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FACTORS AFFECTING THE HYDROLYSIS OF CERAMIDE-3 BY α-GALACTOSIDASE A FROM HUMAN LIVER

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Summary

1. The effect of detergents on the catalytic properties of α-galactosidase from human liver was studied using *p*-nitrophenyl-α-galactoside and galactosyl-α(1→4)-galactosyl-β(1→4)-glucosylceramide (ceramide-3) as substrates.

2. The hydrolysis of *p*-nitrophenyl-α-galactoside by α-galactosidase was inhibited by commercial preparations of sodium taurocholate and by taurocholate purified from these preparations by thin-layer chromatography. The extent of inhibition was dependent on the concentraton of the detergent and on the amount of protein present. The impurities present in the preparation also inhibited the hydrolysis.

3. The inhibition of taurocholate preparations of *p*-nitrophenyl-α-galactoside hydrolysis was pH-dependent.

4. The inhibition by taurocholate of *p*-nitrophenyl-α-galactoside hydrolysis can be partly overcome by adding glycosphingolipids.

5. No significant hydrolysis of ceramide-3 occurs in the absence of detergent. Upon adding increasing concentrations of taurocholate, the rate of hydrolysis increases to a maximum value. At still higher taurocholate concentrations the activity decreases.

6. The concentrations of taurocholate giving a maximal rate of hydrolysis of ceramide-3 is dependent on the amount of protein present and independent of the ceramide-3 concentration.

7. When the pH dependence of the rate of hydrolysis of ceramide-3 was mea-

sured in the presence of a commercially available preparation of pure taurocholate or of crude taurocholate, curves with different shapes were obtained.

Introduction

The neutral glycosphingolipid galactosyl-α(1→4)-galactosyl-β(1→4)-glucosylceramide (ceramide-3 or ceramidetrihexoside) is the major substrate for α-galactosidase A, a lysosomal enzyme present in all human tissues [1]. This enzyme is deficient in Fabry's disease, resulting in the accumulation of ceramide-3 in the lysosomes of many tissues and in body fluids [1—3].

To measure the activity of α-galactosidase with ceramide-3 as substrate in vitro, the glycosphingolipid which is insoluble in water, has to be made accessible to enzymic hydrolysis. Ceramide-3 is a class II * polar lipid. The most frequently used method of studying the interaction of such lipids with enzymes is to employ detergents to solubilize the substrate, which results in the formation of mixed micelles between the lipid and detergent. Non-ionic detergents like Triton X-100 or anionic detergents like cholate and taurocholate have proved very suitable for this purpose. For example, sphingomyelin hydrolysis by sphingomyelinase is performed in the presence of Triton X-100 [5,6].

In studying the hydrolysis of ceramide-3, most authors have used an anionic detergent, sometimes in combination with the non-ionic detergent Triton X-100 [7—14]. Apart from the fact that different detergents have been used, the assay conditions have been different, so that comparison of the results obtained by different authors is difficult. In addition, no information is available on the effect of the detergent on the enzyme itself.

We have studied the effect of detergent on the hydrolysis of ceramide-3 by α-galactosidase from human liver in relation to the pH of the medium and the relative concentrations of detergent, substrate and protein. In addition, by making use of the fact that α-galactosidase is able to hydrolyse the water-soluble artificial substrate *p*-nitrophenyl-α-galactoside [1] the effect of the detergent on the activity of the enzyme could be studied. The results obtained, some of which have been reported in a preliminary form [15], are described in this paper. After this manuscript was submitted for publication, a paper by Poulos and Beckman [16] appeared in which the interaction is described between ceramide-3, taurocholate and amount of protein in determining the rate of hydrolysis of the glycolipid.

Materials and Methods

α-Galactosidase was purified from normal human liver using concanavalin A-Sepharose 4B (Pharmacia) and carboxymethyl-cellulose (Whatman) chromatography [17]. The specific acticity of the preparation was about 0.08 U/per mg protein, using *p*-nitrophenyl α-galactoside (12 mM) as substrate at pH 4.6.

