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THE EFFECT OF THE INTERDEPENDENCE OF PROTEIN AND BILE SALT CONCENTRATIONS ON THE MEASUREMENT OF CEREBROSIDE SULPHATE SULPHATASE ACTIVITY IN HUMAN LEUCOCYTE AND FIBROBLAST EXTRACTS

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Key words: Protein concentration; Bile salt; Cerebroside sulfate sulfatase; (Human)

Summary

The previously observed differences in properties of human leucocyte and fibroblast cerebroside sulphate sulphatase (cerebroside-3-sulphate 3-sulphohydrolase, EC 3.1.6.8) measured in vitro have been found to be due to subtle differences in incubation conditions.

Maximum enzyme activity was observed with either crude sodium taurocholate or with pure sodium taurodeoxycholate. The optimum bile salt concentration of the enzyme in leucocyte or fibroblast extracts, but not the pure ox liver enzyme, was critically dependent on protein concentration. At low concentrations of the latter (less than 0.1 mg/ml), maximum activity was observed at taurocholate concentrations less than 0.5 mg/ml; at protein concentrations greater than 0.20 mg/ml substantially more bile acid (more than 1.3 mg/ml) was required to stimulate maximum activity. Addition of Triton X-100 or bovine serum albumin to the incubation mixtures increased the optimum taurocholate concentration. The dependence of the bile salt optimum on protein concentration appears to be related to the binding of the lipid substrate to membranous protein present in the tissue extracts. Release of the bound lipid is effected either by increasing the bile salt concentration or by adding Triton X-100. In the presence of excess bile salt human leucocyte, fibroblast and liver cerebroside sulphate sulphatase activity is stimulated by Triton at low protein concentrations; under identical conditions the pure or crude ox-liver enzyme is substantially inhibited.

Our data also show that cerebroside sulphate sulphatase activity measured in extracts from leucocytes and fibroblasts, the tissues normally used to effect a diagnosis of metachromatic leucodystrophy, is the result of a complex interac-

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Abbreviations: CS, cerebroside sulphate; CT, crude sodium taurocholate; TD, sodium taurodeoxycholate.

tion of bile salt, protein, Triton X-100 and probably the substrate itself. Any slight alteration in any of those factors, without a corresponding change in any or all of the others, can have a marked effect on the measured enzyme activity, and may lead to errors in the diagnosis of metachromatic leucodystrophy.

Introduction

The determination of cerebroside sulphate sulphatase activities of leucocyte and fibroblast extracts is important in the diagnosis of metachromatic leucodystrophy [1,2]. Biochemical diagnosis of the disease is initially based on the measurement of the enzyme in leucocyte extracts although an analysis of fibroblast extracts offers a valuable confirmation. Conditions chosen for the assay of the enzyme from the two sources are frequently similar, even though differences in properties may be theoretically possible.

During the course of a recent study of the residual cerebroside sulphate sulphatase activity of tissue extracts derived from patients with metachromatic leucodystrophy, we observed that considerable differences existed in the properties of the enzyme in normal leucocyte and fibroblast extracts [3]. Later work indicated that these observations were more apparent than real and were related to subtle differences in incubation conditions. Our data suggest that the anionic detergents, taurodeoxycholate and taurocholate, and the non-ionic Triton X-100 play separate roles in regulating the enzyme activity in vitro and moreover, that these effects are critically dependent on the protein concentration. A preliminary report of those observations has been published [3].

Materials and Methods

[^{3}H]Cerebroside sulphate, (galactosyl[^{3}H]-ceramide sulphate) labelled in the ceramide moiety, was prepared as described previously [4]. The unlabelled lipid was obtained from Applied Science Laboratories. [*phenyl*-^{3}H]-Triton X-100 (1.58 mCi/mg) and [2,4-^{3}H]cholic acid (16 Ci/mmol) were obtained from New England Nuclear. Sodium taurocholate and taurodeoxycholate were obtained from British Drug Houses and Sigma Chemical Co., respectively. The former contained in addition a number of other bile acids [5] and will be referred to as crude taurocholate. Triton X-100 was obtained from Rohm and Haas. Bovine serum albumin (Fraction V) was purchased from the Armour Pharmaceutical Co.

