CEREBRAL LYSOSOMES. IV.
THE REGIONAL AND INTRACELLULAR DISTRIBUTION OF ARYLSULFATASE AND EVIDENCE FOR TWO POPULATIONS OF LYSOSOMES IN RAT BRAIN

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INTRODUCTION

Following the original observation of Beaufay *et al.*[3], the presence of lysosomes in brain has been amply documented and a lysosomal localization for the following acid hydrolases established[15,18,21,27,28]: acid phosphatase (EC 3.1.3.2), β-2-acetylamino-2-deoxy-D-glucohydrolase (*N*-acetylglucosaminidase) (EC 3.2.1.30), β-galactosidase (EC 3.2.1.23), β-glucuronidase (EC 3.2.1.31), acid ribonuclease (EC 2.7.7.16), acid deoxyribonuclease and cathepsin. Less is known, however, about the intracellular localization of several other hydrolases, also detected in nervous tissue, *e.g.* arylsulfatase (EC 3.1.6.1) (refs. 2, 8), neuraminidase (EC 3.2.1.18) (ref. 22), and β-glucosidase (EC 3.2.1.21) (ref. 10).

Regionally, the lysosome has been seen in the electron microscope, *inter alia*, in peripheral nerve[13], the pineal gland[1], the cerebellum[11] and the cerebral cortex[18], while histochemical[11,19] and ultramicrochemical[23,31] techniques have suggested its presence in several other brain regions and brain tumors[12], as well. Functionally[7], lysosomes have been implicated in the response of the nervous tissue to various injurious agents or treatments such as nerve crush[13], nerve transection[17], convulsants[29] and viral infection[15]. A specialized role for lysosomes in the neonatal period has also been suggested[4].

The present study approaches the possibility of qualitative differences among lysosomes of rat brain cortex, hypothalamus and thalamus. Secondly, it reports on the activity of two lysosomal hydrolases, arylsulfatase (sulfatase) and *N*-acetylglucosaminidase (NPG-ase), in a total of 6 rat brain regions, the premise for this aspect of the study being that the existence of interregional differences in enzyme activity and/or properties may reflect a microheterogeneity of lysosomes which in turn may aid in the biochemical characterization of a given brain structure known to be endowed with specific functions.

A preliminary report has appeared[30].

EXPERIMENTAL

Materials

Chemicals. *o*-Nitrocatechol, *o*-nitrocatechol sulfate (K salt), pyruvic acid (K salt) and NADH: Sigma Co., St. Louis, Mo.; *o*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (NPG): Pierce Chemical Co., Rockford, Ill.; Triton X-100: Rohm and Haas, Philadelphia, Pa.; acetylthiocholine iodide (ACTH) and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5 phenyltetrazolium chloride (INT): Dajac Laboratories, Philadelphia, Pa; 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB): K and K Labs., Plainview, N.Y.

Animals. Male rats, weighing between 150 and 250 g were used.

Methods

Analytical

Protein was determined according to Lowry *et al.*[20] with bovine serum albumin as standard.

Enzymic

Arylsulfatase. This was measured with 6 m*M* *o*-nitrocatechol sulfate[24] as substrate at pH 5 in 0.05 *M* pyridine buffer. Free and total activities are distinguished, as described previously[27]. Specific details of incubation conditions are referred to in the legends to the tables. Enzyme action was stopped by the addition of 1 ml of 4% trichloroacetic acid and the resulting precipitate was removed by centrifugation at 2000 rev./min for 15 min. Samples (1.5 ml) of the supernatant fluid were pipetted into 2.5 ml of 2.5 *N* NaOH and the resulting color was read at 515 mμ in a Gilford microsample spectrophotometer, model 300. One unit of activity refers to the release of 1 μmole of *o*-nitrocatechol/min.

N-acetyl-β-D-glucosaminidase (NPG-ase). This was measured as previously described[25,27] and, as for sulfatase, the specific incubation details are given in the legends. One unit refers to the release of 1 μmole of *o*-nitrophenol/min.

