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THE INTRACELLULAR LOCALIZATION OF CARBONIC ANHYDRASE AND A CARBONIC ANHYDRASE INHIBITOR IN THE BRAINS OF MICE

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Abstract—Homogenates prepared from the perfused brains of mice were differentially centrifuged in 0·25 M sucrose. On the basis of activity per mg of nitrogen, only the supernatant showed a concentration of carbonic anhydrase activity greater than that of the original homogenate (about two- to three-fold). No concentration of activity was found in the particulate fraction, in any of its components studied, or in the washes of the various residues. Partition of per cent activity varied with the procedure used, but approximately two-thirds of the total activity of the original homogenate were in the supernatant fraction; the total particulate matter accounted for about one-third. No differences of any consequence were found amongst whole brain, cortex, and brain with the cortex removed. The data are believed to demonstrate the intracellular localization of carbonic anhydrase in the soluble fraction of cells; the activity of particulate matter is considered to be the result of contamination.

The inhibitor of carbonic anhydrase found in the brains of mice following the intravenous administration of methazolamide was almost entirely localized in the supernatant fractions; an intercellular localization in the soluble fraction is also postulated.

The localization of both carbonic anhydrase and carbonic anhydrase inhibitor in the soluble fraction of homogenates of the brains of mice does not constitute evidence of the inhibition of the enzyme in the brain *in vivo*. However, the concentrations of inhibitor found in the whole homogenates 15 min and 2 hr after the administration of doses of methazolamide at the level of the ED_{95} for anticonvulsant effect were theoretically capable of causing maximum inhibition of carbonic anhydrase in the brains of mice. Anticonvulsant action and inhibition of carbonic anhydrase are probably related.

INTRODUCTION

CONSIDERABLE circumstantial evidence has been published in support of the belief that the anticonvulsant action of inhibitors of carbonic anhydrase is attributable to inhibition of this enzyme in the brain.[1, 2] No direct proof of the inhibition of carbonic anhydrase in brain *in vivo* has been presented. Furthermore, since the times of the maximum concentration of inhibitor in the brain and the maximum anticonvulsant action are not coincident,[2] anticonvulsant action independent of carbonic anhydrase remains a possibility.

The present state of the art makes a direct demonstration of the degree of activity of carbonic anhydrase in brain *in vivo* following the administration of inhibitors extremely difficult, if not impossible. An indirect approach to the problem would be a distribution study with labeled inhibitor in conjunction with a histochemical method for the localization of carbonic anhydrase. Unfortunately, a reliable histochemical method for the detection of carbonic anhydrase is not available.[3] A crude but possibly

useful approach would be the differential centrifugation of brain homogenates. Location of the enzyme and an inhibitor in separate and discrete fractions would suggest that the anticonvulsant action is a pharmacodynamic effect independent of any action on carbonic anhydrase. It would also be of interest to calculate the possible degrees of inhibition of the enzyme in brain at various times following the administration of amounts of methazolamide causing a maximum anticonvulsant effect. The results of such a study are presented in this report.

METHODS

The mice used were male Swiss albino, from 6 to 9 weeks of age and weighing from 20 to 30 g. All reagents and solutions used in assay, perfusion and homogenization were prepared with double (glass)-distilled water. Except where indicated, perfused brains from mice anesthetized with sodium pentobarbital were used in the study of enzyme localization; unanesthetized mice sacrificed by cervical fracture furnished brains for the study of inhibitor distribution. These were rinsed in saline and blotted free of blood. Perfusion of the brain was carried out by placing small artery clamps on the abdominal aorta and on the inferior vena cava, then inserting a 20-gauge hypodermic needle into the left ventricle. Except where indicated, the perfusion fluid was 0·85 per cent saline. Immediately after perfusion had started, the right atrium was snipped. Perfusion was continued until no discoloration due to blood was observed on a piece of filter paper; usually a period of from 3 to 5 min was required. All brains, perfused and not perfused, were blotted dry and weighed on a torsion balance to the nearest milligram. Experiments showed no important differences in the localization of carbonic anhydrase in homogenates of perfused brains and of brains that had not been perfused.