Preparation of tissue extracts

Leucocyte and fibroblast extracts. Human leucocytes were isolated from 10 ml heparinised blood samples and the enzyme released by repeated freeze-thawing in either Triton X-100 [6] or water. Cellular debris was removed by centrifugation at 600 × *g* for 10 min at room temperature and the supernatant was used in most experiments. Occasionally a high speed supernatant, prepared by centrifuging the 600 × *g* supernatant at 95 000 × *g* for 30 min at room temperature in a Beckman airfuge, was used in place of the low speed supernatant. Culture fibroblasts were grown in basal Eagle's medium containing 10% foetal-

calf serum. Cells were harvested by trypsinization as described by Tomkins and Pye [8], suspended in water and then freeze-thawed and centrifuged as described for the leucocytes.

Human and ox liver extracts. Human and ox liver (2 g) was homogenised for 1 min at 0°C in 20 ml distilled water using a Sorvall Omni-Mixer Homogeniser. The homogenate was freeze-thawed and centrifuged as described for leucocytes.

Pure ox liver cerebroside sulphate sulphatase. A sample of the pure enzyme was generously provided by Dr. A.B. Roy.

Protein. Protein estimations were performed as described by Lowry et al. [7].

Cerebroside sulphate sulphatase activity

Standard assay. The standard incubation conditions for the measurement of CT-stimulated cerebroside sulphate sulphatase and TD-stimulated cerebroside sulphate sulphatase activities were essentially those described elsewhere [9] except for the substitution of 19.2 nmol substrate for 9.6 nmol and the omission of Triton X-100. Chloroform-methanol (2 : 1, v/v) solutions of CT (260 μg, 0.48 μmol) or TD (150 μg, 0.29 μmol) and CS were mixed in the desired amounts and the solvent evaporated off at 40°C under nitrogen. Triton X-100 (also in chloroform-methanol; 2 : 1, v/v) was added either at this stage or was added later as a 0.1% (v/v) aqueous solution. Sodium acetate buffer, pH 4.6 (1.26 μmol acetate) and either leucocyte extract (40—60 μg protein) or pure cerebroside sulphate sulphatase (0.04—0.22 μg) were added to make up a total volume of 100 μl. The mixtures were then gently vortexed prior to incubation at 37°C. Incubation periods varied from 10 to 120 min depending on the enzyme activity. Unless otherwise stated all incubations were performed using these conditions.

Measurements of the enzyme activity in fibroblast extracts were performed as described above. However, because of the higher specific activity smaller amounts of cell extract (5—10 μg protein) were used. Enzyme activity in liver extracts was estimated as described for fibroblasts. [^{3}H]Galactosylceramide production was measured by chromatography of incubation mixture extracts on small DEAE-cellulose columns [4].

Binding experiments

The binding of CS, Triton and cholate was studied by incubating each separately with leucocyte extracts. The composition of the incubation mixtures is shown below.

CS binding. The same composition as the incubation mixtures used for measuring leucocyte CT-activated cerebroside sulphate sulphatase activity except for the use of 50 μg rather than 260 μg CT. The final protein concentration was 0.50 mg/ml.

Triton X-100 binding. The same composition as above except for substituting the same amount of unlabelled CS for the labelled lipid and including [^{3}H]-Triton X-100 (50 μg, 130 000 dpm).

[^{3}H]Cholate binding. The same composition as used for CS binding except for substituting unlabelled CS for the labelled lipid. Sodium [^{3}H]cholate (50 μg, 250 000 dpm), was used in place of CT.

Leucocyte extract (7—70 μg protein) was then added and the mixtures incubated at 37° C for 30 min. Under these conditions 10% of the substrate was converted to product. Samples were then centrifuged at 95 000 × *g* for 15 min at ambient temperature in a Beckman airfuge. The sediment, which was resuspended by ultrasonication for 30 s in 100 μl distilled water, and the supernatant, were both counted in 10 ml PCS (Amersham, IL, U.S.A.) with a Nuclear Chicago Series 3000 liquid scintillation spectrometer.