Acetylcholine esterase (EC 3.1.1.7). The assay[9] was performed as follows: to 3 ml of 0.2 *M* Na-phosphate buffer (pH 8.0) was added 0.1 ml of DTNB (40 mg in 10 ml of 0.1 *M* K-phosphate buffer, pH 7, containing 15 mg of $NaHCO_3$) and 25 μl of ACTH (375 mg/5 ml of water), the absorption at 412 mμ was determined and enzyme added to initiate the reaction. Absorption at 412 mμ was monitored for 3 min of linear reaction course. The net change in absorption expresses enzyme activity.

Succinate-INT-reductase. This activity, used as a mitochondrial marker[15], was assayed as follows: 0.5 *M* Na-succinate, 0.1 ml, and 0.5% (w/v) INT, 0.2 ml, were brought to 0.5 ml with 0.5 *M* K-phosphate buffer, pH 7.4. Suitable volumes of enzyme were added, the tubes incubated at 37°C (see Table V for details) and the reaction terminated by the addition of 1 ml of a 1 : 1 mixture of ethyl acetate and ethyl alcohol containing 10% (w/v) trichloroacetic acid. The tubes were buzzed briefly and absorption at 490 mμ determined.

Lactic dehydrogenase (EC 1.1.1.27). This was determined[16] by monitoring the disappearance of NADH at 340 mμ. Blanks consisted of all incubation components, minus K-pyruvate.

Surgical dissection of rat brain

This was performed manually on freshly excised brains kept on an ice-cold surface. When desired, up to 6 regions were separated: cerebral cortex, cerebellum, hippocampus, corpus striatum, hypothalamus and thalamus. The wet tissue weight was determined and homogenization in 0.25 *M* sucrose accomplished so as to yield 10–15% homogenates.

Centrifugal fractionation of rat cerebral cortex

The centrifugation schedules outlined in detail previously[26] were used without modification. These resulted in the isolation of 5 fractions (N, M, L, P, S), of the combined fraction M + L or of fraction L alone. The latter 2 fractions (containing predominantly nerve endings, mitochondria and lysosomes) were used for density gradient centrifugation. Additional details are given in the legends to the tables.

Density gradient centrifugation

Continuous gradients of sucrose in distilled water were set up directly and also by allowing discontinuous gradients to equilibrate (16–24 h, 0°C). The gradients were carefully loaded and, after centrifugation, fractions were collected either by manual

TABLE I

TOTAL ACTIVITY OF NPG-ASE AND SULFATASE IN BRAIN REGIONS OF THE RAT

Tissue homogenates (10–15%, w/v) were prepared in 0.25 *M* sucrose and were preincubated in the presence of 0.1% Triton X-100 and 0.05 *M* pyridine buffer, pH 5 at 37°C for 120 min, a treatment found necessary[27] to maximize enzyme activity. Then, samples were withdrawn and pipetted into the respective assay medium and incubation carried out at 37°C for 10 min. The actual ranges of values (units/g) were: Sulfatase: cortex, 0.95–1.58; cerebellum, 0.80–1.67; hippocampus, 0.96–1.90; corpus striatum, 1.14–1.80; hypothalamus, 0.48–1.54; thalamus, 1.92–3.04. NPG-ase: cortex, 4.45–6.20; cerebellum, 4.05–4.60; hippocampus, 4.50–6.03; corpus striatum, 4.87–5.10; hypothalamus, 5.50–11.2; thalamus, 4.15–7.15. Numbers in parentheses indicate number of rats.