Ten per cent homogenates (w/v) were prepared in 0·25 M sucrose by means of a glass homogenizer fitted with a Teflon®* pestle (A. H. Thomas Co., No. B-3235, clearance from 0·005 to 0·007 in., 220 rev/min, approximately ten strokes per min for from 3 to 8 min). When polyvinylpyrrolidone was used, the concentration in the sucrose was 7·3 per cent. Homogenization was carried out in an ice-bath. This precaution was omitted in the determination of inhibitor concentration. Lusteroid tubes were used throughout the centrifugation procedure. All residues, supernatant fractions and washes were made up to 10 ml with the suspending medium and stored at 3 ° until assayed. Equipment (tubes, etc). and suspending media were refrigerated at the same temperature. Assays for enzyme activity and inhibitor were carried out within 24 hr. Under these conditions, storage for 1 week had no effect on enzyme activity or concentration of inhibitor. Homogenates and the first and second residues were examined for intact cells and erythrocytes; counts of the latter were made when possible.

Localization of carbonic anhydrase

Of the 10 % homogenate, 12 ml were differentially centrifuged in a Spinco model "L" ultracentrifuge (no. 40 rotor) at 5000 **g** for 10 min, 12,000 **g** for 15 min, and 45,000 **g** for 60 min; calculations of centrifugal force were made from the midpoint of the tube. These centrifugal forces were chosen arbitrarily since morphological characterization of the various fractions was not our main concern.

* Registered trademark of the E. I. duPont de Nemours and Company (Inc.).

The first two residues were washed twice with the suspending medium. The final residue was washed once. In one experiment, the first centrifugation was 1000 **g** for 10 min. The effect of single high-speed centrifugation was also investigated.

An homogenate prepared in the usual way from the brains that had not been perfused was used to study the localization of enzyme following the addition of hemolysed mouse blood. It was divided into three 20-ml aliquots. One received an amount of hemolysed mouse blood containing about 150–160 units of carbonic anhydrase activity. The second sample was heated at 100° for 5 min. After cooling, the identical amount of hemolysed mouse blood was added. The third sample received an equal volume of the same hemolysed mouse blood which had been heated to 100° for 5 min and allowed to cool; all three samples were agitated by hand over a period of 5 min. Differential centrifugation was carried out in the manner previously described.

Localization of inhibitor

Methazolamide* was solubilized by the addition of one equivalent of sodium hydroxide per mole of compound. Fifty milligrams per kilogram in terms of the free acid, were given intravenously to mice; this is the ED_{95} for anticonvulsant effect in this species. Fifteen and 120 min later, the mice were sacrificed by cervical fracture. Homogenates were prepared as previously described. On the basis of the data obtained on the localization of carbonic anhydrase, 12-ml aliquots of these homogenates were partitioned into particulate and supernatant fractions by centrifugation at 45,000 **g** for 60 min. The residues were resuspended and recentrifuged under the same conditions. Homogenates prepared from the brains of untreated mice were centrifuged in the same manner; the particulate matter, wash, and supernatant fractions were heated at 100° for 5 min to furnish appropriate blanks.

Analytical methods

Enzyme activity and concentration of inhibitor were measured essentially in the manner described by Maren.[4] Three millilitres of phenol red were used instead of five. Control experiments showed that the assay of homogenates and fractions thereof in distilled water resulted in a loss of enzyme activity; there was also a spurious increase in the concentration of inhibitor. Consequently, the assays were carried out in such manner that the reaction volume of 7 ml contained amounts of sucrose or sucrose plus polyvinylpyrrolidone equivalent to 0·5 ml of the suspending medium.

The assay of enzyme activity was conducted as follows: aliquots of homogenates, residues, washes and supernatant fraction were heated at 100° for 5 min to furnish blanks in volumes of 0·1 ml to 0·5 ml for the construction of reference curves. Using arithmetic co-ordinates, the reciprocal of the uncatalysed reaction time in the presence of a known volume of the blank and the reciprocal of one-half this reaction time (equivalent to one unit) were used to plot a theoretical reference curve covering from 0 to 2 units of enzyme activity. A selected volume of the unknown, usually 0·1–0·2 ml, was then assayed. If the reciprocal of the reaction time was within the limits of the reference curve, two additional measurements were made. To be considered valid, these three reaction times could vary no more than 1 sec. The mean was used for the determination of enzyme units; the error was estimated to be $\pm$ 2 per cent. By definition, one unit of carbonic anhydrase activity is the amount necessary to reduce the uncatalysed reaction time by one-half in 7 ml of solution at 0.4°.