Arylsulphatase A. 4-Nitro-catechol sulphate sulphatase was measured as described by Snyder and Brady [10].

Results

The effect of Triton X-100 on enzyme activity

The rate of the leucocyte enzyme reaction in the presence of an amount of CT, previously found to produce optimum activity (2.6 mg/ml), [9] or TD was linear with time, both in the presence or absence for at least 90 min. Under similar incubation conditions except for the substitution of smaller quantities of protein to compensate for its higher specific activity, the fibroblast enzyme was also linear up to about 90 min. TD-activated cerebroside sulphate sulphatase activity also displayed linearity with time under the above conditions.

Leucocyte CT-activated cerebroside sulphate sulphatase activity was linearly related to protein concentration up to about 0.60 mg/ml in the presence of 0.5 mg/ml Triton. In the absence of Triton an apparent activation was observed at concentrations greater than approx. 0.1 mg/ml. A similar activation was observed with fibroblast extracts (Fig. 1). No such activation was observed for TD-activated cerebroside sulphate sulphatase activity; the reaction rate was linear up to approx. 0.25 mg/ml.

The effect of increasing Triton concentrations on the leucocyte enzyme activity measured under the standard conditions is shown in Fig. 2a. Leucocyte

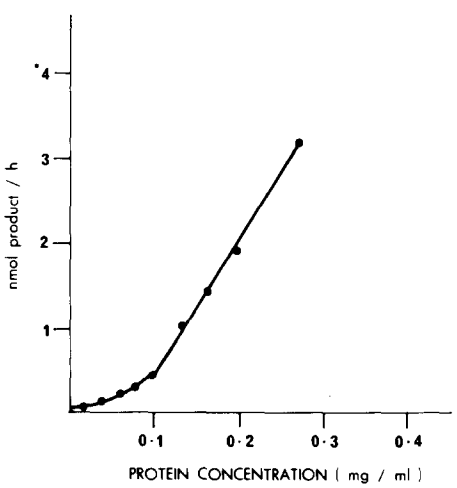

Fig. 1. Effect of protein concentration on fibroblast CT-activated cerebroside sulphate sulphatase activity. Enzyme activity was measured in the absence of Triton X-100 as described in the Materials and Methods section.

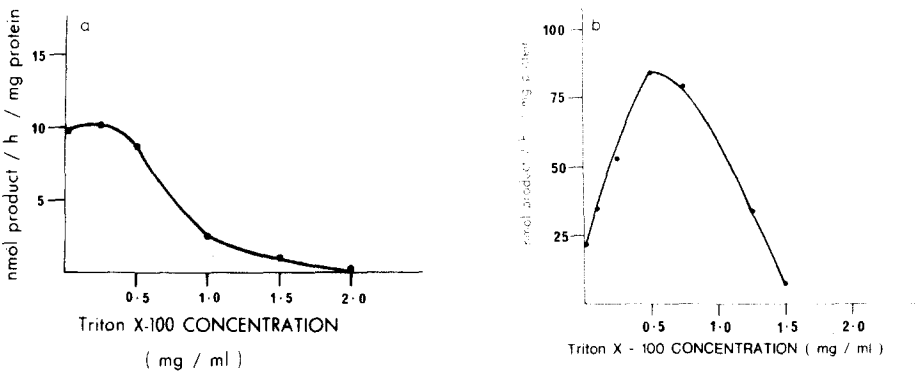

Fig. 2. Effect of Triton X-100 on leucocyte and fibroblast CT-activated cerebroside sulphate sulphatase activity. Enzyme activity was measured as described in the Materials and Methods section. (a) leucocyte protein, 0.6 mg/ml; (b) fibroblast protein concentration, 0.08 mg/ml.

CT-activated cerebroside sulphate sulphatase activity is inhibited by Triton at concentrations greater than 0.25 mg/ml; maximum fibroblast enzyme activity however, was obtained in 0.50 mg/ml Triton (Fig. 2b). Similar data were obtained for TD-activated cerebroside sulphate sulphatase activity.