Brain region	*NPG-ase* (*u/g*)	*Sulfatase* (*u/g*)	$\frac{\textit{Sulfatase}}{\textit{NPG-ase}} \times 10$
C. cortex	5.08 (6)	1.26 (12)	2.48
Cerebellum	4.32 (2)	1.17 (8)	2.70
Hippocampus	5.26 (2)	1.43 (5)	2.70
C. striatum	4.98 (2)	1.52 (5)	3.05
Hypothalamus	7.82 (5)	1.04 (11)	1.32
Thalamus	5.69 (5)	2.37 (7)	4.17

TABLE II

INITIAL FREE ACTIVITY OF NPG-ASE AND SULFATASE IN 6 REGIONS OF THE RAT BRAIN

Suspensions of freshly prepared tissue homogenates in 0.25 *M* sucrose were incubated for 10 min in the assay medium described in the text which was also 0.25 *M* in sucrose and the activities compared to total values determined simultaneously (see legend, Table I). The values refer to percentages [100 × (free/total)]. Numbers in parentheses indicate number of rats.

Enzyme	*Cortex*	*Cerebellum*	*Hypothalamus*	*Hippocampus*	*C. striatum*	*Thalamus*
NPG-ase	26.9 (3)	27.0 (3)	37.6 (2)	27.4 (3)	25.7 (3)	27.1 (3)
Sulfatase	34.4 (2)	37.1 (2)	33.2 (3)	34.7 (2)	40.1 (2)	36.5 (2)

TABLE III

INTRACELLULAR DISTRIBUTION OF ARYLSULFATASE AND NPG-ASE IN THE CORTEX, THALAMUS AND CORPUS STRIATUM OF RAT BRAIN

The values are from 1 of 4 experiments (cortex), 3 experiments (thalamus) and 2 experiments (striatum). Absolute values (homogenates) are in mg/g for protein and units/g for enzymes. N, nuclear fraction; M, heavy mitochondrial fraction containing besides, nerve endings and lysosomes; L, light mitochondrial fraction containing besides, nerve endings and lysosomes; P, microsomal fraction and S, final supernatant.

	Absolute values	*Percentage values*					*Recovery (%)*
		N	*M*	*L*	*P*	*S*	
Cortex							
Protein	80.3	16.9	27.5	7.2	23.9	24.5	87.2
Sulfatase	1.47	14.5	46.9	18.5	16.5	3.6	99.0
NPG-ase	4.52	15.8	38.4	10.4	21.7	13.7	93.4
Thalamus							
Protein	91.1	20.1	31.5	5.8	16.3	26.3	—
Sulfatase	2.56	20.8	45.6	9.4	9.0	15.2	—
NPG-ase	4.70	17.5	37.2	15.3	9.9	20.1	—
C. striatum							
Protein	60.0	21.1	29.0	6.0	14.1	29.9	—
Sulfatase	1.60	17.5	45.2	10.5	9.9	16.9	—
NPG-ase	4.87	14.4	28.7	10.1	18.8	28.0	—

aspiration or by pumping the gradient through a hole in the bottom of the centrifuge tube into an LKB UltroRac fraction collector at a rate of 15 drops/tube.

RESULTS

Total activity of NPG-ase and sulfatase. This was determined in 6 regions of rat brain (Table I). NPG-ase activity was highest and sulfatase activity lowest in the

TABLE IV

RELATIVE SPECIFIC ACTIVITY* OF ARYLSULFATASE AND NPG-ASE IN SUBCELLULAR FRACTIONS DERIVED FROM THE CORTEX, THALAMUS AND CORPUS STRIATUM OF RAT BRAIN

Brain region		*Subcellular fraction*				
		N	*M*	*L*	*P*	*S*
Cortex	Sulfatase	0.86	1.70	2.57	0.69	0.14
	NPG-ase	0.93	1.39	1.45	0.95	0.56
Thalamus	Sulfatase	1.03	1.44	1.62	0.55	0.58
	NPG-ase	0.85	1.13	2.15	0.53	0.98
C. striatum	Sulfatase	0.83	1.56	1.75	0.71	0.56
	NPG-ase	0.77	1.14	1.47	1.22	0.84

* Relative specific activity (RSA): percent of total activity/percent of total protein.