* The classification of lipids is according to Small [4].

To determine *p*-nitrophenyl-α-galactosidase activity, the reaction mixture contained 125 mM sodium acetate (pH 3.5), 12 mM *p*-nitrophenyl-α-galactoside (Koch Light), enzyme preparation (25 μg protein unless otherwise stated) and water to 0.5 ml. After incubation at 37°C for 10—60 min, the reaction was stopped by adding 1 ml 0.3 M glycine-NaOH (pH 10.6). The liberated *p*-nitrophenol was estimated spectrophotometrically at 405 nm, using a molar extinction coefficient of $18.5 \cdot 10^6$ cm/mol [18]. One unit of enzyme activity is defined as 1 μmol substrate hydrolysed/min at 37°C.

To determine α-galactosidase activity with ceramide-3 as substrate, the reaction mixture contained 100 mM sodium acetate (pH 3.5). ^{3}H-labelled ceramide-3 (spec. act. 22 400 dpm/nmol), enzyme preparation (5—15 μg protein), detergent, and water to a final volume of 0.1 ml. The detergents used were a preparation of 'pure' taurocholate (Calbiochem Grade A; Cataloque No. 580217; Lot No. 410106), crude taurocholate (Mann Research Laboratory; Catalogue No. 1505; Lot No. U-3741), Triton X-100 (Koch Light) or a combination of taurocholate and Triton X-100.

After incubation at 37°C for 5—20 min, the reaction was stopped by adding 2 ml $CHCl_3/CH_3OH$ (2 : 1, v/v) and 0.4 ml 0.4 mM galactose. Product formation was linear for at least 20 min. After mixing and centrifuging at $1000 \times g$ for 2 min, 250 μl of the upper phase, containing liberated [^{3}H]galactose, was transferred to a scintillation vial and mixed with 10 ml scintillation fluid containing toluene/ethanol (3 : 1, v/v), 2 g/l 2,5-diphenyl oxazole and 25 mg/ml 1,4-bis-(4-methyl-5-phenyl-oxazole-2-yl)-benzene. Radioactivity was measured with a Nuclear Chicago scintillation counter (Isocap 300). The counting efficiency was 25—35%.

Ceramide-3 was purified from human erythrocyte membranes by the method of Vance and Sweely [19] and characterized by chromatography. It was specifically labelled with ^{3}H in the terminal galactose residue essentially as described by Radin et al. [20] for galactosylceramide. The specific activity was $11 \cdot 10^6$ dpm/nmol.

The effect of the following two preparations of sodium taurocholate on the activity of α-galactosidase was tested using *p*-nitrophenyl-α-galactoside as substrate.

(1) Preparation 1; sodium taurocholate from Calbiochem designated as pure (Grade A, catalogue No. 580217; lot number unknown). This preparation, from Dr. Mae Wan Ho, contained two impurities as revealed by thin-layer chromatography [21] (Fig. 1).

(2) Preparation 2: sodium taurocholate from Calbiochem designated as pure (Grade A, catalogue No. 580217, lot No. 410106). This preparation was used in the experiments with ceramide-3 as substrate. It contained 3 impurities in freshly prepared solutions as shown by thin-layer chromatography [21] (Fig. 1). After storage of solutions for 2 weeks or longer, a fourth impurity appeared (Fig. 1).

(3) Sodium taurocholate purified from preparation (2) by preparative thin-layer chromatography [21]. Fraction I is purified taurocholate (Fig. 1; see Ref. 21), and is designated as preparation 3.

Protein was measured as described by Eggstein and Kreuz [22], using crystallized egg albumin as standard.