In an attempt to determine whether the apparent activation of the fibroblast enzyme activity by Triton was related to the fact that the protein concentrations employed in the assay were generally lower than those used for the leucocyte enzyme, the CT- and TD-activated cerebroside sulphate sulphatase activities of leucocyte and fibroblast extracts were measured in the presence of varying amounts of Triton at three different protein concentrations (0.08, 0.25 and 0.50 mg/ml). The data shown in Fig. 3 demonstrate that the activation effect is also observed for the leucocyte enzyme but only at the lower protein concentrations (0.08 and 0.25 mg/ml). Similar results were obtained with fibroblast extracts and by using a high speed (Materials and Methods) rather than a low speed supernatant.

Triton (0.5 mg/ml) inhibited CT- and TD-activated cerebroside sulphate

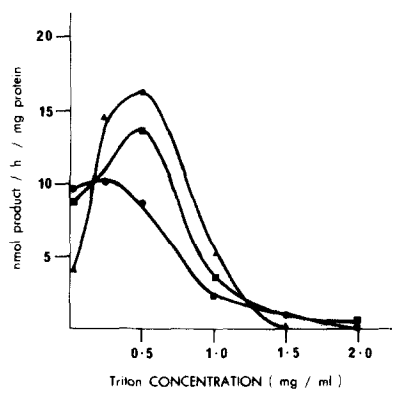

Fig. 3. Effect of Triton X-100 on leucocyte CT-activated cerebroside sulphate sulphatase activity measured at different protein concentrations. Leucocyte protein: ▲———▲, 0.08 mg/ml; ■———■, 0.25 mg/ml; and ●———●, 0.50 mg/ml.

sulphatase activities in bovine liver extracts quite substantially (Table I); the purified ox-liver enzyme was also inhibited. In contrast, CT- and TD-activated cerebroside sulphate sulphatase activities in human-liver extracts were both stimulated significantly by Triton (Table I).

Effect of taurocholate, cholate and taurodeoxycholate on cerebroside sulphate sulphatase activity

The enzyme activity from both sources was activated by a number of bile salts [9]. Triton X-100 could not be substituted for either of the bile salts. The optimum CT, cholate or TD concentrations were directly related to the protein content of the incubation mixtures. For example the CT optimum varied from 0.44 mg/ml (0.8 mmol/l) at 0.07 mg/ml protein to 1.3 mg/ml (2.4 mmol/l) at 0.40 mg/ml protein (Fig. 4). The CT optimum for pure cerebroside sulphate sulphatase however was 0.5 mg/ml (0.93 mmol/l) and showed little variation in the protein range studied (0.04—1.3 μg/ml).

Addition of Triton (0.5 mg/ml) shifted the CT optimum of leucocyte cerebroside sulphate sulphatase activity from approx. 0.5 to 2.0 mg/ml. This effect was observed at low protein levels (0.06 mg/ml); little change was noted at protein concentrations greater than 0.40 mg/ml. Triton (0.25 mg/ml) also shifted the CT optimum of pure cerebroside sulphate sulphatase (Fig. 5). Similar changes were observed for TD.

The effect of bovine serum albumin

Albumin stimulated fibroblast CT-activated cerebroside sulphate sulphatase activity. In the presence of a fixed amount of total protein (0.5 mg/ml, fibroblast protein plus albumin) the enzyme activity was directly proportional to the concentration of fibroblast protein up to approx. 0.10 mg/ml. (Fig. 6). A deviation from linearity at high protein levels was thought to be due to exhaustion of substrate. The stimulatory effect of albumin was not observed however at low bile salt concentrations i.e., 0.26 mg CT or 0.50 mg/ml TD; under these conditions albumin markedly reduced the enzyme activity. The pure enzyme was also stimulated by albumin under normal assay conditions and inhibited at low bile salt concentrations.

TABLE I

THE EFFECT OF TRITON X-100 ON LIVER CT-ACTIVATED CEREBROSIDE SULPHATE SULPHATASE ACTIVITY

CT-activated cerebroside sulphate sulphatase activities were measured in the absence and in the presence of Triton X-100 (0.5 mg/ml) under normal assay conditions. Total incubated protein, 0.07 mg/ml.