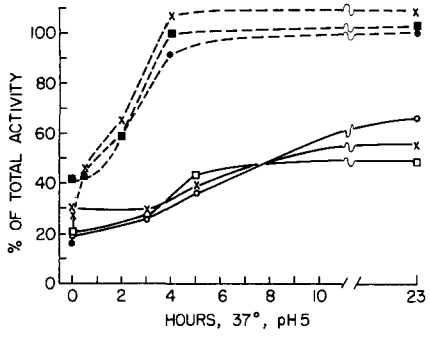

Fig. 1. Thermal activation of sulfatase (dotted lines) and NPG-ase (full lines) in homogenates of cortex (circles), thalamus (crosses) and hypothalamus (squares). Total activity was determined, as described in the legend to Table I, and free activities, as described in the legend to Table II. The free activities are expressed as percentages of the total activity values obtained after 2 h of exposure to Triton X-100 followed by 17 h of storage at 4°C.

TABLE V

CENTRIFUGATION OF FRACTION M + L (CORTEX) IN A CONTINUOUS DENSITY GRADIENT: RSA* OF SUCCINATE-INT-REDUCTASE, NPG-ASE AND SULFATASE

The cerebral cortex from 1 rat (641 mg) was fractionated to yield fraction M + L (ref. 27). This was suspended in 8 ml of 0.25 *M* sucrose and 2 ml each were placed on top of three 32–52% sucrose gradients prepared by layering successive volumes (7 ml) of 52, 47, 42, 37 and 32% sucrose 16 h before centrifugation. Centrifugation was for 2.5 h in Spinco rotor SW-25.1 at 25,000 rev./min. Deceleration was without brake. The tube contents were aspirated to give 5 fractions. No pellet was collected.

Fraction	*INT-reductase*	*NPG-ase*	*Sulfatase*
1	0.38	0.53	0.56
2	0.39	0.33	0.96
3	2.82	0.64	1.16
4	0.79	5.72	3.25
5	0.38	2.90	2.24

* RSA = Relative specific activity. See Table IV for definition.

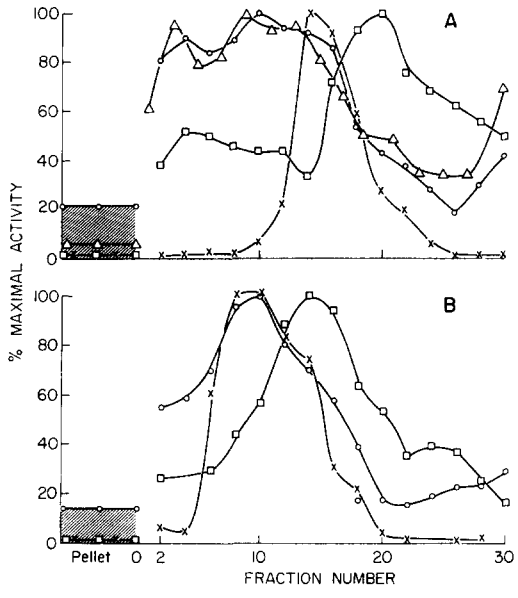

Fig. 2. Centrifugation of fraction L (cortex) in a continuous sucrose density gradient. Tissue (676 mg) was homogenized and subjected to differential centrifugation. Fraction L (ref. 27) was isolated as a pellet and suspended in 3 ml of 0.25 *M* sucrose. 2 ml (2.45 mg of protein) were placed on top of a continuous 32–52% sucrose gradient (28 ml) and centrifugation carried out for A 2.5 h and B 20 h, in the Spinco SW-25.1 rotor at 25,000 rev./min. Deceleration was without brake. The tube was punctured slightly off-center so as not to disturb the pellet and 30 fractions were collected automatically. The pellets (hatched areas) were suspended in 0.25 *M* sucrose. The ordinate, as it refers to the pellets, indicates the actual percentages of total recovered activity (pellet + gradient contents) found in the pellet. Sulfatase (○——○), NPG-ase (△——△), succinate-INT-reductase (×——×) and acetylcholine esterase (□——□). Protein, also analyzed, plateaued in tubes 15–17 (A) and tubes 9–12 (B).

hypothalamus. Conversely, sulfatase was highest in the thalamus, the activity elsewhere not exceeding 65% of the thalamic levels.

