piston homogenizer. The homogenate was first centrifuged at 900 g for 10 min. The pellet was resuspended and re-centrifuged twice and the final pellet was designated as the nuclear-cell debris fraction. The combined supernate was centrifuged at 11,000 g for 20 min. The pellet was washed twice to yield the crude mitochondrial fraction. The combined 11,000 g supernates were centrifuged at 105,000 g for 60 min. This procedure gave to the microsomal (pellet) and cytosol (supernate) fractions. The particulate fractions were then subjected to the procedures described above for leukocyte homogenate to obtained the enzyme source for subsequent studies.

ACTH admininstation and endocrine studies: The patients were 8 cases who were treated with ACTH for the control of infantile spasms at the Department of Pediatrics, Tokyo Women's Medical College Hospital, during the period between June 1982 and January 1983. The patients were all diagnosed as having infantile spasms. The age of onset ranged from 2 to 18 months of age. The age at commencement of treatment was between 4 and 20 months of age. The ACTH therapy was performed according to the protocol which consists from an 8 weeks course with gradual tapering method as described before[21]. It could be modified as to dosage and duration when side effects were severe. The dosage of ACTH (tetracosactide acetate-Zn, Cortrosyn-Z®, Daiichi Seiyaku Co. Tokyo; Organon NV, The Netherlands) was 12.5 or 25.0 μg per kg body weight per shot, and it was injected intramuscularly. One mg of tetracosactide acetate corresponds to 40 IU natural ACTH.

The serum levels of cortisol and thyroxine (T_4) were determined by radioimmunoassay procedure; cortisol was assayed with a Gamma Coat

[^{125}I] Cortisol Kit (Japan Travenol Co, Tokyo), and T_4 was assayed with a Konsul T_4 RIA Kit (Daiichi RI Research Lab, Tokyo). For this assay, blood samples were collected between 7:30 am and 8:30 am before breaktast.

Enzyme assay: The substrates, 4-methylumbelliferyl α-mannopyranoside and other glycosides, were kept frozen as a 4 mM suspension in 10 mM sodium citrate-20 mM phosphate buffer, pH 4.0. The reaction mixture consisted of 0.1 ml of appropriately diluted enzyme source, 0.1 ml of the substrate suspension, 0.1 ml of 0.1 M sodium citrate-0.2 M phosphate buffer of appropriate pH, and 0.1 ml of water, or a solution of metal ions of an appropriate concentration. During early phases of this study, it was noted that neutral α-mannosidases were very unstable and rapidly lost its activity during storage or experimental manipulation. When studied the effects of ACTH, the activity of neutral α-mannosidase was assayed with the addition of 1 mM Mn^{2+} to protect and stimulate this enzyme. After incubation for 20 min at 37°C with gentle shaking, the reaction was stopped by the addition of 2 ml of 0.2 M sodium glycine buffer, pH 10.7 and the liberated 4-methylumbelliferone was determined in a Hitachi 203 fluorescence spectrophotometer. The protein content of the enzyme source was determined by the Bio-Rad dye-binding protein assay kit.

Commercial materials: The following commercial materials were purchased from the indicated sources. All fluorogenic enzyme substrates (Koch-Light Lab, Colnbrook Berks, England); Concanavalin A-Sepharose 4B (Pharmacia Fine Chemicals, Pharmacia-Japan, Tokyo); Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA).

Results

Whole homogenate of leukocytes yielded a bimodal pH curve when it was assayed for mannosidase activities in the absence of any additional divalent ions or EDTA (Fig. 1). The 11,000 g supernate also gave a bimodal pH curve. This acidic activity at pH below 5 was then completely

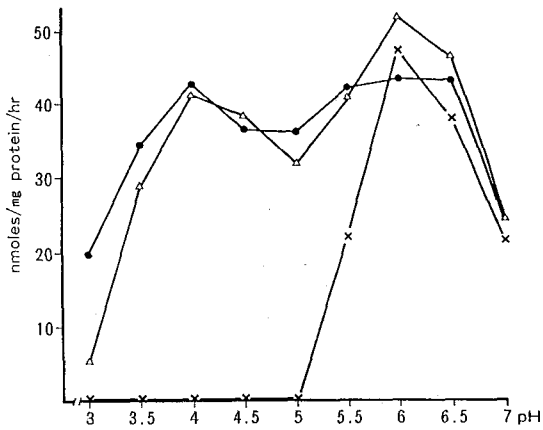

Fig. 1 ConA-Sepharose column chromatography and effects of pH on human leukocytes α-mannosidase activity
Assays were carried out as described in the text. No exogenous divalent ions were added to the assay mixture. ●—● whole homogenate, △—△ 11,000 g for 20 min supernate, ×—× ConA-Sepharose unbound fraction.

