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CEREBROSIDE GALACTOSIDASE: A METHOD FOR DETERMINATION AND A COMPARISON WITH OTHER LYSOSOMAL ENZYMES IN DEVELOPING RAT BRAIN[1]

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Abstract—(1) A method is described for assaying brain for cerebroside galactosidase activity. The enzyme was liberated by sonication and addition of sodium taurocholate, then by digestion with pancreatic enzymes. It was further purified by precipitation at pH 3. The enzyme was then incubated with an emulsion of galactose-labelled cerebroside in taurocholate and oleate at pH 4·5, and the liberated galactose was determined by scintillation counting.

(2) The content of cerebroside galactosidase in rat brain at various ages has been determined. The enzyme was present before cerebroside appears in noticeable amounts (4 days) and the amount rose considerably during the period of active cerebroside deposition and myelination. The amount then remained at a high concentration even in the adult.

(3) Comparison with other lysosomal brain enzymes was made in the age study. Nitrophenyl galactoside hydrolase also increased during myelination but levelled off earlier; its activity paralleled the amount of ganglioside. Nitrophenyl glucoside hydrolase started at a lower level and decreased with age. Sulphatase activity rose during myelination, then decreased somewhat after 15 days. Ceramidase followed a pattern similar to that of nitrophenyl galactoside hydrolase; it is suggested that both of these enzymes reflect ganglioside metabolism.

(4) The relative amounts of brain enzymes in different states were determined as a function of age in the case of cerebrosidase, nitrophenyl galactoside hydrolase and sulphatase. The proportion found in the high speed supernatant fraction was low but increased after myelination. The proportion that could be 'solubilized' by sonication decreased after myelination but the values differed greatly for the three enzymes. This treatment solubilized one-seventh of the cerebrosidase, half the nitrophenyl galactosidase and three-quarters of the sulphatase.

IN THE course of developing a method for purification of rat brain cerebroside galactosidase (cerebrosidase), we observed that there was a positive correlation between the concentration of enzyme and the concentration of cerebroside (BOWEN and RADIN, 1968*a*). The subcortical region of rat brain contained more of both than did the cortex, and older rats contained more of both than did younger rats. It seemed of interest to obtain further details on the changes with age, especially with regard to the question whether or not cerebrosidase appears in brain before the appearance of cerebroside. This lipid is essentially indetectable before 9 days in rat brain, then increases rapidly (KISHIMOTO, DAVIES and RADIN, 1965).

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Abbreviations used: NPGalH, *p*-nitrophenyl β-D-galactoside hydrolase; NPGluH, *p*-nitrophenyl β-D-glucoside hydrolase; Buffer A, 0·05 M-Tris–HCl, pH 7·4 (at room temperature), + 0·01 M-$MgCl_2$ + 8 mM-mercaptoethanol.

Determination of cerebrosidase is most easily performed with radioactive cerebroside by measuring the release of labelled galactose, but the substrate specific activity is drastically affected by the content of endogenous cerebroside in the tissue. It was therefore necessary to develop an assay method which involves removal of the endogenous lipid. Complicating the assay was the finding in the preparative studies (BOWEN and RADIN, 1968*a*) that digestion with pancreatic enzymes considerably increased the activity of the cerebrosidase, and for this reason a digestion step was included in the assay. The method finally developed is described in this paper.

By way of comparison, we assayed additional acid hydrolases which seem to be involved in sphingolipid breakdown. Ceramidase (GATT, 1966*a*) acts on the lipoidal product of cerebrosidase action. NPGalH is probably a group of galactosidases, most of which do not act on cerebroside (BOWEN and RADIN, 1968*b*), but which may act on ceramide dihexoside and tetrahexoside (GATT, 1967). The enzyme which hydrolyses cerebroside sulphate also acts on 5-nitrocatechol sulphate (MEHL and JATZKEWITZ, 1968); the more convenient synthetic substrate was used here. NPGluH activity was also measured; perhaps this reflects activity toward glucosyl ceramide (GATT, 1966*b*).