* The trademark of the American Cyanamid Company for methazolamide is Neptazane®.

In the measurement of the concentration of inhibitor, the reaction time of the unknown was adjusted by the reaction time of the same volume of appropriate blank. All measurements were made in triplicate and, to be considered valid, could vary no more than 1 sec; the error was calculated to be $\pm$ 3 per cent.

Nitrogen was determined by the direct nesslerization procedure of Hoffman and Osgood,[5] modified by the use of 30 per cent peroxide to reduce caramelization. All measurements were made in duplicate with a Beckman model "B" spectrophotometer; the error was $\pm$ 5 per cent.

Relative concentration of enzyme and S/P ratio were calculated as:

$$\frac{\text{units/mg N fraction or supernatant } (S)}{\text{units/mg N homogenate or total particulate matter } (P)}$$

Units of enzyme activity and percentages were rounded off to the nearest whole number; units of activity per mg of nitrogen were calculated to the first decimal place.

Calculation of enzyme inhibition

The theoretically possible degrees of enzyme inhibition in brain 15 min and 2 hr after the administration of doses of methazolamide at the level of the ED_{95} for anticonvulsant effect were calculated from the means of the number of enzyme units and inhibitor contents of the original homogenates given in Tables 1 and 3. From the data published by Maren *et al.*,[6] one enzyme unit in the carbonate buffer system was calculated to be $1{\cdot}68 \times 10^{-11}$ moles of carbonic anhydrase. The reaction conditions used in the present study and those of Maren and co-workers did not differ in important details. The equilibrium expression used was

$$K_I = \frac{[(E)-(EI)][(I)-(EI)]}{(EI)}$$

where (E) and (I) are the initial molar concentrations of free enzyme and inhibitor in intracellular water. The latter was calculated considering total water to be 78 per cent of the weight of the brains of mice.[7] On the basis of the estimate of extracellular space in brain made by Davson and Spaziani,[8] 85 per cent of the total water was considered to be intracellular. Acceptance of the existence of an extracellular space would introduce only a minor error into the calculations. In the above expression, $(I) - (EI)$ was not considered to be equivalent to (I). The value used for the equilibrium constant, K_I, was $0{\cdot}9 \times 10^{-8}$ M. This is the figure given by Maren *et al.*[6] for methazolamide in the carbonate buffer system. The degree of enzyme inhibition was calculated in per cent as

$$\frac{(EI)}{(E)} \times 100$$

RESULTS

1. *Localization of carbonic anhydrase activity in homogenates of whole brain*

Table 1 gives the means and the ranges of the data of three experiments in which the first centrifugation was carried out at 5000 **g** for 10 min. Thirty per cent of the enzyme activity and 52 per cent of the total nitrogen were found in the particulate matter, principally in the first residue. Relatively little enzyme activity and nitrogen were found in the 12,000 **g** and the 45,000 **g** fractions. Within the particulate matter, nitrogen content and enzyme activity appeared to be well correlated. The

supernatant accounted for 19 per cent of the nitrogen and 45 per cent of the total carbonic anhydrase activity of the original homogenate. The enzyme activity and nitrogen content of the combined washes were 20 and 28 per cent. The inclusion of 7·3 per cent of polyvinylpyrrolidone in the suspending medium had no action on the distribution of activity.

TABLE 1. THE LOCALIZATION OF CARBONIC ANHYDRASE FOLLOWING DIFFERENTIAL CENTRIFUGATION OF HOMOGENATES OF THE PERFUSED BRAINS OF MICE