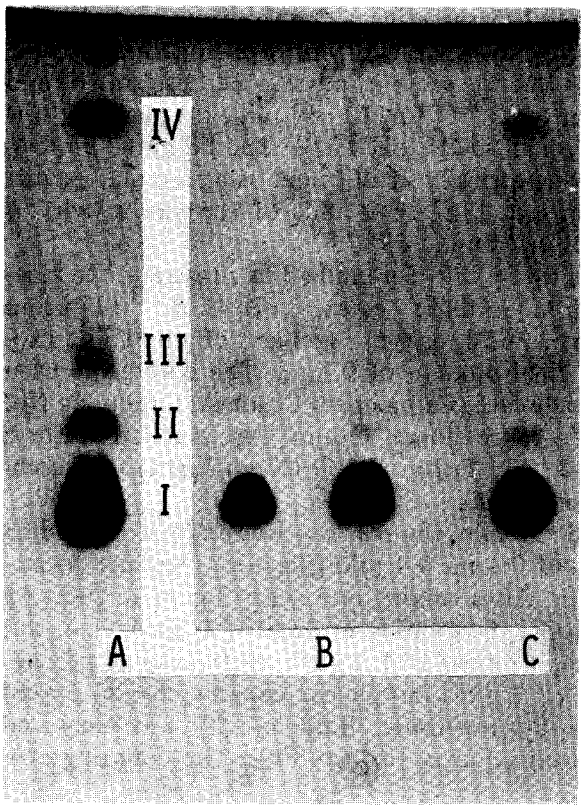

Fig. 1. Thin-layer chromatography of commercial sodium taurocholate preparations. A, taurocholate preparation 2; B, Fraction I, purified taurocholate obtained after thin-layer chromatography of preparation 2; C, taurocholate preparation 1. Fractions II, III and IV represent impurites.

Results and Discussion

Effect of taurocholate on p-nitrophenyl-α-galactosidase activity

Most of the experiments on the effect of sodium taurocholate on the catalytic activity of α-galactosidase A using *p*-nitrophenyl-α-galactosidase as substrate were carried out with taurocholate preparation 2. As shown in Fig. 2 taurocholate inhibits the enzymic activity. This inhibition was dependent on the taurocholate concentration and was maximal at about 4 mM taurocholate in the presence of 25 μg enzyme protein/ml. When the amount of enzyme preparation in the assay system was increased, a curve with a similar shape was obtained, but maximal inhibition was shifted to a higher concentration of taurocholate. These results strongly suggest that adsorption of the inhibitor to protein, including enzyme protein occurs. Indeed, it has been observed that some proteins, e.g. albumin, contain high and low affinity sites for bile salts [23,24].

Inhibition of *p*-nitrophenyl-α-galactoside hydrolysis was also observed with taurocholate preparation 1, with taurocholate preparation 3, and with the three

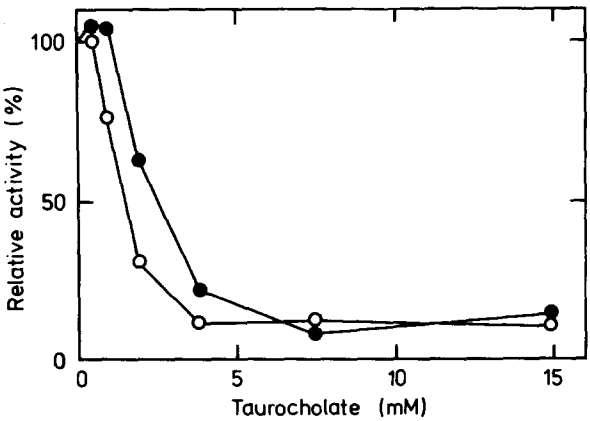

Fig. 2. Effect of 'pure' taurocholate on *p*-nitrophenyl-α-galactosidase activity at different protein concentrations. ○———○, enzyme protein concentration 12.5 μg/0.5 ml reaction mixture; ●———●, enzyme protein concentration 37.5 μg/0.5 ml reaction mixture.

impurities present in freshly prepared solutions of preparation 2 (Table I). However, the maximal extent of the inhibition with the purified taurocholate was less than that observed with the commercial preparations (Table I).

