The Effect of Anoxia on Cerebral Acid Hydrolases in the Five-Day-Old Rat

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1. Five-day-old anaesthetized rats subjected to slow, prolonged asphyxia (50–55 min) were either allowed to die or resuscitated when at the point of death. Activities of various cerebral acid hydrolases known to be associated with lysosomes were determined in these animals and in littermate controls. 2. Asphyxia to death resulted in a significant increase in the activities of acid phosphatase, cathepsin (pH 5.0) and β -glucuronidase in whole-brain homogenates. 3. The effect of asphyxia on β -glucuronidase activity was not apparent when the assay was performed in the presence of Triton X-100 (0.1%, v/v). 4. In resuscitated animals whole-brain-homogenate β -glucuronidase activity showed the greatest increase (31%) 15 min after recovery. After a 60 min recovery period differences between control and asphyxiated animals were no longer apparent. 5. In animals anoxiated to death activities of acid phosphatase and β -N-acetylglucosaminidase in brain high-speed supernatants were significantly higher than in controls. Acid phosphatase activity was similarly increased in asphyxiated animals resuscitated for 5 or 60 min. 6. It is suggested that the response of the immature rat brain to asphyxia involves a disruption or increased fragility of lysosomal particles.

In previous studies (De Souza, 1971) slow prolonged asphyxia in the 5-day-old rat caused cerebral oedema and an accumulation of brain Na^+ with loss of K^+ . These changes are probably a result of cerebral hypoxia causing an impairment of the energy-dependent processes that normally maintain water and electrolyte balance within the brain. It is also likely that considerable cell death occurs.

Anoxia and cell death are often associated with a release of lysosomal acid hydrolases in cytoplasm (Weissman, 1965; de Duve & Wattiaux, 1966) and a corresponding increase in activity of these enzymes as measured in tissue whole homogenates. To determine whether brain electrolyte disturbances in anoxia are accompanied by disruption of lysosomes the activities of a number of acid hydrolases have been studied. Enzymes were chosen whose occurrence within adult rat brain lysosomes is well established (Koenig *et al.*, 1964; Sellinger *et al.*, 1964).

The 5-day-old rat was studied as a possible model for the asphyxiated human newborn infant since at 5 days of age the rat brain has reached approximately the same stage of growth and development as the human brain at birth.

Materials and Methods

Chemicals

β -Glycerophosphate, phenolphthalein glucuronide, bovine haemoglobin and calf liver RNA were purchased from Sigma (London) Chemical Co. Ltd.,

London S.W.6, U.K., and were of the highest grade available. 4'-Nitrophenyl *N*-acetylglucosaminide was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. All other chemicals were of A.R. grade and were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Animals

Rats of a black and white hooded strain were used. On the day of birth litters were decreased to six animals (all males where possible). Young killed at 5 days of age weighed 10–15 g.

Methods

Asphyxia procedure. Rats were anaesthetized by intraperitoneal injection of urethane (0.5 mg/g body wt.) and either were returned to the mother (control) or were asphyxiated in groups of three in an airtight jar (500 ml) at 37°C. Asphyxiated animals died after 50–55 min.

Tissue preparation. Animals were killed by decapitation. Brains (excluding olfactory lobes) were removed, weighed and kept on ice. Homogenates were prepared in either ice-cold 0.14 M-KCl or ice-cold 0.32 M-sucrose by using a Teflon-glass homogenizer (10 strokes at 1200 rev./min, clearance 0.15 mm). High-speed supernatants were prepared by centrifuging homogenates [20% (w/v) in 0.32 M-sucrose] at 50000 g for 1 h at 0°C.