Fraction	Activity			Nitrogen (N)		Relative Concentration
	Units	%	Units/ mg N	mg	%	
Homogenate	119 (114–126)	100 –	4·6 (4·2–5·0)	25·8 (25·2–27·0)	100 –	1·0
5000 g, 10 min	28 (21–35)	23 (18–28)	2·8 (2·1–3·3)	10·2 (9·8–10·5)	39 (36–42)	0·6 (0·4–0·7)
12,000 g, 15 min	5 (4–7)	4 (3–6)	3·1 (2·3–3·7)	1·8 (1·3–2·0)	7 (5–8)	0·7 (0·6–0·7)
45,000 g, 60 min	2 (2–2)	2 (2–2)	1·6 (1·2–1·8)	1·5 (1·3–1·7)	6 (5–6)	0·4 (0·3–0·4)
*Total Particulate (*P*)	36 (27–45)	30 (23–36)	2·6 (2·1–3·2)	13·4 (12·9–13·8)	52 (50–55)	0·5 (0·4–0·6)
Supernatant fraction (*S*)	54 (50–57)	45 (40–50)	11·2 (11·1–11·3)	4·8 (4·5–5·1)	19 (18–19)	2·4 (2·2–2·7)
Washes (*W*)	24 (21–28)	20 (18–24)	3·3 (2·8–3·9)	7·3 (7·2–7·4)	28 (27–29)	0·7 (0·6–0·8)
S – *W*	78 (72–83)	65 (57–70)	6·4 (6·1–6·8)	12·1 (11·8–12·5)	47 (46–47)	1·4 (1·3–1·5)
Recovery	113 (110–117)	95 (93–99)	–	25·5 (25·0–26·0)	99 (96–102)	–

Data in the table are the means and the highest and lowest values (in parentheses) of three experiments. Ten per cent homogenates in 0·25 M sucrose. Discrepancies in balance are due to rounding off the original data (see Methods).

* *S*/*P* ratio = 3·6.

Relative concentration and *S*/*P* ratio $= \frac{\text{units/mg N fraction or supernatant fraction } (S)}{\text{units/mg N homogenate or total particulate matter } (P).}$

The relative concentration of activity in the supernatant fraction was more than two-fold that of the original homogenate and about four times that of the total particulate matter (*S*/*P* ratio); in the particulate matter and in each of its components it was less than that of the original homogenate. The individual washes from each residue showed no concentration of activity and consequently were pooled as shown in the table. In general, the second wash from a residue contained about half the enzyme activity of the first wash on a percent basis; nitrogen content was also diminished.

The substitution of 1000 **g** for 5000 **g** for 10 min did not cause any change in the relative concentration of enzyme activity. Single high-speed centrifugation at 45,000 **g** for 60 min resulted in an equal partition of per cent activity between the particulate matter and the supernatant fraction. The nitrogen content of the particulate matter was increased to 79 per cent, while that of the supernatant fraction was relatively unchanged (21 per cent). Compared with the multiple fractionation procedure, there were no changes in relative concentration in terms of the original homogenate or the particular matter (*S*/*P* ratio). The aliquot of the same homogenate centrifuged at 100,000 **g** for 60 min showed 40 per cent of the enzyme

activity and 82 per cent of the nitrogen in the particulate fraction. The supernatant fraction now contained 60 per cent of the activity; the nitrogen content, 18 per cent, again was little changed. The relative concentration of activity in the supernatant fraction was increased to some degree compared to the single 45,000 g centrifugation or the multiple fractionation procedure. The S/P ratio of 6·8 was a large increase over previous ratios.

When fractionated in the manner described in Table 1, there were no differences of any consequence amongst whole brain, cortex, and whole brain with the cortex removed.

TABLE 2. THE LOCALIZATION OF CARBONIC ANHYDRASE FOLLOWING THE ADDITION OF THE ENZYME TO UNTREATED AND BOILED HOMOGENATES OF THE BRAINS OF MICE