The effect of pH on the inhibition of α-galactosidase by taurocholate prep-

TABLE I

EFFECT OF COMMERCIAL TAUROCHOLATE PREPARATIONS AND OF COMPONENTS ISOLATED FROM THESE PREPARATIONS ON *p*-NITROPHENYL-α-GALACTOSIDE HYDROLYSIS BY α-GALACTOSIDASE FROM HUMAN LIVER

The commercial taurocholate preparations (1 and 2) are described in Materials and Methods. Preparation 2 was purified by thin-layer chromatography. Four fractions were obtained (I, II, III and IV; see Fig. 1). Fraction I is taurocholate.

Expt.	Addition	Concentration (mg/ml)	Activity	
			mU	%
1	None	—	1.31	100
	Preparation 1	5	0.33	25
	Preparation 2	5	0.12	10
	Fraction I	5	0.80	61
2	None	—	2.24	100
	Preparation 1	5	0.42	19
3	None	—	0.95	100
	Preparation 2	5	0.07	8
	Fraction I	5	0.35	37
	Fraction II	5	0.43	44
	Fraction III	5	0.50	53
	Fraction IV	5	0.41	43
4	None	—	1.31	100
	Fraction I	0.25	0.54	41
		0.5	0.58	44
		1	0.67	51
		2	0.68	52
		5	0.80	61

aration 2 is shown in Fig. 3. There was no inhibition at pH 5.0 and as the pH was lowered, the extent of inhibition increased, maximal inhibition being reached at pH 3.5. It is of interest to note that the curve relating enzyme activity to pH in the absence of detergent exhibited a broad plateau between pH 3 and 4.5. This behaviour was observed with three different enzyme preparations.

The inhibition by taurocholate preparation 2 of *p*-nitrophenyl-α-galactoside hydrolysis could be partly overcome by adding glycosphingolipids like ceramide-3 or galactosyl-β(1→4)-glucosylceramide (ceramide-2a) to the medium (Figs. 3 and 4). The extent to which the inhibition is overcome is dependent on the glycosphingolipid used and on its concentration, ceramide-3 being more effective than ceramide-2a (Fig. 4). This effect may be due to formation of mixed micelles between glycosphingolipid and inhibitor, resulting in a decrease in the free inhibitor concentration.

Fig. 4 shows that the inhibition by taurocholate preparation 2 of *p*-nitrophenyl-α-galactosidase activity cannot be entirely overcome by glycosphingolipids even at concentrations of up to 500 μM.

Effect of detergent on ceramide-3 hydrolysis

The effect of varying concentrations of taurocholate preparation 2 on ceramide-3 hydrolysis is shown in Fig. 5; the protein concentration in this experiment was 50 μg/ml. At taurocholate concentrations below 0.5 mM, no significant hydrolysis occurred, possibly because no micelles between glycospingolipid and detergent were formed. As the concentration of taurocholate was increased to 3 mM, the initial velocity increased.

At higher taurocholate concentrations the reaction velocity decreased; this decrease must have been due to inhibition of the enzyme by the detergent (cf. Fig. 2).

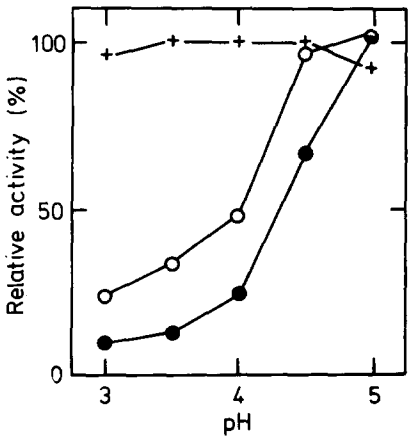

Fig. 3. Effect of pH on *p*-nitrophenyl-α-galactosidase activity in the absence or presence of 'pure' taurocholate. The protein concentration was 25 μg/0.5 ml reaction mixture. +———+, control curve; •———•, in the presence of 3.7 mM taurocholate; ○———○, in the presence of 3.7 mM taurocholate +50 μM ceramide-3. 100% is defined as the activity in the absence of taurocholate at pH 3.5.