Enzyme source	Cerebroside sulphate sulphatase activity (pmol or μmol/min per mg protein)	
	Without Triton	With Triton
Ox liver extracts	89	7
Pure ox liver enzyme	1.1 *	0.58 *
Human liver extracts	43	217

* μmol.

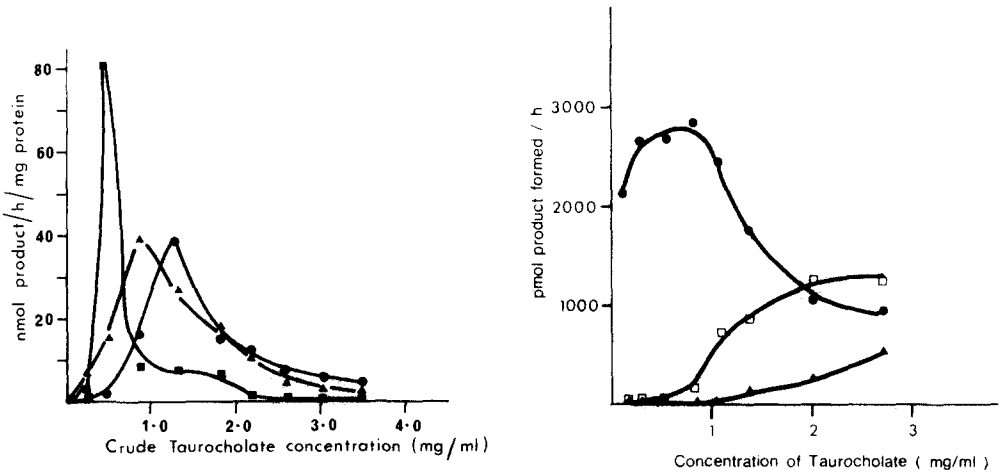

Fig. 4. CT optima at different protein concentrations. Cerebroside sulphate sulphatase activity was measured as a function of crude taurocholate concentration at ▲———▲, 0.07 mg/ml; ■———■, 0.20 mg/ml and ●———●, 0.40 mg/ml leucocyte protein.

Fig. 5. Effect of Triton X-100 on the CT optimum of pure ox-liver cerebroside sulphate sulphatase. Cerebroside sulphate sulphatase activity was measured as a function of CT concentrations in the presence of, ●———●, 0 mg/ml; □———□, 0.1 mg/ml and ▲———▲, 0.25 mg/ml Triton X-100.

The CT optimum of fibroblast cerebroside sulphate sulphatase activity was increased considerably by the addition of 0.42 mg/ml albumin. A new optimum, which appeared to reflect the total protein concentration, was obtained. Similarly, the TD optimum for the leucocyte enzyme activity increased from