eliminated in the ConA-unbound fraction. The α-mannosidase with the pH optimum of 4.0 could be extracted into the 11,000 g supernate at approximately 55.4% recovery when leukocytes were homogenized in 10 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. The acid α-mannosidase thus solubilized was almost completely bound to the ConA-Sepharose column. The neutral α-mannosidase with the pH optimum of 6.0 could be solubilized into the 11,000 g supernate at approximately 75.4% recovery, and recovered at approximately 57.2% in ConA-unbound fraction (Table 1). Thus, the Concanavalin A-Sapharose column provided an effective means of separating the acidic and neutral α-mannosidases from each other.

To detect the other neutral glycosidases in leukocytes, the activities at pH 6.0 were assayed in ConA-unbound fraction (Table 2). The neutral α-mannosidase has specifically high activity compared with other neutral glycosidases in leukocytes. The neutral β-N-acetylglucosaminidase was also noticed and has a pH optimum at 5.5.

Effects of divalent cations on α-mannosidases: Of the divalent cations tested, manganese and

Table 1 ConA-Sepharose column chromatography and recovery of human leukocytes α-mannosidase activity[a]

Fraction	Protein (mg)	Acid		Neutral	
		Specific activity[b]	Recovery%	Specific activity	Recovery%
Whole homogenate	12.0	44.0	100	43.5	100
11,000 supernate	7.8	37.5	55.7	50.5	75.4
ConA-unbound fraction	6.3	0	0	47.4	57.2

(n=4)[c]

a : α-mannosidases in leukocytes from 20 ml peripheral blood
b : Specific activity ; n moles/mg protein/hr
c : n ; *the number of experiments*

Table 2 The activity of neutral glycosidases in ConA-unbound fraction[a]

1.	4MU- α-mannosidase	47.4 n moles/mg protein/hr
2.	β-N-acetylglucosaminidase	16.0
3.	β-N-acetylgalactasaminidase	0.9
4.	β-galactosidase	0
5.	β-glucosidase	0.2
6.	β-glucuronidase	0
7.	α-fucosidase	4.2
8.	α-galactosidase	0
9.	α-glucosidase	4.2

(n=3)[b]

a : *ConA-unbound fraction in leukocytes*
b : n ; the number of experiments

cobalt ions stimulated the activity of leukocytic neutral α-mannosidases in ConA-unbound fraction (Fig. 2). Cupric and zinc ions were strongly inhibitory. On the other hand, zinc ions stimulated the activity of acid α-mannosidase in whole homogenate at pH 4.0. Manganese and cobalt ions were largely without effect to the acid α-mannosidase (Fig. 2).

Subcellular distribution: subcellular fractionation was carried out in the standard procedure in 0.32 M sucrose without any cation. A much greater nuclear cell debris fraction was consistently obtained in leukocytes, even if homogenized more extensively. 27.9% recovery and higher relative specific activity of acid α-mannosidase activity was in the crude mitochondrial fraction, as expected. In contrast, 27.6% of the neutral α-mannosidase activity was recovered in the cytosol fraction with the highest relative specific activity (Table 3).

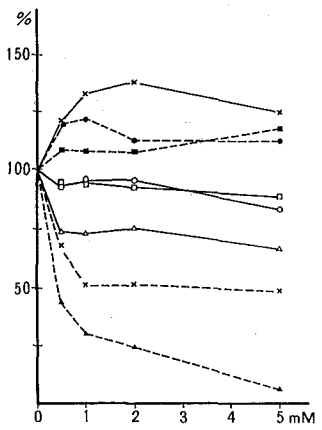

Fig. 2 *Metal effects on acid and neutral α-mannosidase in leukocytes*
Activities were assayed with the whole homogenate, at pH 4.0, as the acid enzyme source, and assayed with the ConA-unbound fraction as the neutral enzyme source with additional divalent ions in chloride form as indicated. The results are expressed as percent of the activity determined without any additions: — acid enzyme, ···· neutral enzyme, —○— ···●··· Mn^{2+}, —□— ···■··· Co^{2+}, —△— ···▲··· Cu^{2+}, —×— ···×··· Zn^{2+}.