Enzyme assays. Acid phosphatase (EC 3.1.3.2) was determined as described by Gianetto & de Duve

(1955) by using either a 10% (w/v) homogenate in 0.32M-sucrose or a high-speed supernatant. The substrate (β -glycerophosphate) concentration was 0.12M. β -N-Acetylglucosaminidase (EC 3.2.1.30) was determined as described (Adlard & Dobbing, 1971) by using a high-speed supernatant. β -Glucuronidase (EC 3.2.1.31) and cathepsin (substrate haemoglobin) were determined by the method of Gianetto & de Duve (1955) by using 10% (w/v) homogenates in 0.14M-KCl. Acid ribonuclease was determined by the method of de Duve *et al.* (1955) by using a 10% (w/v) homogenate in 0.14M-KCl. Sodium acetate buffers (pH 5.0 and 3.0 as indicated) were used for all enzyme determinations. The final buffer concentrations were as indicated in the original methods. The times of incubation for the different enzyme assays were as follows: acid phosphatase, 60 min; β -N-acetylglucosaminidase, 20 min; β -glucuronidase, 60 min; cathepsin, 40 min; acid ribonuclease, 50 min. For all assays the formation of product showed a linear rate with time. All enzymes were assayed at pH 5.0; in addition, cathepsin was assayed at pH 3.0. Acid ribonuclease activity was expressed as the change in E_{260}/h per g fresh wt. of brain. All other enzyme activities were expressed as μ mol of product formed/h per g fresh wt. of brain.

Incubation mixtures for all enzymes except acid phosphatase were approximately iso-osmotic with normal extracellular fluid, having osmolarities in the range 250–300 mosmol/l. The assay mixture for acid phosphatase had an osmolarity of approximately 170 mosmol/l. In this assay, therefore, some disruption of lysosomes may have resulted from osmotic shock, perhaps explaining the small degree of latency found for this enzyme (see the Results section).

Protein determinations. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Statistics. Except where otherwise indicated, results were compared by using Student's *t* test.

Results

Asphyxia to death

Activities of acid phosphatase and β -glucuronidase in whole-brain homogenates (Table 1) were significantly higher in asphyxiated animals than in controls. Acid ribonuclease activity and cathepsin activity at pH 3.0 were not changed by asphyxia. The increase in acid phosphatase activity in asphyxiated animals was not apparent after release of total activity by freeze-thawing (see footnote to Table 1).

There were considerable variations between litters in enzyme activity, emphasizing the importance of using littermate controls. When the response to asphyxia of individual litters was examined changes which occurred were more convincing. For example, the change in cathepsin activity at pH 5.0 was not quite significant (Table 1) when individual animals were considered by using Student's *t* test. However, the increase in activity was significant ($P < 0.005$) when means from a litter were calculated and the results compared by using the paired-comparisons *t* test.

Latency of acid hydrolases

The possibility was considered that acid ribonuclease activity and cathepsin activity at pH 3.0 showed no apparent change in asphyxia because assay conditions

Table 1. *Whole-brain-homogenate acid hydrolase activities in normal 5-day-old rats (control) and those asphyxiated to death (experimental)*

For experimental details see the text. Each value is the mean $\pm$ s.d. of the number of animals indicated in parentheses. Except for cathepsin all assays were performed at pH 5.0 only. N.S. is not significant.

Enzyme	No. of litters	Enzyme activity		<i>P</i>	Change (%)
		Control	Experimental		
Acid phosphatase (μ mol/h per g)	5	50.8 $\pm$ 4.8 (15)*	56.0 $\pm$ 5.5 (15)*	<0.01	+10
Acid ribonuclease ($\Delta E_{260}/h$ per g)	5	112 $\pm$ 11 (13)	117 $\pm$ 8 (15)	N.S.	—
Cathepsin (pH 3.0) (μ mol/h per g)	4	27.6 $\pm$ 1.2 (10)	28.1 $\pm$ 1.3 (12)	N.S.	—
Cathepsin (pH 5.0) (μ mol/h per g)	6	3.99 $\pm$ 1.39 (18)	4.76 $\pm$ 1.27 (18)	N.S.†	+20
β -Glucuronidase (μ mol/h per g)	4	0.394 $\pm$ 0.092 (10)	0.528 $\pm$ 0.101 (12)	<0.01	+34

* Acid phosphatase activities determined in these homogenates after freezing and thawing ten times were as follows: control, 69.0 $\pm$ 12.7 μ mol/h per g; experimental, 72.7 $\pm$ 20.1 μ mol/h per g. The difference was not significant.

† This change was significant ($P < 0.005$) by using a paired *t* test (see the text for further details).