On the basis of subcellular localization studies, cerebrosidase (BOWEN and RADIN, 1968*b*), NPGalH (SELLINGER and RUCKER, 1964) and sulphatase (MEHL and JATZKEWITZ, 1964; SELLINGER and HIATT, 1968) have been characterized as lysosomal. Ceramidase and glucosidase, like these enzymes, have low pH optima and are therefore probably also lysosomal. Lysosomal enzymes are readily released to differing extents (SAWANT, SHIBKO, KUMTA and TAPPEL, 1964) and it seemed possible that the extent of release might be related to age. Several enzymes and age points of special interest in brain maturation were therefore examined for this characteristic.

MATERIALS AND METHODS

Reagents. *N*-Stear-[^{14}C]oylsphingosine (ceramide) was made by Dr. KRYSTYNA KOPACZYK (KOPACZYK and RADIN, 1965). *N*-Stearoylpsychosine (galactosyl 18:0 ceramide or cerebroside) was made with tritium in the 6-position of the galactose (BOWEN and RADIN, 1968*a*). Sodium taurocholate and pig pancreatic 'lipase' (both relatively crude products) were obtained from Mann Research Labs., New York. The artificial substrates were obtained from Sigma Chemical Co., St. Louis. Highly purified bile salts were obtained from Mann Research Labs. and Calbiochem, Los Angeles.

Animals. Female rats of the Sprague–Dawley strain were purchased from Spartan Research Animals, Haslett, Michigan. The three youngest groups of animals were purchased with foster mothers and kept 3 days before killing. The older groups were also kept 3 days before killing.

Preparation of tissues for assay. The rats were anaesthetized with carbon dioxide, decapitated and the brains removed. Groups of brains were homogenized with 3·5 vol. of Buffer A by the procedure of SELLINGER and DE BALBIAN VERSTER (1962) eight up and down strokes being used. The homogenates were transferred to a beaker with an additional 1·5 vol. of Buffer A, then sonicated with an immersion probe (BOWEN and RADIN, 1968*a*).

Cerebrosidase determination. The homogenate (1·5 ml) was mixed with 0·75 ml of 6% sodium taurocholate in Buffer A, 10 mg of pancreatic 'lipase' were added and the mixture was sonicated in a bath of the type used for cleaning (Ultrasonic Industries, Albertson, N.Y.). The mixture was stirred magnetically overnight at 4° and 0·4 ml of 2% taurocholate in Buffer A was added. The mixture was transferred to a cellulose nitrate tube (0·5 × 1·25 in.) and centrifuged at 100,000 *g* for 1 hr. (The extra taurocholate solution was needed to minimize the danger of collapse of the test tube.) A 2 ml portion of the clear supernatant was mixed with 0·33 ml of 1 M-sodium citrate buffer, pH 3, then stirred for 30 min. The precipitated enzyme was collected by centrifugation at 14,000 *g* for 20 min and suspended in 4 ml of Buffer A diluted with 2 parts of water. The enzyme assay was performed with 0·1 and 0·2 ml portions. The enzyme suspension could be stored at least 2 days at 4° and 20 days at −20°.

The substrate was prepared by evaporating to dryness a mixture of solutions of [^{3}H]cerebroside

(1 mg, 350 counts/min/mμmole), Tween 20 (10 mg) and G-2159 (5 mg). The detergent, G-2159, is a polyoxyethylene stearate from Atlas Chemical Industries, Wilmington, Delaware. The cerebroside was stored in chloroform–methanol and the detergents in benzene, both at −20°. The residue was then sonicated in a bath and warmed with 4 ml of sodium taurocholate solution (5 mg/ml) to give a clear emulsion.

'Activator suspension' was prepared by evaporating a hexane solution of oleic acid (3·5 mg) to dryness, then sonicating in a bath at room temperature with 1 ml of tris base (1·65 mg, 1·1 equiv.).