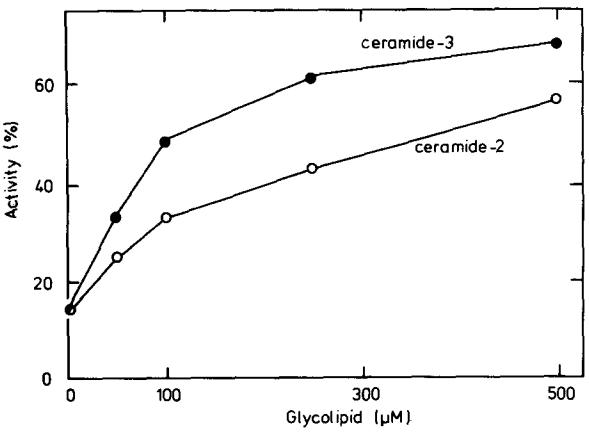

Fig. 4. Effect of added glycolipids on *p*-nitrophenyl-α-galactosidase activity in the presence of 3.7 mM 'pure' taurocholate. 100% is defined as the activity when no taurocholate is present. The protein concentration was 25 μg/0.5 ml reaction mixture.

The taurocholate concentration giving rise to maximal hydrolysis was not significantly altered by increasing the ceramide-3 concentration from 25 μM to 150 μM (Fig. 5). This result shows that the optimum does not simply represent an optimal ratio between detergent and glycosphingolipid, as is the case when sphingomyelin is hydrolysed by sphingomyelinase in the presence of Triton X-100 [6,25]. It represents rather the resultant of two opposing effects occurring when the detergent concentration is increased, an increase in substrate availability and an inhibition of the enzyme.

Further support for the above conclusion is provided by the observation that when the protein concentration was increased from 50 to 150 μg/ml reaction mixture at a constant ceramide-3 concentration, the curve relating the specific

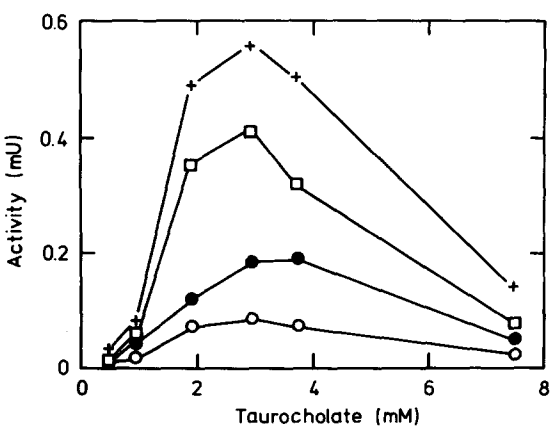

Fig. 5. Effect of 'pure' taurocholate concentration on ceramide-3 hydrolysing activity in the presence of different ceramide-3 concentrations. The protein concentration was 25 μg/0.5 ml reaction mixture. ○———○, 25 μM ceramide-3; ●———●, 50 μM ceramide-3; □———□, 100 μM ceramide-3; +———+, 150 μM ceramide-3.

activity of α-galactosidase was shifted to higher taurocholate concentrations (Fig. 6). Similar observations have been made by Poulos and Beckman [16].

When the rate of ceramide-3 hydrolysis is plotted as a function of ceramide-3 concentration at pH 3.5 and at a constant taurocholate concentration of 3.7 mM, a hyperbolic curve is obtained (Fig. 7). From the Lineweaver-Burk plot (Fig. 7, inset), an apparent 'K_m' for ceramide-3 of 227 ± 45 μM (n = 4) (cf. Ref. 11) is obtained. However, it should be stressed that the hyperbolic relationship is the resultant of two effects, an increase in the number of substrate molecules and a decreased inhibition (Fig. 4) [26,27].