Initial free activity of NPG-ase and sulfatase. This was determined in the absence of Triton X-100 (ref. 27) and in the presence of 0.25 *M* sucrose and was compared to total activities determined simultaneously (Table II). With the exception of the hypothalamus (37%), NPG-ase free activity values were all under, those of sulfatase uniformly over, 30% of the respective total activity values.

Intracellular distribution of sulfatase. Homogenates of cortex, thalamus and striatum prepared in 0.25 *M* sucrose were fractionated and the intracellular distribution of sulfatase compared to that of the NPG-ase marker. Most of the activity was recovered in fraction M (Table III) with little activity being present in fraction L. Nonetheless, as shown by the relative specific activity values (Table IV), both enzymes consistently exhibited a peak in the latter fraction. It should be noted that a relatively high percentage of the thalamic and striatum activity was recovered in fraction S, as compared to the cortical activity.

Differential labilization of NPG-ase and sulfatase. The activation *in vitro*[27] of

both hydrolases was compared in 3 brain regions. Exposure of homogenates to 37°C at pH 5 was used as the labilizing treatment. Fig. 1 indicates a response which is both characteristic of the enzyme and independent of the region.

Density gradient centrifugation of fraction M + L. Crude mitochondrial fractions, subjected to further purification on discontinuous sucrose gradients have yielded preparations[18] harvested as pellets sedimenting through 41% (w/v) sucrose and containing appreciable amounts of several lysosomal hydrolases of high relative specific activity. The mitochondrial marker, succinate dehydrogenase, associated with granules of lesser density. Similarly, a relative specific activity peak inside the limits of a discontinuous gradient and a second peak in the pellet fraction have been observed[28].

In the present study the useful range of densities within which granule populations may equilibrate has been expanded by adoption of continuous sucrose gradients. The results (Table V) show that, in a 32–52% (w/v) gradient, mitochondria collect at lower densities than lysosomes, which again show a tendency toward a bimodal distribution (RSA $>$ 2 in fractions 4 and 5). Additional evidence for the presence of more than one population of lysosomes was obtained when cerebral cortex fraction L was centrifuged in a 32–52% gradient for 2.5 *vs.* 20 h. A virtually complete equilibration of both lysosomal populations was achieved by the end of 2.5 h (Fig. 2), for no migration of the main lysosome peak (tube 10, Fig. 2A) toward regions of higher density occurred upon prolonged centrifugation. Mitochondria, on the contrary, did not equilibrate equally readily, as shown by the shift of the succinate-INT-reductase peak from tubes 14 and 15 (Fig. 2A) to tubes 8–10 (Fig. 2B). The membranous structures bearing acetylcholine esterase (peaks in tubes 20 and 14, Figs. 2A and B) behaved similarly.

The lysosomal population found in the pellet at the end of 2.5 h of centrifugation was characterized by a high relative specific activity (sulfatase: 3.8; NPG-ase: 4.0) as well as by the virtual absence ($>$ 0.5%) of both succinate-INT-dehydrogenase and acetylcholine esterase. Lactic dehydrogenase, not shown in graphic form in Fig. 2, was also absent from the pellets yet it contaminated the less dense population. A rather strong adsorption of the soluble enzyme onto lysosomes is indicated[6,14], since the degree of this contamination failed to diminish as a result of prolonged centrifugation.

A bimodal distribution similar to that shown for the cortex in Fig. 2A was observed when fraction L derived from the thalamus or the hypothalamus was subjected to 2.5 h of centrifugation in a 32–52% continuous sucrose density gradient. The existence of two distinct populations of lysosomes in these regions is therefore also apparent.