To each incubation tube were added 0·4 ml of substrate, 0·1 ml of 'activator suspension', enzyme suspension, Buffer A–water 2:1 and 0·1 ml of 1 M-citrate buffer, pH 4·5; total volume, 1 ml. The turbid mixture was shaken gently at 37° for 3 hr, after which time 5 ml of chloroform–methanol 2:1 and 0·1 ml of galactose (0·1 mg) were added. The mixture was agitated by ten inversions (Teflon-lined screw cap) and centrifuged and the upper layer was washed with 1·5 ml of 'Folch lower layer' (FOLCH, LEES and SLOANE-STANLEY, 1957). A 1·5 ml portion of the upper layer was evaporated to dryness in a scintillation vial at 50° with a stream of air. The residue was dissolved in 0·5 ml of water and counted in 15 ml of scintillation solvent (BRUNO and CHRISTIAN, 1961). The mixture is turbid initially but clears when the insoluble portion settles out. The observed activities drop slightly with time and stabilize after several hours.

Ceramidase determination. This method is based on the procedure of GATT (1966*a*). The substrate emulsion was prepared by evaporating to dryness solutions of [^{14}C]ceramide (0·92 mg), Tween 20 (10 mg) and G-2159 (5 mg), adding 2 ml of sodium taurocholate solution (10 mg), sonicating in a bath and warming. Sodium acetate buffer, pH 5 (0·5 M), was mixed just before use with γ-D-galactonolactone, 16 μmoles/ml.

The incubation tubes contained 0·2 ml of substrate (50,000 counts/min), 0·1 or 0·2 ml of the sonicated brain homogenate (diluted with an equal volume of water) and 0·1 ml of acetate–lactone solution; total volume = 0·5 ml. The mixture was shaken gently at 37° for 3 hr and partitioned by a modification of the method of DOLE (1956). The extraction mixture contained more concentrated sulphuric acid, 3 N, and hexane instead of heptane. Also, the hexane added to form the second liquid phase contained 0·1 mg carrier stearic acid. The stearic acid was isolated from the hexane for counting by the method of GATT (1966*a*). The assay procedure was shown to be linear with respect to time for 3 hr.

Galactonolactone was included in the incubations to prevent hydrolysis of endogenous cerebroside, with consequent dilution of the radioactive ceramide. However, later tests showed it had little effect on the observed values.

Colorimetric enzyme assays. A portion of the sonicated brain homogenate was diluted with an equal volume of Buffer A and 0·2 and 0·4 ml portions were used for determination of activity upon *p*-nitrophenyl-β-D-glucoside (5 μmoles). The incubation mixture contained 100 μmoles of sodium acetate buffer, pH 4·5 (GATT and RAPPORT, 1966) in a total volume of 1 ml. Incubation was for 1 hr at 37° then the reaction was stopped and the *p*-nitrophenol content determined according to the method of HUNTER and MILLSON (1966).

The diluted homogenate described above was further diluted with 2 vol. of water for NPGalH determination. Conditions were as above, the corresponding galactoside and sodium citrate buffer, pH 3·1, being used (GATT and RAPPORT, 1966). Smaller portions of this diluted homogenate were used for sulphatase assay, 0·05 and 0·1 ml, according to the method of SELLINGER and HIATT (1968). The buffer was 100 μmoles sodium acetate, pH 5, (BLESZYNSKI, 1967), and incubation was carried out for 1 hr.

The specific activities for the three colorimetric assays were independent of sample weight and duration of incubation up to 1 hr. Preincubation of the sonicated brain homogenates with 0·1% Triton X-100 for 10–30 min did not result in increased activity.

Glucosidase was determined on the day the animals were killed, as it was found to be relatively unstable. Sulphatase and the galactosidases were determined the next day, and ceramidase on the fourth day (with frozen homogenate).

RESULTS

Development of the cerebrosidase assay. It had been found that crude brain fractions did not yield linear results with respect to amount of tissue (BOWEN and RADIN, 1968*a*). This effect was apparently due to dilution of the radioactive substrate by endogenous cerebroside, as shown by the breakdown of endogenous cerebroside during incubation (HAJRA, BOWEN, KISHIMOTO and RADIN, 1966) and the improvement in linearity on purification of the enzyme. For this reason a simple method of separating the cerebrosidase from the accompanying substrate was devised. In developing a method for isolating this enzyme, we found that extraction with sodium

cholate and digestion with pancreatic enzymes brought the enzyme into soluble form while considerably increasing the total apparent activity.