The curves of Fig. 8 show the relationship between rate of hydrolysis of ceramide-3 and the concentration of ceramide-3, at a constant molar ratio between the two compounds. Curves with different shapes are obtained, depending on the molar ratio. At taurocholate/ceramide-3 ratios of 10, 20 or 40 the curves are sigmoidal, and are displaced to lower ceramide-3 concentrations as the molar ratio is increased. These curves are similar to those observed by Yedgar and Gatt [6] in a system utilizing Triton X-100 and sphingomyelin. However, in the taurocholate/ceramide-3 system, a complicating factor is that inhibition occurs at high ceramide-3 concentrations due to the fact that concomitantly the concentration of the taurocholate preparation is increased; this is particularly evident at a molar ratio of taurocholate/ceramide-3 of 80.

From the curves of Fig. 8, it may be concluded that the minimum tauro-

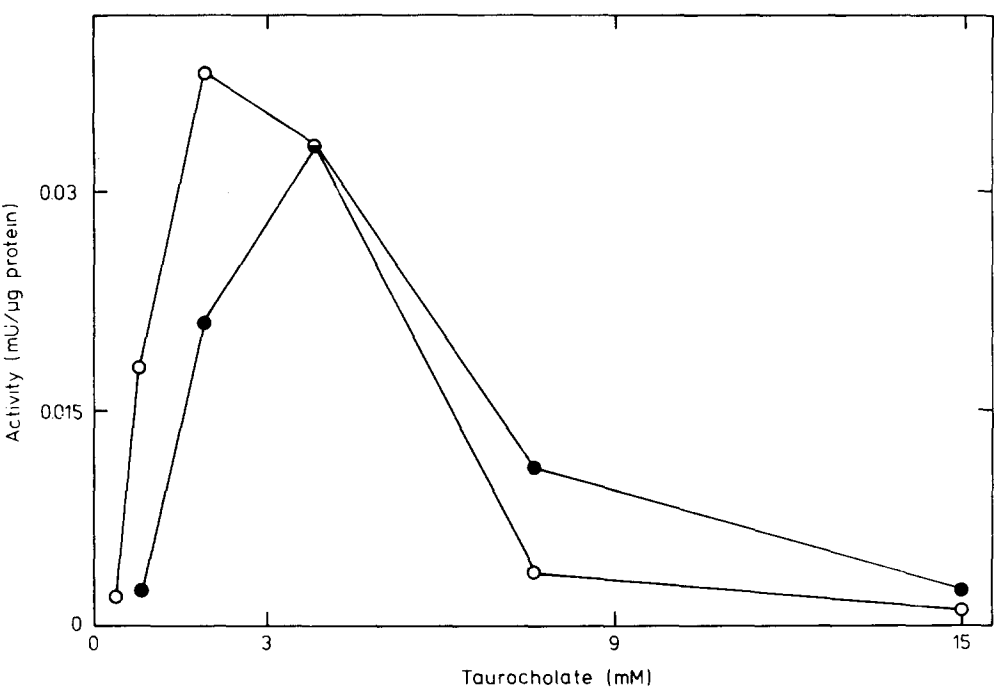

Fig. 6. Effect of 'pure' taurocholate preparation on the specific activity of α-galactosidase with ceramide-3 as substrate, at two protein concentrations and constant ceramide-3 concentration (100 μM). ○———○, enzyme protein concentration 25 μg/0.5 ml reaction mixture; ●———●, enzyme protein concentration 75 μg/0.5 ml reaction mixture.