Table 2. Latency of different acid hydrolases in homogenates of normal 5-day-old rat brain

Each value is the mean $\pm$ S.D. of the number of animals indicated in parentheses. Each homogenate was assayed both with no treatment and after either freeze-thawing (see the footnote to Table 1) or in the presence of Triton X-100. In all cases these treatments resulted in a significant ($P < 0.001$) increase in enzyme activity.

Enzyme	No. of litters	Treatment of homogenate ...	Enzyme activity		
			None	Freeze-thaw ten times	0.1 % (v/v) Triton X-100
Acid phosphatase ($\mu\text{mol/h per g}$)	5		50.8 $\pm$ 4.8 (15)	69.0 $\pm$ 12.7 (15)	
Cathepsin (pH 3.0) ($\mu\text{mol/h per g}$)	2		26.8 $\pm$ 1.7 (6)		46.9 $\pm$ 1.0 (6)
Acid ribonuclease ($\Delta E_{260}/\text{h per g}$)	2		141 $\pm$ 6 (6)		198 $\pm$ 9 (6)
	2		101 $\pm$ 5 (6)	161 $\pm$ 12 (6)	

Table 3. Whole-brain-homogenate β -glucuronidase assayed with or without Triton X-100 in 5-day-old rats asphyxiated to death

Two litters were used. Three animals in each litter were asphyxiated to death (experimental); the remainder were used as controls. Each brain was used for enzyme assay in both the presence and absence of Triton X-100. Each value is the mean $\pm$ S.D. for six animals. N.S. is not significant.

Concentration of Triton X-100	β -Glucuronidase activity ($\mu\text{mol/h per g}$)		P	Change (%)
	Control	Experimental		
None	0.347 $\pm$ 0.035	0.444 $\pm$ 0.042	<0.005	+28
0.1 % (v/v)	0.612 $\pm$ 0.082	0.624 $\pm$ 0.064	N.S.	+2

alone led to complete lysosome disruption. However, this was probably not the case since both enzymes showed latency (Table 2) as conventionally defined (de Duve & Wattiaux, 1966). For example, acid ribonuclease activity was increased 40% by Triton X-100 and 60% by freeze-thawing. The small degree of latency showed by acid phosphatase was probably a result of the hypo-osmotic assay medium.

β -Glucuronidase activity was increased by 76% by incubation with Triton X-100, compared with a 28% increase after asphyxia (Table 3). If both changes were due solely to disruption of lysosomes and assuming that Triton X-100 causes complete disruption (Wattiaux & de Duve, 1956), 30–40% of the total latent enzyme activity was released by anoxia. After activation by Triton X-100 β -glucuronidase activity did not differ between control and asphyxiated animals (Table 3).

β -Glucuronidase after resuscitation

It is conceivable that enzyme changes after asphyxia to death (Table 1) were due to post-mortem autolysis

occurring in the time-interval between observing cessation of gasping and performing decapitation (not more than 2 min) rather than to the preceding period of asphyxia (50–60 min). Experiments with animals asphyxiated to the point of death and resuscitated were carried out to examine this question (Table 4). Resuscitated animals showed a significant increase in β -glucuronidase activity up to 30 min after the asphyxial episode. At 15 min the degree of increase was comparable with that found in animals asphyxiated to death (Table 1).

High-speed-supernatant enzyme activities

Activities of both acid phosphatase and β -N-acetylglucosaminidase in brain high-speed supernatants were significantly increased by asphyxia to death (Table 5). The response of the latter enzyme may have been a post-mortem phenomenon since activity was not changed in resuscitated animals. In contrast supernatant acid phosphatase activity was significantly increased both 5 min and 60 min after recovery from asphyxia.

Table 4. *Whole-brain-homogenate β -glucuronidase activity in 5-day-old rats asphyxiated to the point of death and allowed to survive for different periods*

Each value is the mean $\pm$ S.D. of the number of animals indicated in parentheses. All asphyxiated rats from any one litter were killed at the same time-interval after resuscitation. From each litter equal numbers (where possible) of animals were used as either control or experimental animals. N.S. is not significant.