The large amount of cholate and residual cerebroside at this stage interfered with subsequent assay and it was found that at pH 3 the enzyme was precipitated, leaving cerebroside in the supernatant. It was necessary to use taurocholate instead of cholate, since the latter is very insoluble at this pH.

In a study of these factors, whole rat brain (female, 9–12 months old) was homogenized in 9 vol. of 2% taurocholate in Buffer A; (line 1, Table 1). A portion of the homogenate was centrifuged at 80,000 *g* for 2 hr. This treatment improved the

TABLE 1.—EFFECT OF BRAIN PROCESSING ON CEREBROSIDASE ACTIVITY

Brain fraction	Enzyme activity (units/5 mg brain)	Linearity factor*
1. Total homogenate	192	0·60
2. Taurocholate extract, supernatant	236	0·80
3. (2) treated with 'lipase' (16 mg/g brain)	430	0·96
4. pH 3 precipitate from (3)	397	0·98
5. Supernatant from (4)	7	—
6. Insoluble material from (2), treated with 'lipase' as above, supernatant portion	115	1·05
7. Insoluble portion from (6)	16†	0·67
8. (1) after 'lipase' treatment as above, supernatant portion	507	0·99
9. Insoluble portion from (8)	64†	0·68
10. Like (8) but treated with 32 mg 'lipase'/g brain	510	1·05
11. pH 3 precipitate from (10)	453	0·98
12. pH 3 supernatant from (10)	7	—
13. Insoluble portion from (10)	63†	0·68

Oleate was not included in the incubations, but the substrate was made up to double the usual specific activity. Enzyme units refer to mμmoles of galactose released per hr per g of wet brain.

* Ratio of specific activities observed with 10 and 5 mg of brain.

† Pellet contained about 0·4 ml of supernatant, out of 9 ml total supernatant.

linearity in the supernatant (line 2) and total activity rose a little, apparently as the result of removal of some cerebroside in the pellet. Treating the supernatant liquid with 'lipase' led to a great increase in activity (line 3). While in this experiment the linearity was rather good, the effect was somewhat erratic. Lines 4 and 5 show that only slight loss in activity resulted from the pH 3 precipitation. (The supernatant from this precipitation was dialysed against Buffer A containing 2% taurocholate to remove the citrate and correspondingly less taurocholate was used in making up the substrate.) A control incubation without brain showed that the 'lipase' was essentially devoid of cerebrosidase activity.

Examination of the taurocholate-insoluble fraction of brain, by incubation with 'lipase' in taurocholate, showed that an appreciable amount of activity had remained insoluble (lines 6 and 7). The digestion solubilized most of the residual activity and most of the endogenous cerebroside apparently remained insoluble, as indicated by the linearity factors. Lines 8 and 9 show that omitting the initial centrifugation produced a supernatant after digestion which had about 89 per cent of the total activity (the activity in the pellet was not corrected for inclusion of soluble enzyme or for dilution of the radioactive substrate). The activity in the supernatant at this point, 507 units, was 93 per cent of that found in the separately digested extract and

pellet (lines 3 and 6). A test of the complete procedure with double the amount of 'lipase' (lines 10–13) gave very similar results. The final value, 453 units, seems to represent about 79 per cent of the total brain activity.

A portion of the suspended pH 3 precipitate was examined for cerebroside content by adjusting the pH to 8·5 with NaOH, extracting with 5 vol. of chloroform–methanol 2:1, and centrifuging. The lower layer was analysed by TLC with known amounts of cerebroside, chloroform–methanol–water 24:7:1 being used. It was estimated that the precipitate derived from 10 mg of brain would contain 2 μg of hydroxy cerebroside and 0·5 μg of nonhydroxy cerebroside; this is derived from an estimated 68 μg of hydroxy and 37 μg of nonhydroxy cerebroside in the original sample (KISHIMOTO and RADIN, 1959). Since the radioactive cerebroside in each incubation tube weighed 100 μg, the dilution by residual cerebroside was only 2·5 per cent at the higher level of tissue. The amount of cerebroside in the supernatant remaining after pH 3 precipitation was 4–5 times that in the precipitate.