DISCUSSION

The regional survey of the total activity of sulfatase and NPG-ase (Table I) reveals that the two activities are present in all of the brain regions tested and that certain, mutual differences in topographical distribution exist between them. Thus, as indicated by the ratio of sulfatase to NPG-ase, the hypothalamus is characterized

by the highest levels of NPG-ase and the lowest levels of sulfatase while a seemingly converse relationship obtains for the thalamus. The regional variations of arylsulfatase have been examined previously in calf brain[8], where white matter had the highest and gray matter the lowest activity levels. Other studies have revealed that arylsulfatase activity varies by at least a factor of three over a distance of 20 μ from the cortical surface inward[31]. Similarly, it has been noted that the respective patterns of change of β-galactosidase and β-glucuronidase during development are different[23]. While it is not possible to interpret the findings of Table I definitively at this time, it may be speculated that the regional differences in the levels of hydrolase activity are to be correlated with regional differences in lysosomal enzyme content and, ultimately, with the regional peculiarities of metabolic patterns.

As noted by several workers[15,18,27], the initial free activities of lysosomal hydrolases in brain may vary from low levels of approximately 10% to high levels of approximately 40%. The present results (Table II) suggest that there is a constancy in the level of initial free activity characteristic for a given hydrolase and which is manifested irrespective of the region. Sulfatase of brain is thus to be compared to acid phosphatase[21,27] (high initial free activity), while NPG-ase is to be compared with β-glucuronidase[21] (low initial free activity).

Equally enzyme-, rather than region-specific appeared to be the response of NPG-ase and sulfatase to thermal activation *in vitro*, sulfatase exhibiting a rather rapid, NPG-ase a gradual, activation (Fig. 1). It should be noted that, unlike previously[27], NPG-ase activation was determined on whole homogenates rather than on suspensions of fraction M + L.

The intracellular distribution of both hydrolases was examined in three rat brain regions (Table III). The central feature of the distribution of sulfatase is its particulate association (about 50% of the total activity in fraction M alone). Regional differences in the respective partition of sulfatase and NPG-ase may be discerned, particularly with regard to the percentages of activity in fractions P and S. Thus, cortical soluble NPG-ase (13.7%) was about 4 times higher than the corresponding soluble sulfatase (3.6%) while, in the other two regions, this ratio was well below 2. It should be recognized that these differences may result from the differential influence of the homogenizing medium on the adsorption of hydrolases to structure[5].

As with arylsulfatase of three different lymphoid tissues (spleen, thymus and lymph nodes)[5], it was possible to obtain relative specific activity peaks for this hydrolase in all three of the brain regions examined.

The separation of the granule population rich in succinate-INT-reductase from the population rich in hydrolase activity was readily accomplished in continuous sucrose density gradients (Table V, Fig. 2A). A compact distribution characterized the mitochondria, whereas a more diffuse pattern obtained for lysosomes. With regard to the minority population of lysosomes found in the pellet, different proportions of the two hydrolases (20% of fraction L for sulfatase and 6% for NPG-ase) were contained in it. Presumably, these lysosomes, unlike their less dense counterparts (tubes 2–12 inside the gradient) are characterized by proportionately higher levels of sulfatase than of NPG-ase.

The nature of the cell types giving rise to the two populations of lysosomes, segregated by centrifugal means, is unknown. A more comprehensive analysis of the two populations, preferably subsequent to a preliminary separation of cerebral cell types (neurons *vs.* glia), is necessary before this can be revealed.

SUMMARY

1. The activities of arylsulfatase and *N*-acetyl-β-D-glucosaminidase were determined in 6 regions of rat brain.

2. The degree of structural latency of the two hydrolases was estimated.

3. The intracellular distribution of arylsulfatase was studied in the cortex, thalamus and striatum and a lysosomal localization could be established.

4. Centrifugation of the cortical crude lysosomal fraction on continuous sucrose density gradients revealed the existence of a minority population of lysosomes which, unlike the bulk, sedimented through the gradient and could be recovered in pellet form. Proportionately more sulfatase than glucosaminidase was found in this lysosomal population.

5. The main lysosomal population was well separated from mitochondria but was contaminated by the membranes bearing acetylcholine esterase and soluble lactic dehydrogenase. The pellet fraction was virtually free of contamination.

6. Evidence for the presence of more than one population of lysosomes in the thalamus and the hypothalamus of rat brain was also obtained.

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