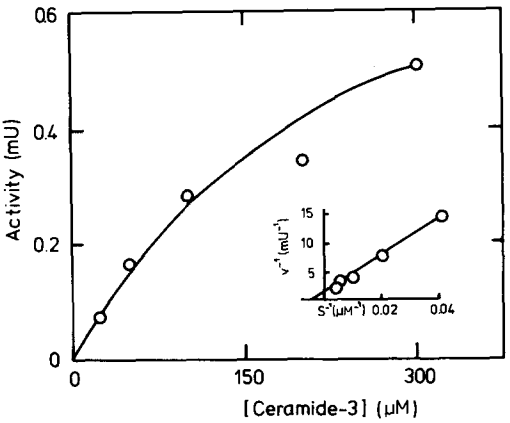

Fig. 7. Effect of ceramide-3 concentration on the ceramide-3 hydrolysing activity in the presence of 3.7 mM 'pure' taurocholate. The ceramide-3 concentration was varied from 25 to 300 μM. The protein concentration was 25 μg/0.5 ml reaction mixture.

cholate concentration required to obtain micelles in which the ceramide-3 is in a form suitable for hydrolysis is 0.5—1 mM. This detergent concentration may perhaps represent the critical micellar concentration.

The effect of pH on the rate of hydrolysis of ceramide-3 by α-galactosidase is shown in Fig. 9. In the presense of taurocholate preparation 2, maximal hydrolysis was obtained at pH 3.0—3.5 (Fig. 9A). The activity declined as the pH was increased and reached zero at a pH of 6.0 (Fig. 9A). When a crude taurocholate preparation was used instead of preparation 2, a sharp pH optimum at 4.5 was observed (Fig. 9B).

When Triton X-100 was present as well as taurocholate preparation 2, the activity was much lower at all pH values than in the presense of taurocholate alone (Fig. 9A). Addition of Triton X-100 in the presence of crude tauro-

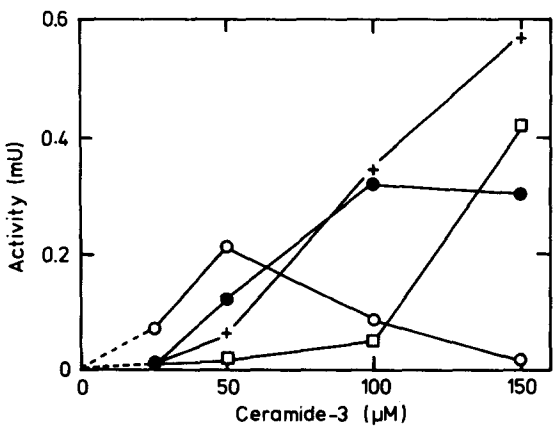

Fig. 8. Effect of ceramide-3 concentration on ceramide-3 hydrolysing activity at a constant molar ratio between 'pure' taurocholate and ceramide-3. □———□, ratio of 10; X———X, ratio of 20; ●———●, ration of 40, ○———○, ratio of 80. Data obtained from the experiment of Fig. 4.