The pancreatic enzymes were added as dry material rather than as a suspension in water because the latter was too heterogeneous for pipetting, despite sonication of the suspension.

The activating effect of digestion. The apparent increase in cerebrosidase activity that results from 'lipase' digestion (Table 1, lines 2 and 3) might be due to partial destruction of endogenous cerebroside during the incubation. That this is a minor factor was shown by repeating the comparison with less tissue. With 4·8 mg of brain, the taurocholate extract (after centrifugation) showed an activity of 245 units and a linearity factor of 0·93, when compared with 2·4 mg brain. When the corresponding supernatant liquid was treated with 'lipase' overnight, the activity was 502 units and the linearity was 1·01. Thus a doubling of activity ensued as the result of incubation while the effect of endogenous cerebroside was shown to be almost negligible. These assays, and those in Table 1, were carried out without oleate in the incubation medium.

Since the digestion process probably hydrolyses some of the brain lipids, we thought the activation might be due to formation of free fatty acid. To test this, we treated the pH 3 precipitate in citrate buffer (pH 3) with 3 $\times$ 5 vol. of diethyl ether, with a VirTis homogenizer to ensure mixing. Nitrogen was bubbled through the aqueous layer to remove ether and the enzyme activity in the suspension was compared with that of an untreated control. The extraction procedure reduced the cerebrosidase activity by 24 per cent; this supported the idea that part, at least, of the lipase activation was due to formation of an acidic lipid. Two-thirds of this activity loss was restored on adding back the lipids in the ether extract.

The lipid remaining in the ether-treated enzyme was removed with chloroform–methanol and the lipids in this and the ether extract were analysed by TLC with hexane–ether–acetic acid 80:20:3. Comparison with standards showed that the ether extract contained much fatty acid and that the residual enzyme contained none. (The ether extract also appeared to contain cholesterol and a nonpolar material.)

On the basis of these experiments and our earlier finding (BOWEN and RADIN, 1968*b*) that palmitic acid stimulated the activity of a crude cerebrosidase preparation, we investigated the effect of adding oleic acid to the assay system. Figure 1 shows that the observed activity was almost doubled by adding 250–500 μg of oleic acid (as the tris salt).

When the assay was carried out as in the Methods section, the observed specific activities were independent of sample weight and duration of incubation, in the absence or presence of oleate.

Comparison of the bile salts. In our initial study with partially purified cerebrosidase from rat brain supernatant liquid (HAJRA *et al.*, 1966), we showed that little enzyme activity could be demonstrated unless cholate was present. Taurocholate was more effective, while deoxycholate was only slightly stimulatory. To find optimal conditions for assay with the pH 3 precipitate, we compared a wider assortment of

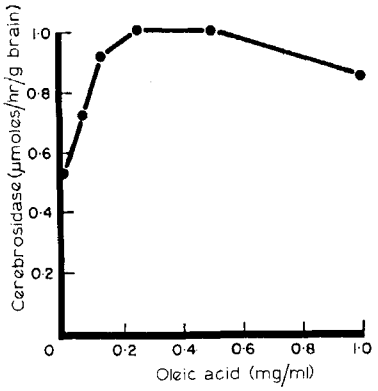

FIG. 1.—Effect of oleic acid on observed cerebrosidase activity. Incubation was as described in the text, but with varying amounts of tris oleate, and enzyme derived from 4·8 mg of brain by the digestion–precipitation method.

bile salts. The data (Table 2) show that the crude taurocholate gave the best activity and a mixture of pure taurodeoxycholate and taurocholate gave second best. The pH 3 precipitate contained some taurocholate from the digestion step, enough to give a relative activity of about 30 per cent of the maximum. It is apparent that taurochenodeoxycholate and deoxycholate were actually inhibitory. In almost every case an increased level of bile salt was inhibitory.