Time after initiating resuscitation (min)	No. of litters	β -Glucuronidase activity (μ mol/h per g)		<i>P</i>	Change (%)
		Control	Experimental		
5	4	0.367 $\pm$ 0.046 (12)	0.452 $\pm$ 0.036 (12)	<0.001	+23
15	3	0.314 $\pm$ 0.056 (9)	0.412 $\pm$ 0.072 (9)	<0.01	+31
30	4	0.359 $\pm$ 0.085 (12)	0.432 $\pm$ 0.064 (9)	<0.05	+20
60	3	0.410 $\pm$ 0.089 (9)	0.401 $\pm$ 0.063 (9)	N.S.	—

Table 5. *Acid phosphatase and β -N-acetylglucosaminidase activities in high-speed supernatants of brain homogenates from control or asphyxiated 5-day-old rats*

Each value is the mean $\pm$ S.D. of the number of animals indicated in parentheses. A minimum of three litters was used for each experimental treatment. From each litter equal numbers of animals were used as either control or experimental animals. N.S. is not significant.

Experimental treatment	Acid phosphatase activity (μ mol/h per g)		<i>P</i>	β -N-Acetylglucosaminidase activity (μ mol/h per g)		<i>P</i>
	Control	Experimental		Control	Experimental	
Asphyxia to death	6.55 $\pm$ 1.69 (12)	8.19 $\pm$ 2.86 (12)	<0.05	1.18 $\pm$ 0.12 (9)	1.52 $\pm$ 0.19 (9)	<0.001
Asphyxia to point of death; resuscitation for 5 min	5.28 $\pm$ 0.97 (12)	6.21 $\pm$ 1.21 (12)	<0.05	0.79 $\pm$ 0.18 (12)	0.81 $\pm$ 0.11 (12)	N.S.
Asphyxia to point of death; resuscitation for 60 min	4.99 $\pm$ 0.65 (14)	5.52 $\pm$ 0.48 (14)	<0.025	1.11 $\pm$ 0.13 (14)	1.17 $\pm$ 0.19 (14)	N.S.

Table 6. *β -Glucuronidase activity in 5-day-old rat tissues other than brain*

Experimental animals were asphyxiated to the point of death, resuscitated and killed after either 5 min (liver, thymus) or 15 min (heart, blood, plasma). Enzyme activity is expressed as μ mol of phenolphthalein released/h either per g fresh wt. of tissue (liver, thymus, heart) or per ml (blood, plasma). Whole homogenates of liver, thymus and heart were used. Each value is the mean $\pm$ S.D. of the number of animals indicated in parentheses. N.S. is not significant.

Tissue	No. of litters	β -Glucuronidase activity		<i>P</i>
		Control	Experimental	
Liver	3	39.3 $\pm$ 13.1 (9)	42.6 $\pm$ 10.2 (9)	N.S.
Thymus	3	6.25 $\pm$ 1.42 (9)	6.19 $\pm$ 1.00 (9)	N.S.
Heart	2	0.265 $\pm$ 0.036 (6)	0.266 $\pm$ 0.018 (6)	N.S.
Blood	3	0.298 $\pm$ 0.126 (9)	0.323 $\pm$ 0.079 (8)	N.S.
Plasma	3	0.162 $\pm$ 0.066 (9)	0.195 $\pm$ 0.058 (8)	N.S.

β -Glucuronidase activity in tissues other than brain

Of the various tissues examined β -glucuronidase activity was highest in the liver (Table 6). Blood concentrations were comparable with those of brain.

Anoxia did not appear to change β -glucuronidase activity in homogenates of liver, thymus or heart, nor was a substantial amount of enzyme released into blood.

Supernatant total protein concentrations

The protein concentration in supernatant from 5-day-old rat brain was unaffected by asphyxia to death. Results (mean $\pm$ s.d.) for three litters were as follows: control, 21.1 ± 1.6 mg of protein/g fresh wt., $n = 9$; experimental, 20.0 ± 1.3 mg of protein/g fresh wt., $n = 8$.