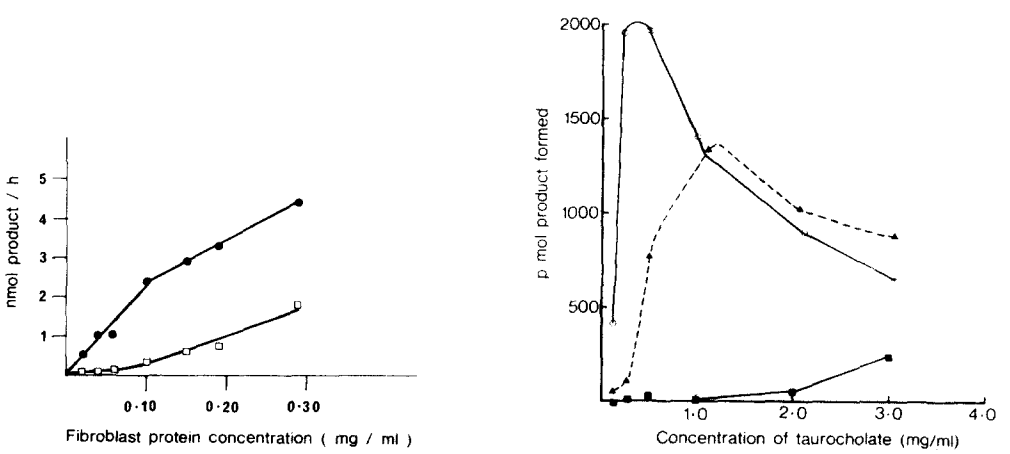

Fig. 6. The effect of bovine serum albumin on fibroblast CT-activated cerebroside sulphate sulphatase activity. Cerebroside sulphate sulphatase activity was measured as a function of fibroblast protein concentration both in the presence (●———●) and absence (□———□) of bovine serum albumin. The former incubations containing varying amounts of fibroblast and albumin which together made up a total protein concentration of 0.5 mg/ml.

Fig. 7. The effect of leucocyte extracts on the CT-optimum of pure ox liver CS sulphatase. Cerebroside sulphate sulphatase activity was measured in the presence of: ○———○, 0 mg/ml; ▲- - - - -▲, 0.06 mg/ml and ■———■, 0.60 mg/ml leucocyte protein.

1.5 mg/ml in the absence of albumin to approx. 2.5 mg/ml in the presence of 0.5 mg/ml albumin. Addition of a fixed amount of pure enzyme (1 μg/ml) to increasing amounts of leucocyte protein or albumin also resulted in an increase in the bile salt optimum (Fig. 7).

Binding of [^{3}H]cerebroside sulphate

It was considered initially that the apparent interdependence of detergent and protein concentration may have been due to binding of either the substrate or the detergents to protein components of the tissue extracts. To test this hypothesis radiolabelled substrate, bile salt and Triton X-100 were separately incubated with tissue extracts (see Materials and Methods). For these experiments radiolabelled cholate was used in place of either CT or TD mainly because of its ready availability. Earlier experiments had demonstrated that the optimum cholate concentration was similarly dependent on the incubated protein concentration. The degree of binding was assessed by counting the sediment produced by centrifugation at high speed (95 000 × *g* for 30 min). More than 90% of the substrate was sedimentable under these conditions; in the absence of tissue extract less than 5% of the lipid was sedimented. The addition of increasing amounts of bile salt (0.5—4.0 mg/ml) or Triton X-100 (0.25—1.0 mg/ml) resulted in a progressive decrease in binding (Figs. 8a, 8b). Neither cholate or Triton X-100 were bound to any significant degree under these conditions.