TABLE 2.—EFFECT OF BILE SALTS ON CEREBROSIDASE ACTIVITY

Bile salt (Na)	Relative activity	
	2 mg bile salt	4 mg bile salt
Taurocholate (crude)	100	80
Taurocholate	61	40
Taurodeoxycholate	66	43
Taurocholate + taurodeoxycholate (equal parts)	82	—
Taurolithocholate	57	59
Taurochenodeoxycholate	23	10
Deoxycholate	20	21
Cholate	40	21

Data expressed as percentage of activity obtained with 2 mg of crude taurocholate; all samples assayed in the absence of added oleate. Except for the crude taurocholate, all bile salts were highly purified.

Enzyme changes with age. The activity of the five hydrolases was determined in brain of rats of 4, 7, 15, 24, 90 and 320 days old. (The ages of the last two groups are approximate.) Four brains were pooled for each analysis and the average value

obtained from three such groups was used to plot Figs. 2 and 3. The vertical line shown at each time point indicates the two extreme values.

Of particular interest is the wide diversity of changes between the different enzymes. Ceramidase and sulphatase reached maxima at the point of most active myelination, while NPGalH reached its maximum a little later, and cerebroside galactosidase reached its maximum even later. In contrast, NPGluH remained rather

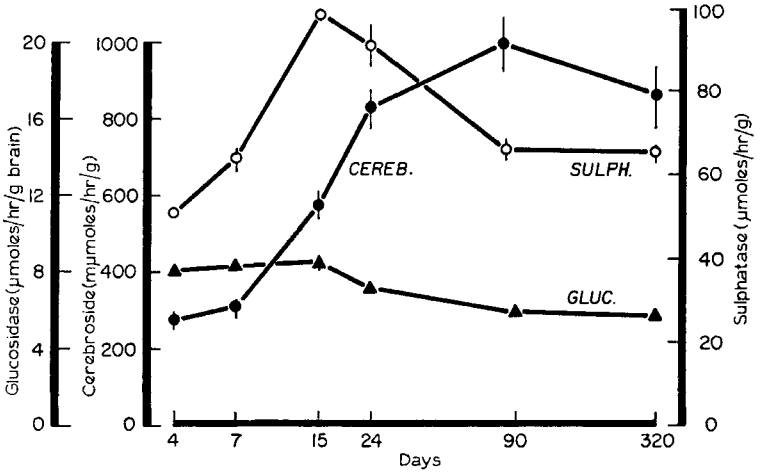

FIG. 2.—Developmental profile of cerebrosidase, sulphatase and glucosidase, with age shown logarithmically.

constant until 15 days, then declined moderately. The enzyme showing the most rapid decline, following its maximum, was sulphatase and the enzyme showing the greatest *percentage* increase was cerebrosidase.

Since some cerebrosidase is discarded in our assay method, we examined the two

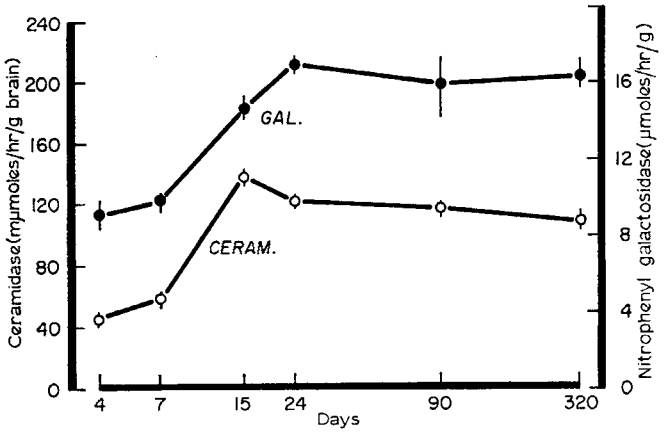

FIG. 3.—Developmental profile of nitrophenyl galactosidase and ceramidase, with age shown logarithmically.