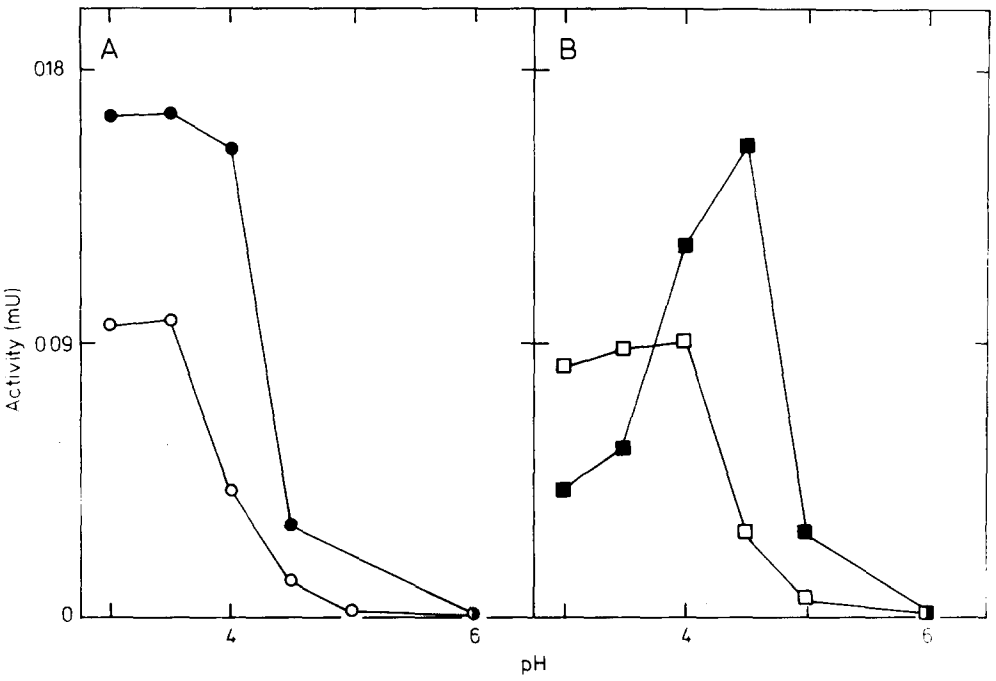

Fig. 9. A. Effect of pH on ceramide-3 hydrolysing activity in the presence of 3.7 mM 'pure' taurocholate (i.e. 0.2%) ●———●; in the presence of 3.7 mM 'pure' taurocholate and 0.1% Triton X-100 (○———○). B. Effect of pH on ceramide-3 hydrolysing activity in the presence of 0.2% crude taurocholate (■———■) or in the presence of 0.2% crude taurocholate and 0.1% Triton X-100 (□———□). The ceramide-3 concentration was 100 μM and the protein concentration 25 μg/0.5 ml reaction mixture.

cholate led to an increase of activity at pH 3.0 or 3.5 and to a decrease at pH 4.0 to 5.0 (Fig. 9B). No activity was observed in the presence of Triton X-100 alone. Furthermore, Triton X-100 had no effect on the hydrolysis of *p*-nitrophenyl-α-galactoside.

It is of interest to note that the results shown in Fig. 9A are in apparent contradiction to those of Ho [11] who found that, at pH 3—4, both Triton X-100 and 'pure' taurocholate were required for ceramide-3 hydrolysis. The taurocholate used by this group was Calbiochem preparation 1, which contained at least two impurities, whereas the Calbiochem preparation used by us (preparation 2) contained three impurities. It is obvious that the apparent discrepancy between our results and those of Ho [11] must be due to the differences in the two batches of taurocholate.

Conclusion

The results reported in this paper show that great caution must be taken in interpreting data on the hydrolysis of ceramide-3 by α-galactosidase. The rate of hydrolysis is dependent on a complicated interaction between enzyme, other proteins present, detergent, and substrate. Obviously, the rate of hydrolysis will depend on the concentration of mixed micelles containing ceramide-3 in a form

suitable for hydrolysis by α-galactosidase. However, taurocholate and the impurities present in the commercial preparations bind to protein, including enzyme protein, leading to inhibition of hydrolysis. The extent of the inhibition is dependent on the preparation of taurocholate used, on the concentration of detergent, on the amount of protein present and on the glycosphingolipid concentration.

Careful analysis of the system has to be made in order to choose the optimal detergent concentration in relation to the amount of substrate, enzyme and non-enzymic protein introduced into the assay system. Thus the same amount of enzyme (as measured with the artificial substrate) obtained from different sources or at different stages of purification will give different rates of hydrolysis of ceramide-3 under otherwise identical conditions.

It is clear that the pH optimum for hydrolysis of artificial substrate can be quite different from that for hydrolysis of ceramide-3 and that it depends on the nature of the detergent or detergents used.

It is of interest that the activity of several lysosomal enzymes, as measured with *p*-nitrophenyl glycosides, is inhibited by taurocholate. Thus in a concanavalin A eluate of human liver [17], the activity of *N*-acetyl-β-hexosaminidase is inhibited by 90% by 4.6 mM taurocholate (Preparation 2), β-galactosidase is inhibited by 75%, α-glucosidase by 90% and *N*-acetyl-α-galactosaminidase by 70%.

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