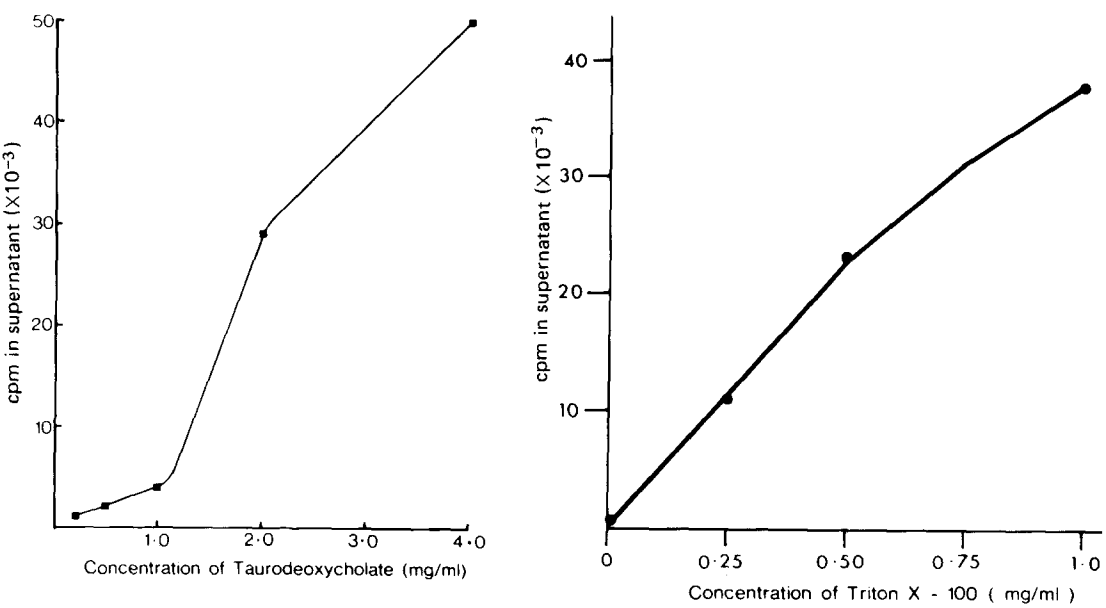

Fig. 8. The effect of CT and Triton X-100 on the binding of CS to leucocyte membranes. Varying amounts of (a) CT (0—4 mg/ml) and (b) Triton X-100 (0—1 mg/ml) were added to incubation mixtures whose composition is described in the text. Radioactivity measurements were performed on the 95 000 × *g* supernatants.

Effect of Triton X-100 and CT on arylsulphatase A activity

Triton X-100 (up to 2 mg/ml) and CT (1—5 mg/ml) had no discernible effect on nitrocatechol sulphate sulphatase activity at the three protein concentrations examined (0.045, 0.13, 0.40 mg/ml).

Discussion

These investigations were prompted by earlier work which indicated that considerable differences existed in the properties of leucocyte and fibroblast cerebroside sulphate sulphatase [3]. The large differences in K_m were later shown to be largely due to the presence of Triton X-100 which appeared to increase both the apparent K_m and V of the leucocyte enzyme [9]. Of particular interest was the earlier observation concerning the apparent activation of the fibroblast enzyme at protein concentrations in excess of 0.1 mg/ml [3], a property apparently not shared by the leucocyte enzyme. Our data demonstrate that, in the absence of Triton, CT-activated cerebroside sulphate sulphatase from both sources displays an activation at protein concentrations between 0.05 and 0.10 mg/ml. As this effect is not shown in the presence of TD, it suggests that the crude bile salt may contain an inhibitor which is neutralised at higher protein concentrations. Some evidence supporting this view is provided by the data shown in Fig. 6, which demonstrate that this effect is abolished by the addition of bovine serum albumin.

The amount of CT required to produce optimum activity increased as protein content of the incubation mixtures increased (Fig. 4). A similar effect was observed for both cholate and TD. Since CT had no readily discernible effect on enzyme activity when measured with nitrocatechol sulphate as substrate, it suggests that this effect is related to the nature of the substrate. The increase in detergent optimum with increasing protein concentration has been observed with other enzymes [5,11,12]. In the present instance, it appears to be related to the binding of the lipid substrate to the sedimentable membrane particles (Figs. 8a, 8b). At low bile salt and high protein concentrations most of the [^{3}H]cerebroside sulphate added to incubation mixtures is bound. At these protein concentrations much greater amounts of bile salt are necessary to release the bound lipid. It seems probable that, in this form, the lipid is a less efficient substrate for the enzyme. The lower bile salt concentrations employed (less than 1 mg/ml, less than 2 mmol/l) may be below their critical micellar concentrations [13] and, thus may explain the marked binding of the [^{3}H]cerebroside sulphate, particularly at high protein levels. As the bile salt concentration is increased the critical micellar concentration is exceeded, thereby leading to an enhancement of detergent action. Our data suggest that excess bile salt, which has been shown to inhibit the pure bovine liver enzyme [14], also inhibits the enzyme in crude leucocyte and fibroblast extracts (Fig. 4). Whether the inhibition of the enzyme in the latter is due to an interaction with protein, or whether it is caused by changes in lipid-bile salt micelles is not known. However, it appears that, in in vitro systems, the addition of bile salts assists both in the solubilisation of the lipid, and in its release from binding with membrane components, probably proteins, present in tissue extracts.