remaining fractions. The insoluble portion remaining after 'lipase' treatment contained the following specific activities at the respective age points: 23, 26, 64, 98, 98 and 78 units (mμmoles/hr/g). These figures were calculated from 5 mg brain equivalents and the linearity factors were 1·05, 0·98, 1·00, 0·83, 0·79 and 0·73. The good

linearity in this residue of the first three age points is due to the very low concentration of cerebroside in such young rat brains. It is evident that the insoluble enzyme accumulates with age according to the pattern for the major part of the enzyme. The activity in the supernatant fraction following pH 3 precipitation was in all cases less than 5 per cent of the total.

The ceramidase values showed an interesting progression with age with respect to the effect of tissue weight on the observed activity. The linearity factors, based on 16·7 and 8·3 mg brain, were 0·84, 0·83, 0·92, 0·89, 0·98 and 1·03. These results suggest that there is an appreciable amount of endogenous ceramide in the youngest animals.

The states of lysosomal enzymes as a function of age. Studies of lysosomal enzymes have shown that a certain proportion of the tissue activity is to be found in the cytosol (high speed supernatant fluid obtained from homogenates) and that additional activity can be brought into the supernatant fraction by a variety of particle-disrupting procedures (DE DUVE, 1963; SAWANT *et al.*, 1964). Thus one can distinguish three types of activities: cytosol, dispersible and nondispersible. It seemed of interest to compare these three types as a function of age, so three groups of rats were chosen for analysis for cerebrosidase, sulphatase and NPGalH. Duplicate runs were made, each with four pooled brains from 5-day-old rats and two pooled brains from 15- and 320-day-old rats.

The brains were homogenized as in the Methods section, but with six up and down strokes and in 0·25 M-sucrose. The homogenate was diluted with 2·5 vol. of sucrose (total dilution seven-fold) and centrifuged at 100,000 *g* for 1 hr; this yielded the cytosol fraction. The pellet was suspended in Buffer A (6 ml/g brain), sonicated as described before and re-centrifuged; this yielded the dispersible fraction. The pellet from this step, containing the nondispersible fraction, was suspended in Buffer A and portions were taken for analysis. Because of the large amount of endogenous cerebroside in this fraction, the portions for cerebrosidase assay had to be treated by the 'lipase'-pH 3 procedure.

The data for total activity in Table 3 show changes with age that are very similar to those of Figs. 2 and 3. The percentage of activity in the cytosol fraction was small for all three enzymes, rising somewhat after 15 days of age. The percentage in the dispersible fraction was characteristic of the individual enzyme, being highest with sulphatase and intermediate with NPGalH. The percentage in this fraction decreased with age for all three enzymes. The nondispersible fraction showed relatively small changes with age.

The linearity factor in the cytosol fraction for cerebrosidase decreased with age, being 0·93, 0·83 and 0·74 at the three age points. Since this fraction was assayed without prior removal of endogenous cerebroside, the relative activities for the older animals were even higher than indicated in Table 3. Cerebroside does occur in this fraction (HAJRA *et al.*, 1966) and apparently its concentration increases with age. The linearity factors in the dispersible fraction were little affected by age (0·95, 0·91, 0·91) and apparently little cerebroside enters this material.

DISCUSSION

Brain contains several distinctly different kinds of cells, and it would not be surprising to find that each cell produces lysosomes of different enzymic composition.