Condition	Fraction	Activity (units)	Activity (%)	Activity (units/mgN)	Nitrogen (N) (mg)	Nitrogen (N) (%)	Relative concentration
Untreated plus enzyme (A)	Homogenate	295	100	12·8	23·0	100	1·0
	5000 g, 10 min	112	38	16·6	6·8	30	1·3
	12,000 g, 15 min	4	1	2·7	1·5	6	0·2
	45,000 g, 60 min	3	1	2·2	1·6	7	0·2
	*Total particulate (P)	120	41	20·0	9·8	43	1·6
	Supernatant fraction (S)	128	43	22·0	5·8	25	1·7
	Washes (W)	43	15	5·8	7·3	32	0·5
	$S + W$	171	58	13·0	13·1	57	1·0
	Recovery	291	99	—	22·9	100	—
Boiled plus enzyme (B)	Homogenate	166	100	7·6	21·8	100	1·0
	5000 g, 10 min	85	51	13·9	6·1	28	1·8
	12,000 g, 15 min	1	1	0·6	1·8	8	0·1
	†45,000 g, 10 min	2	1	0·6	3·3	15	0·1
	Total particulate (P)	88	53	7·9	11·2	52	1·0
	Supernatant fraction (S)	67	40	9·3	7·2	33	1·2
	Washes (W)	12	7	3·4	3·6	16	0·4
	$S + W$	79	47	7·3	10·8	49	1·0
	Recovery	167	100	—	22·0	101	—
Untreated plus boiled blood (C)	Homogenate	149	100	6·8	22·0	100	1·0
	5000 g, 10 min	46	31	6·1	7·5	34	0·9
	12,000 g, 15 min	6	4	4·1	1·5	7	0·6
	45,000 g, 60 min	4	3	2·5	1·5	7	0·4
	Total particulate (P)	56	38	5·3	10·5	48	0·8
	Supernatant fraction (S)	62	42	12·2	5·0	23	1·8
	Washes (W)	35	24	5·2	6·7	30	0·8
	$S + W$	97	66	8·3	11·7	53	1·2
	Recovery	152	102	—	22·2	101	—
	Original homogenate	145	100	5·9	24·4	100	—

The brains were not perfused. A 10% homogenate in 0·25 M sucrose was divided into three 20-ml aliquots. A and B received 150–160 units of carbonic anhydrase activity (hemolyzed mouse blood); B received the enzyme after being heated at 100°C for 5 min; C received the same amount of hemolyzed mouse blood heated at100°C for 5 min.

Relative concentration and S/P ratio calculated as shown in Table 1.

* S/P ratio = 1·1.

† S/P ratio = 1·2.

S/P ratio = 2·3.

2. *Localization of activity following the addition of enzyme to brain homogenate*

The particulate matter of both untreated and boiled homogenates showed a great capacity to carry down carbonic anhydrase activity upon centrifugation; these data are summarized in Table 2. The added activity in each instance was about equally

partitioned between the supernatant fraction and the total particulate matter. The first residue (5000 g, 10) was the sole carrier. Only the boiled homogenate differed importantly from the untreated aliquot receiving only boiled blood. The first residue accounted for about 51 per cent of the total activity and the combined washes for only 7 per cent. The distribution of nitrogen differed to some degree from the established pattern. The addition of enzyme to untreated homogenate resulted in a slight increase in the relative concentration of activity in the first residue, with no change in that of the supernatant fraction. An increase was found in the particulate matter; there was no difference between it and the supernatant fraction ($S/P = 1{\cdot}1$). The relative concentration of activity in the 5000 g residue of the boiled homogenate was significantly increased, being nearly twice that of the whole homogenate. The concentrations of activity in the latter and in the total particulate and supernatant fraction were practically identical.

Compared with the aliquot receiving boiled blood, the concentrations of activity in the 12,000 g and the 45,000 g residues of untreated and boiled homogenates were reduced to some degree.

TABLE 3. THE LOCALIZATION OF CARBONIC ANHYDRASE INHIBITOR FOLLOWING CENTRIFUGATION OF HOMOGENATES OF THE BRAINS OF MICE

Fraction	A		B	
	(μg)	(%)	(μg)	(%)
Homogenate	11·1 (9·6, 12·6)	100	5·6 (6·0, 5·3)	100
45,000 g, 60 min	1·0 (1·2, 0·8)	9 (12, 6)	1·1 (1·2, 1·0)	20 (20, 19)
Supernatant fraction (S)	9·4 (7·3, 11·4)	83 (76, 90)	3·8 (4·0, 3·6)	68 (67, 68)
Wash (W)	1·2 (0·9, 1·4)	10 (9, 11)	0·6 (0·7, 0·5)	11 (13, 9)
$S + W$	10·5 (8·2, 12·8)	93 (85, 101)	4·4 (4·7, 4·1)	78 (78, 77)
Recovery	11·5 (9·5, 13·6)	104 (99, 108)	5·5 (6·0, 5·0)	97 (100, 94)

Ten per cent homogenates in 0·25 M sucrose; the brains were not perfused. A was from mice sacrificed at 15 min and B from mice sacrificed at 2 hr after the intravenous administration of 50 mg of methazolamide per kg. The data are the means and individual values obtained in two separate experiments. With the exception of the original homogenate (12 ml), the various fractions and washes made up to 10 ml with 0·25 M sucrose; conversion of the data to μg/ml can be readily made.