An explanation for the exact role of Triton X-100 at the molecular level in

such a highly complex system is difficult. However, it is clear that the detergent is potentially capable of acting in a number of different ways. Firstly, it may compete with the bile salt for the enzyme either as a result of its incorporation into existing bile salt-lipid micelles [15] or by forming separate Triton-lipid micelles. The increase in K_m of both the crude leucocyte enzyme [9] or the pure bovine liver enzyme (Poulos, A and Beckman, K. (1980), unpublished data) tends to support this view. Secondly, Triton is thought to drastically alter the physical state of micellar lipids [16,17]; thus its interaction with the lipid substrate may result either in mixed lipid-Triton micelles, which we have found to be unsatisfactory substrates, or by its incorporation into lipid-bile salt micelles it could produce structural changes, which result in an altered susceptibility of CS to enzyme atack. A third possible effect concerns the demonstrated affinity that Triton has for certain classes of protein [18]. The nonionic detergent may associate with enzyme and thus reduce the inhibitory effect of the bile salt [19]. This is supported to some extent by the apparent shift in the bile salt optimum of both the crude and pure enzyme, and by the markedly different effects of the detergent in the human and the bovine enzyme. Whether the effect of Triton is due, as seems likely, to the hydrophobic interaction is at present unknown. Some evidence to support this view is provided in the totally differing effects of Triton on cerebroside sulphate sulphatase activity at high and low protein concentrations (Fig. 3). Presumably the great excess of Triton at low protein concentrations would greatly assist the formation of Triton-cerebroside sulphate sulphatase complexes; at high protein concentrations such interactions may be inhibited by the much greater concentrations of nonspecific protein.

These data demonstrate the critical dependence of cerebroside sulphate sulphatase activity on a number of parameters. In particular, the dependence of enzyme activity on the bile salt/protein ratio, and the modifying effect of Triton X-100 on this requirement indicate that the measured enzyme activity is the result of a complex interaction of factors. Moreover, these observations show that any change in concentration of either bile salt, protein or Triton X-100 can have a marked effect on enzyme activity. Our observations have particular relevance to the diagnosis of metachromatic leucodystrophy, and almost certainly have application to the diagnosis of some of the other sphingolipidoses [20]. Unless care is exercised in the choice of incubation conditions, diagnostic errors may result.

Acknowledgements

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References

1 Percy, A.K., Farrell, D.F. and Kaback, M.M. (1972) J. Neurochem. 19, 233—236
2 Booth, C.W., Chen, K.K. and Nadler, H.L. (1975) J. Pediat. 86, 560—564
3 Poulos, A. and Beckman, K. (1978) Proc. Eur. Neurochem. Soc. 1, 570
4 Poulos, A. and Beckman, K. (1978) Clin. Chim. Acta 89, 417—420

5 Poulos, A. and Beckman, K. (1978) Clin. Chim. Acta 89, 35—45
6 Poulos, A. and Pollard, A.C. (1976) Clin. Chim. Acta 72, 327—335
7 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
8 Tomkins, G.A. and Pye, D. (1968) J. Nat. Canc. Inst. 40, 91—96
9 Poulos, A. and Beckman, K. (1979) Clin. Chim. Acta 95, 113—121
10 Snyer, R.A. and Brady, R. (1969) Clin. Chim. Acta 25, 331—38
11 Mapes, C.A., Suelter, C.H. and Sweely, C.C. (1973) J. Biol. Chem. 248, 2471—2479
12 Roelfzema, H., Broekhuyse, R.M. and Veerkamp, J.H. (1973) Biochim. Biophys. Acta 306, 329—339
13 Small, D.M. (1973) in 'The Bile Acids', Chemistry, Physiology and Metabolism, (Nair, P.P. and Krit chevsky, D., eds.) Vol. 1, pp. 249—355, Plenum Press, New York
14 Jerfy, A. and Roy, A.B. (1973) Biochim. Biophys. Acta 239, 178—190
15 Shankland, W. (1970) Chem. Phys. Lip. 4, 109—130
16 Dennis, E.A. (1973) J. Lip. Res. 14, 152—159
17 Kaplan, A. and Teng, M. (1971) J. Lip. Res. 12, 324—330
18 Clarke, S. (1977) Biochem. Biophys. Res. Commun. 79, 46—52
19 Mueller, O.T. and Rosenberg, A. (1979) J. Biol. Chem. 254, 3521—3525
20 Poulos, A. and Beckman, K. (1980) Clin. Chim. Acta., in the press