Table 3 Subcellular fractions of acid and neutral α-Mannosidases in leukocytes n=3

Fraction	Protein (%)	Acid			Neutral		
		Specific activity[a]	RSA[b]	recovery%	Specific activity[a]	RSA[b]	recovery%
Whole homogenate	100	62.1	1.0	100	37.9	1.0	100
Nuclear cell debris	50.4	61.8	0.99	50.1	22.7	0.60	30.2
Mitochondria	18.2	95.8	1.53	27.9	25.3	0.66	12.1
Microsome	5.3	22.3	0.36	1.9	21.5	0.57	3.0
Cytosol	23.3	23.9	0.39	9.0	44.7	1.18	27.6

n : the number of experiments
Specific activity[a] : nmoles/mg protein/hr
RSA[b] : Relative specific activity ; % of recovered activity/% of recovered protein

Mitochondria fraction showed a single pH curve with a pH optimum at 3.5 (Fig. 3). Cytosol fraction has a large pH optimum curve at 5.5 (Fig. 3). Although the initial cytosol fraction yielded only 27.6% of the neutral α-mannosidase, it was approximately 65% of the recovered activities except nuclear cell debris. Therefore, the concanavalinA-unbound neutral α-mannosidase of leukocytes appears to be primarily localized in the cytosol fraction.

The serum levels of cortisol and thyroxine during ACTH therapy was described in Fig. 4. The serum cortisol levels increased rapidly with the successive daily administration of ACTH and reached the maximum on the 11th day of ACTH therapy. At the end of the 2nd week, it began to decrease slightly in spite of daily administration. A high level (approximate 80 μg/100 ml) of serum cortisol, however, still persisted until the twice a week administration at the end of the 6th week (Fig. 4). On the other hand, serum T_4 levels rapidly decreased in contrast to the serum cortisol levels. The lowest level T_4 was noticed on the 11th

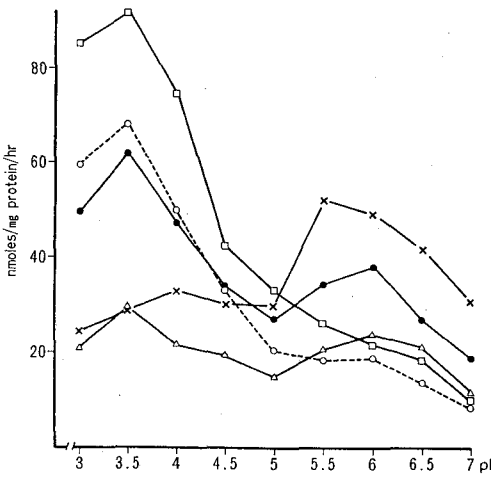

Fig. 3 Subcellular fractions and their pH reaction of α-mannosidases
Assays were carried out as described in the text. No exogenous divalent ions were added to the assay mixture: ●—● whole homogenate, ○---○ nuclear cell debris fraction, □—□ mitochondria fraction, △—△ microsome fraction, ×—× cytosol fraction.

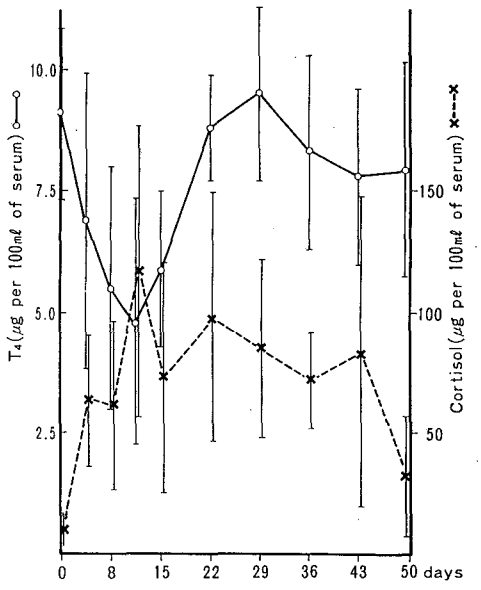

Fig. 4 Serum T_4 and cortisol levels during ACTH therapy
Assays were carried out as described in the text. Values correspond to mean values whereas one standard deviation of the mean is indicated by the thin line. ○—○ T_4, ×—× cortisol.

day of the therapy when the serum cortisol levels reached the maximum. The serum T_4 values of five cases in this study were as a low level as often found in patients with clinical hypothyroidism, below 5 μg/100 ml, during ACTH therapy (Fig. 4). The relation between the clinical response to ACTH and the endocrine changes was described in more detail in other[21].