3. *Localization of carbonic anhydrase inhibitor*

Fifteen minutes after the intravenous administration of methazolamide, 83 per cent of the total inhibitor activity was found in the supernatant; the particulate matter accounted for 9 per cent (Table 3). The supernatant fraction and the wash accounted for over 90 per cent of the total amount of inhibitor present. Essentially similar results were obtained with an homogenate prepared from perfused brains. The concentration of inhibitor in the particulate matter is not due to residual blood. The inclusion of 7·3 per cent of polyvinylpyrrolidone in the suspending medium had

no effect. Two hours after administration, 68 per cent of the inhibitor present was found in the supernatant fraction; the particulate matter contained 20 per cent. The absolute amount of inhibitor in this fraction did not change with time.

When methazolamide (sodium salt) was added to brain homogenate, the amount found in the particulate matter was the same as that found after *in vivo* administration.

Perfusion of the brains of mice with normal saline up to 15 min had no action on the concentration of inhibitor. In two experiments, sucrose perfusion for a period of 15 min caused reductions of 19 and 28 per cent; in one experiment it was without effect. All perfusions were equally effective in reducing the erythrocyte count.

4. *The calculated degrees of the inhibition of carbonic anhydrase in brain*

On the basis of the procedure given in the section on Methods, the molar concentration of carbonic anhydrase in the brains of mice was calculated to be $2{\cdot}534 \times 10^{-6}$ M. (*EI*) was $2{\cdot}534 \times 10^{-6}$ M at both 15 min and 2 hr after the intravenous administration of amounts of methazolamide estimated to have an anticonvulsant effect in 95 per cent of a population of mice. Consequently, maximal inhibition (100 per cent) of the enzyme in brain appears to be possible at these times, provided that all of the enzyme and all of the inhibitor are in immediate contact.

DISCUSSION

The heterogeneity of the cell population of the brain restricts the usefulness of the technique of differential centrifugation. It is not known if the carbonic anhydrase in brain is located in certain specific cells or in all cells. Conceivably, the enzyme and the inhibitor could be located in different cells, but following centrifugation both could end up in the supernatant fraction of the whole homogenate. Conclusions drawn about the situation *in vivo* would be entirely spurious. A further difficulty with this technique is the likelihood of the removal of water-soluble substances from the particulate matter into the aqueous phase. The localization of an agent in the extracellular space would also be misinterpreted as location in the soluble fraction. However, in view of the rather unsettled state as to the existence of extracellular space in brain,[8, 9] there is not much to be gained in pursuing this possibility further.

The inhibitor found in brain has not been identified; however, there is no reason to believe that it is not methazolamide. At pH 7·3, the solubility of methazolamide in water is about 3·5 mg/ml; at pH 2·0 it is about 1 mg/ml. The free acid is relatively insoluble. Theoretically, all inhibitor present in brain could be leached out of the particulate matter into the suspending medium. However, solubility alone does not dictate distribution in biological systems. Maren *et al.*[10] found that methazolamide was not readily washed out of human and canine red blood cells, even though the solubility of methazolamide greatly exceeds the amounts of inhibitor present. The unionized moiety most probably is the form that enters into the brain; the p*Ka* of methazolamide is 7·4. Consequently, at physiological pH, about one-half of the amount in brain could exist in the ionized state. If methazolamide were actually located in the particulate matter, a considerable amount, perhaps one-half, might not be able to cross boundaries at the pH of the suspending medium (6·5). The binding of agents of this type by tissue components is an additional factor to be taken into consideration (Maren *et al.*[10]). The above considerations support the belief that the

presence of nearly all of the inhibitor in the supernatant fraction represents intracellular localization in the soluble fraction; its presence there does not appear to be an artifact due to the leaching of methazolamide out of the particulate matter into the aqueous phase. If an extracellular space exists in the brain, methazolamide does not appear to be predominantly localized there. Perfusion with saline or 0·25 M sucrose had no significant action on the concentration of inhibitor. The small amount of inhibitor found in the particulate matter most probably represents contamination. The constancy of the amount found in the particulate matter at different times following intravenous administration further suggests that its presence there is not fact, but artifact. The distribution of inhibitor following the addition *in vitro* of the water-soluble sodium salt of methazolamide to brain homogenate may not be pertinent. However, it does show that the particulate matter can carry down an amount of inhibitor of the same order of magnitude as found when methazolamide is given intravenously.