TABLE 3.—STATES OF LYSOSOMAL ENZYMES AS A FUNCTION OF AGE

Age group (days)	Cerebrosidase					Sulphatase					Nitrophenyl galactosidase				
	% Distribution			Total activity	% Recovery	% Distribution			Total activity	% Recovery	% Distribution			Total activity	% Recovery
	C	D	ND			C	D	ND			C	D	ND		
5a	2	17	81	129	108	1	80	19	33,000	—	4	57	40	7000	—
5b	2	16	82	132		1	77	21	34,000	91	4	52	45	7700	94
15a	2	14	84	228	115	1	77	23	69,000	105	2	51	47	13,200	101
15b	1	12	87	240		1	72	27	67,000	105	2	46	52	13,000	102
320a	5	7	88	344	93	3	75	20	44,000	102	12	40	48	13,500	104
320b	4	7	89	332		3	68	27	39,000	86	11	38	53	12,500	96

C = cytosol fraction of brain homogenate; D = dispersible fraction from particulate matter; ND = nondispersible residual matter. Total activity, in mμ moles/hr/g wet brain, is obtained by summing the activities in the three brain fractions. Recovery values were obtained from portions of the initial total homogenate, following sonication (in the case of cerebrosidase, equal portions of the homogenates from both duplicate groups of rats were pooled before assay). The cerebrosidase assays were carried out as in Table 1. The sulphatase was assayed according to the method of SELLINGER and HIATT (1968).

Since the number, size and state of each cell type follow different patterns in the developing brain, one would expect to find different developmental patterns for their enzymes. Perhaps this explains the different curves of Figs. 2 and 3 and the different states of binding (Table 3).

An additional factor is the matter of true substrate, since three of the five enzymes we studied were assayed with unnatural materials. There appear to be at least three galactosidases in brain, acting on different galactolipids (BOWEN and RADIN, 1968*b*) and we also know that a substantial part of the nitrophenyl galactosidase activity can be separated from cerebrosidase activity. Gangliosides have a higher turnover rate than cerebrosides, at least in weanling rats (RADIN, MARTIN and BROWN, 1957). Thus, if the lysosomal enzymes take part in the normal degradative processes, one would expect to find that the NPGalH reflects the hydrolase activity for ganglioside intermediates more than for cerebroside.

This interpretation is supported by the data in this paper. These show considerable parallelism between deposition rate of cerebroside and cerebrosidase, and deposition rate of ganglioside and NPGalH (KISHIMOTO *et al.*, 1965). Deposition of cerebroside and its hydrolase is most rapid between 15 and 25 days of age while that of ganglioside and NPGalH is most rapid during an earlier period of life. In further support is the recent finding by OKADA and O'BRIEN (1968) that patients with generalized gangliosidosis have a marked deficiency in the galactosidase which attacks ganglioside GM_1(gal-galNAc-(sialyl)gal-gluceramide) and, to a lesser extent, in NPGalH.

The pattern for ceramidase changes with age follows somewhat the pattern for NPGalH; this suggests that the former measurement represents the ceramidase acting on ceramide from ganglioside breakdown rather than on ceramide from cerebroside breakdown.

These findings can be interpreted reciprocally as supporting the idea that lysosomes function in normal metabolic turnover rather than in pathological processes alone.

Cerebrosidase is present in appreciable quantities at 4 days, even though its substrate is barely detectable at this point. The large amount of cerebrosidase in mature rats is consistent with the observation that myelin cerebroside (the major site for this lipid) undergoes turnover (SMITH and ENG, 1965). It also suggests that cerebroside turnover in myelin is higher than isotope experiments might indicate, owing to efficient reutilization of the hydrolysis products prior to dilution with adjacent metabolic pools.

ROBINS, FISHER and LOWE (1961) reported a similar age study with rat brain and the fluorogenic substrate, methylumbelliferone galactoside. Their curve resembles that of ours with the unnatural substrate (Fig. 3) in shape and absolute activities, but their curve shows some decrease in the mature animal. Perhaps their substrate, with its larger aglycone group, resembles cerebroside more than it does ganglioside intermediates in its susceptibility to the action of the various brain galactosidases.

The data in Table 3 for relative activity in the cytosol fraction are more interesting when calculated on an absolute basis. Cerebrosidase goes from 3·5 units to 15 units between 15 and 320 days of age; sulphatase goes from 680 units to 1250 units and NPGalH goes from 131 to 1500 units. Perhaps these changes mean that lysosomes become somewhat more fragile with increasing age and, if the cytosol form is the physiologically active form, that it is important for the mature brain to minimize the accumulation of additional lipid.

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