Discussion

A number of noxious stimuli are known to cause disruption of lysosomes *in vivo* or *in vitro* (Weissman, 1965). Such a process results in a release into cytoplasm of acid hydrolases capable of degrading a variety of cellular constituents. The present paper reports an approach to the problem of whether disruption of brain lysosomes occurs in immature rats subjected to slow prolonged asphyxia. It is hoped that the results may be relevant to discussions of the mechanisms of cerebral injury after human birth asphyxia.

After asphyxia to death certain brain acid hydrolases showed an increase in activity as measured in whole homogenates. There are a number of possible explanations of this phenomenon: (1) increased enzyme synthesis during anoxia; (2) a discharge of acid hydrolases from peripheral organs (particularly liver) causing increased enzyme activity in the blood within the brain; (3) inefficient cellular disruption during the homogenization process combined with a preferential lysis of brain cells of asphyxiated (compared with control) rats either *in vivo* or during incubation; (4) increased permeability of the lysosomal membrane to enzyme substrates; (5) disruption of lysosomes or increased fragility of these organelles during anoxia.

The possibility of increased enzyme synthesis during asphyxia seems to be ruled out for acid phosphatase and β -glucuronidase, by the observations (Tables 1 and 3) that 'total' activities of these enzymes were unaffected by asphyxia.

Blood concentrations of β -glucuronidase were not greater than those of brain and were not markedly altered in anoxia. It seems inconceivable, therefore, that the apparent change in brain enzyme activity was due to the blood within the brain. Of the tissues examined liver had much the highest β -glucuronidase activity. Since the enzyme activity in liver was unaffected in anoxia it was not surprising that plasma values were similarly unchanged, at least in resuscitated animals. The failure of β -glucuronidase activity in non-neural tissues to be altered by asphyxia suggests that brain lysosomes may be particularly vulnerable to anoxic damage.

The possibility that the increased cerebral acid hydrolase activity in anoxia represented enzyme release from cells rather than from lysosomes cannot be ruled out. However, the homogenization con-

ditions were quite severe for a soft tissue. Indeed, in normal animals the acid hydrolases showed relatively little latency compared with that observed by other workers (Koenig *et al.*, 1964), suggesting that homogenization itself resulted in considerable disruption of lysosomes.

The substrates used for β -glucuronidase and acid phosphatase assays are of relatively small molecular weight and it is possible that an increase in lysosomal permeability would result in substantial intralysosomal hydrolysis of these substrates. In contrast it is unlikely that either RNA or haemoglobin (the substrate of cathepsin) will cross the lysosomal membrane. The lack of effect of asphyxia on acid ribonuclease activity would therefore support a hypothesis that enzymic changes in anoxia are a result of increased lysosomal permeability. If this were the case, however, enzyme activities would not have been increased in high-speed supernatants (Table 5). Further, the activity of cathepsin, with a substrate of high molecular weight, was increased by asphyxia, at least when assayed at pH 5.

The evidence thus points to a disruption or increased fragility of lysosomes as the major causes of alterations in cerebral acid hydrolase activities in asphyxia. Since the assay techniques necessarily involve mechanical treatment of the brain it is not possible to determine whether release of acid hydrolases into cellular cytoplasm occurs *in vivo* or *in vitro* (during homogenization or incubation).

β -Glucuronidase activity in brains of asphyxiated rats appears to return to normal values 30–60 min after beginning resuscitation. If this decrease represents a loss of enzyme it could result from (1) destruction of enzyme through normal mechanisms of protein turnover, (2) loss of β -glucuronidase to blood and/or cerebrospinal fluid or (3) re-uptake of cytoplasmic enzyme into lysosomes. It is not possible at present to decide between these alternatives. However, the results do imply that, if release of acid hydrolases into cytoplasm has deleterious effects on the cell, then these effects will continue for some time after return to adequate oxygen supply.

Different acid hydrolases showed various degrees of response to asphyxia. This may reflect either a heterogeneity of lysosomes with respect to their enzyme content or a different neuroanatomical distribution of acid hydrolases. The very low activity of β -glucuronidase in brain compared with other enzymes or organs suggests that this enzyme may be confined to certain lysosomes or certain brain regions and that either of these compartments is particularly affected by anoxia.

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