When enzymes preparations were assayed without any divalent cation on acid glycosidases and assayed with additional 1 mM Mn^{2+} on neutral α-mannosidase, both activities of the acid β-galactosidase and the acid α-mannosidase dropped during ACTH therapy. On the other hand, both activities of the acid β-N-acetylgalactosaminidase and the neutral α-mannosidase remained at the same level or dropped to the level slightly lower than those of the above two enzymes (Fig. 5). When neutral α-mannosidase of the two cases were assayed in ConA-unbound fraction, the neutral enzyme remained at same activities in spite of the suppression of the acid α-mannosidase.

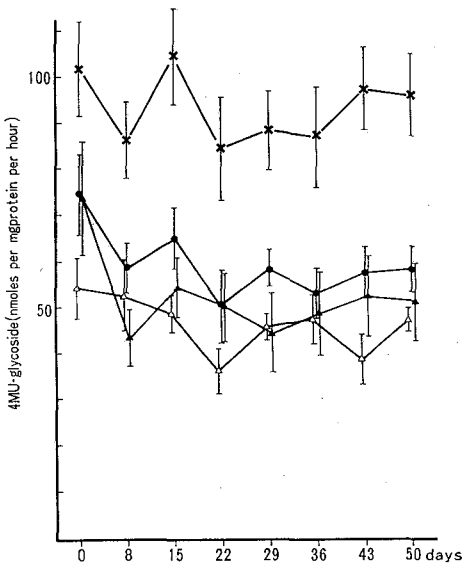

Fig. 5 Glycosidase activities in leukocytes during ACTH therapy
Specific activities of acid β-N-acetylgalactosaminidase (pH 3.5, ×—×), acid β-galactosidase (pH 4.0, ●—●), acid α-mannosidase (pH 4.0, ▲—▲) and neutral α-mannosidase (pH 6.0, assayed with 1 mM Mn^{2+} △—△) were assayed as described in the text. Values correspond to mean values whereas half standard deviation of the mean is indicated by the thin line.

Discussion

The results of this initial phase of our studies on leukocytic neutral glycosidases demonstrated specitically high activity of neutral α-mannosidase. The neutral α-mannosidase is primarily localized in the cytosol, do not bind to concanavalinA, has a pH optimum more neutral than that of the acid lysosomal α-mannosidase, protected and activated by some divalent cations; by the manganese and cobait ions in tested. Cupric and zinc ions are strongly inhibitory and ACTH has not any effect. Hepatic neutral α-mannosidase have been studied in some detail in several laboratories. Divalent ions, such as Mn^{2+}, Co^{2+}, stabilize the hepatic enzyme and cause a small shift in the pH optimum[5)8)22)]. The hepatic neutral α-mannosidase appears to be involved in the processing steps of glycoprotein biosynthesis[7)]. The leukocytic enzyme may conceivably function in similar manner.

Horowitz et al and Oberkotter et al described age-related differences in reactivity of acid lysosomal glycosidases in rat liver and kidney to thyroxine and cortisone[16)18)23)]. A common response pattern was found that crotisone decreased and thyroxine increased enzyme activity. The ACTH therapy increased cortisol and decrease thyroxine in our study[21)]. Our present data was almost consistent with their findings[16)18)23)] but the acid lysosomal β-N-acetylgalactosaminidase was unaffected. This difference could conceivably be due to the difference of species, tissues and/or involved.

Corticosteroids have been used in various diseases; when used in leukemia, it acts as cell-lysis. Thus, corticosteroids and thyroid hormones regulate proteolysis and some lysosomal enzyme activities in leukocytes.

The reason why neutral α-mannosidase is unaffected by ACTH therapy is little known at the present time. The ultimate goal of these studies is to understand the physiological function of neu-

tral glycosidases. Further studies are in progress to elucidate these problems.

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人白血球グリコシダーゼ：中性 α-マンノシダーゼの存在確認と，酸性および中性 α-グリコシダーゼに対する ACTH の影響

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中性 α-マンノシダーゼ(EC3, 2, 1, 24)が人白血球の原形質分画に高濃度活性で存在していることを確認した．この酵素はコンカナバリン A-セファロースに結合せず，至適 pH は6.0で，酸性リソゾーム α-マンノシダーゼよりもより中性側にあり，Mu^{2+} と Co^{2+} の 2 価陽イオンで活性は保護され，活性化された．銅と亜鉛は強力に抑制性に作用した．この中性 α-マンノシダーゼ活性は点頭てんかん患児に対する ACTH 療法では変化しなかったが，酸性 α-マンノシダーゼや酸性 β-ガラクトシダーゼは ACTH にて抑制された．