The carbonic anhydrase activity in the supernatant fraction appears to represent intracellular localization in the soluble fraction. The evidence is as follows: only in the supernatant fraction is the concentration of carbonic anhydrase activity (in terms of units per mg of nitrogen) significantly greater than that of the original homogenate. The activity in the supernatant fraction does not appear to be due to the leaching or the leaking of the enzyme out of the particulate matter; the concentration of activity in the washes of the various residues was always less than that of the original homogenate or of the supernatant fraction. Furthermore, with reference to the residue washed, the second wash contained a much smaller per cent of the residual activity than did the first. The inclusion of polyvinylpyrrolidone, which is considered to stabilize biochemical and morphological characteristics,[11, 12] was without effect.

The carbonic anhydrase activity in the particulate matter is the result of its great ability to carry down the enzyme, as shown by: (1) the absence of any concentration of activity in the total particulate matter or any of its fractions studied, relative to the original homogenate; (2) the nitrogen content of the individual residues was closely correlated with enzyme activity (within the particulate fraction, the partition of carbonic anhydrase on a per cent basis was directly related to the amount of material present as measured by nitrogen content); (3) the activity of the particulate matter was not independent of the force of centrifugation, i.e. 100,000 **g** for 60 min reduced the per cent of the total activity in the particulate matter; (4) erythrocytes and other unbroken cells that could contribute a considerable amount of carbonic anhydrase activity were only occasionally seen in the 5000 **g** residue, and never were found in the 12,000 **g** fraction; and (5) the direct demonstration of the great capacity of the particulate matter to carry down enzyme following addition *in vitro*.

Datta and Shepard[12] have also concluded that carbonic anhydrase is localized in the soluble fraction of the livers and the kidneys of rats. Data published recently by Karler and Woodbury[14] are claimed to show that the enzyme is located in both the soluble fraction and mitochondria of the kidneys and cerebral cortices of rats. Examination by the light microscope showed that the 5000 **g** residue of the present study was mostly nuclei, debris and occasionally an unbroken cell. The 12,000 **g** residue consisted of particles morphologically similar to mitochondria[15] and could be stained with Janus Green B. Functional studies as an aid to identification were not

carried out. This residue of the cerebral cortices of the brains of mice might correspond to what Karler and Woodbury consider to be the mitochondrial fraction of the cerebral cortices of rats. The activity was only 7 per cent of the total, in relative concentration was not materially different from the original homogenate, and is considered to be the result of contamination.

The technique of differential centrifugation has produced evidence, admittedly gross, that the carbonic anhydrase in the brains of mice and the inhibitor found there following the administration of methazolamide are very probably localized intracellularly in the soluble fraction. This, of course, does not constitute proof of the inhibition of the enzyme *in vivo* or even of location in the same cell. Theoretically, at both times studied, there would appear to be sufficient inhibitor present to cause a maximum inhibition of all the carbonic anhydrase in the brains of mice *if* all of the enzyme and all of the inhibitor were in intimate contact. These calculated degrees of enzyme inhibition should be received with considerable reservation and extrapolated to the situation *in vivo* with caution because of: (1) the uncertainty regarding the equilibrium constants (K_I) (for example, the K_I of acetazolamide determined by Keller *et al.*[16] in carbonate buffer is about one-sixth of that found by Maren *et al.*[6]; (2) the concentration of "free" inhibitor (I) may be seriously overestimated because of (a) the known binding of inhibitors by other tissue components,[10] and (b) a different anatomical distribution pattern of enzyme and inhibitor; (3) the demonstration by Keller *et al.*[16] that carbonic anhydrase itself apparently has an additional binding site besides the active centre (this second binding site has been neglected in the equilibrium expression); (4) the estimations of the degrees of enzyme inhibition have not been extended to 37 °C; and (5) pending the purification of carbonic anhydrase, the possibility of error in expressing the concentration of enzyme in molar terms should be kept in mind.

With all of its limitations, the results of this study cannot be considered conclusive. However, no evidence was found that the anticonvulsant action of this class of compounds is unrelated to the inhibition of carbonic anhydrase in brain.

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