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ANTIEPILEPTIC PROPERTY OF INHIBITORS OF CARBONIC ANHYDRASE

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Abstract—Mechanism of the anticonvulsant action of the inhibitors of carbonic anhydrase was investigated in rats treated with an intraperitoneal or intraventricular injection of the drugs. The duration of the maximal electroshock seizure was taken as an indicator of the intensity of convulsions, and the activity of carbonic anhydrase in the brain and erythrocytes was determined by a pH-changing method.

The inhibitors of carbonic anhydrase displayed an anticonvulsant effect without exception when they inhibited the activity of carbonic anhydrase in the brain. Thiazide derivatives, while potent inhibitors of the enzyme *in vitro*, failed to show any anticonvulsant action when they were injected by the intraperitoneal route, because of their inability to inhibit the brain carbonic anhydrase *in vivo*, although they exhibited the anticonvulsant effect after intraventricular administration.

A parallel was demonstrated between the degree of anticonvulsant action and the degree of inhibition of the brain carbonic anhydrase.

The distribution pattern of the activity of carbonic anhydrase in the brain after administration of the inhibitors was also studied. Three kinds of patterns were obtained in accordance with the difference in the clinical indications of the drugs. This might suggest that the clinical indication of inhibitors of carbonic anhydrase for the seizure patterns of epilepsy may be related to the site of the drug action.

SOME types of inhibitors of carbonic anhydrase, acetazolamide,[1, 2] ethoxzolamide,[3] sulthiame (N-[4'-sulfamylphenyl]-butansultam [1–4]),[4–6] and disamide,[7, 8] have been reported to be effective for suppression of epileptic seizures, and the antiepileptic action of acetazolamide has been attributed to its inhibitory effect on carbonic anhydrase.[9, 10] Mechanism of the antiepileptic action of the inhibitors has been studied by some investigators. Millichap *et al.*[9] reported that the antiepileptic action of acetazolamide was not related to diuresis or general acidosis but to direct inhibition of the brain carbonic anhydrase. Gray *et al.*[10] and Tanimukai *et al.*[11] independently studied the time-course changes of the maximal electroshock seizure threshold and of the concentration of the inhibitors in the brain after the drug administration, and reached the same conclusion proposed by Millichap *et al.*[9] The conclusion, however could not be fully accepted because of the limited number of inhibitors used.

Thiazide derivatives, such as benzthiazide, cyclopenthiazide, polythiazide, etc., on the other hand, lack antiepileptic action in spite of their potent inhibitory effect on carbonic anhydrase *in vitro*.

The present study was undertaken to learn the difference in the mechanisms of action of the inhibitors of carbonic anhydrase with or without antiepileptic properties; and

some evidence of the positive correlation between anticonvulsant action of the drugs and the inhibition of the brain enzyme is presented.

MATERIALS AND METHODS

Adult male Wistar rats, 120–160 g body weight, were used. Among known inhibitors of carbonic anhydrase, acetazolamide, sulthiame, ethoxzolamide, disamide, diphenylmethane-4,4′-disulfonamide, benzthiazide, cyclopenthiazide, and polythiazide were used. The first four compounds are known to be effective drugs for epileptic seizures, and the last three are known as diuretics of the thiazide group. Sulfonamide derivatives newly synthesized for this study were benzylaniline-4,4′-disulfonamide; phenylacetanilide-4,4′-disulfonamide; *p*-sulfamoylbenzoic acid; 5-(*p*-sulfamoyl)-oxazolidine-2,4-dione; ethyl N-(*p*-sulfamoylphenyl)-aminoacetate; and *p*-sulfamoylphenylglycine. These compounds were synthesized by Ono Pharmaceutical Co. Ltd., Osaka, Japan, and were identified by elementary analysis and melting points.

Intraperitoneal administration

Animals were given a controlled electrostimulation (first electrostimulation). One hour or more later, the drugs were injected i.p. All the drugs were used in a dose of $2{\cdot}5 \times 10^{-4}$ moles/kg body weight, except disamide which was used at 1×10^{-3} moles/kg.

Anticonvulsant effect and inhibition of carbonic anhydrase in the brain and erythrocytes were examined 2 hr after drug administration, because in preliminary experiments the peak of the anticonvulsant effect was found for all the tested drugs 1–3 hr (2-hr average) after the injection. The control animals were injected with physiological saline in the same way.

Intraventricular administration

This experiment was performed with acetazolamide and benzthiazide. For each drug the dosage was $2{\cdot}5 \times 10^{-7}$ moles/g brain weight and the injected volumes were less than 0·03 ml for an animal. One hour or more after the controlled electrostimulation, the animal was affixed with cellophane tape to a board, the scalp was opened (local anesthesia) and the drug injected into the left lateral ventricle. The animal was freed from the binding and placed in a glass cage, and the anticonvulsant effect was examined 1, 1·5 and 2 hr after the drug injection. The site of the injection was examined macroscopically by autopsy at the end of the experiment.

Anticonvulsant effect

Anticonvulsant effect was judged by disappearance or shortening of the duration of the maximal electroshock seizure, which was characterized by tonic extension of the hindlimbs. In practice, the animals were fitted with Spiegel's corneal electrodes[12] under ether anesthesia, and the experiment was performed the next day. Stimulation was given by Woodbury's electrostimulator[13] before (control) and after the drug administration. The conditions of the electrostimulation were 100 mA for 0·2 sec. The duration of the tonic extensor phase induced by the electrostimulation was measured by a stopwatch, and the anticonvulsant effect was expressed by the shortening ratio (%) calculated from the formula $[(tc-ti)/tc] \times 100$, where *tc* and *ti* represent the duration of the tonic extensor phases before and after the drug administration respectively.

Inhibitory rates of carbonic anhydrase in the tissues

Shortly after the second electroconvulsion ceased, the animal was anesthetized with ether and the thorax opened. The blood was collected by heart puncture with a syringe containing 3·8% of sodium citrate, and the residual blood was removed by a vital perfusion method with physiological saline in order to prevent the contamination of the tissues by the drug in the blood. The brain was then dissected out. The erythrocytes were precipitated by centrifugation from the collected blood and were washed twice with physiological saline.

Direct method. After rinsing the cerebrospinal fluid from the ventricles, the brain tissue was homogenized with 24 volumes (for newly synthesized inhibitors) or 49 volumes (for known inhibitors) of distilled water, and 0·1 ml of the supernatant was used for the determination of the activity of carbonic anhydrase. The erythrocytes were hemolyzed with 49 volumes of distilled water, and 0·1 ml of the hemolysate was applied to determine the enzyme activity. All these procedures were performed at 5°.

Carbonic anhydrase activity was determined by a modification[14] of Maren's pH-changing method.[15] The enzyme activities of the whole brain and erythrocytes of the control animals were 42·7 ± 4·2 units/100 mg wet weight and 325 ± 17 units/0·1 ml respectively.

Inhibitory rates of carbonic anhydrase in the tissues were calculated from the formula $[(Ec-Ei)/Ec] \times 100$, where *Ec* and *Ei* represent the enzyme activities in the tissues taken from the animals treated with saline or the drug respectively.

The activity of carbonic anhydrase in the tissues was proved in a preliminary experiment not to change by the electrostimulation applied or by anesthesia with ether.

Indirect method. Degrees of inhibition of carbonic anhydrase in the tissues *in vivo* were also examined from the concentrations of the drugs in the tissues. The materials were treated in the same way as for the direct method (but the volumes of the added water for homogenization were half those in the direct method respectively) and were boiled for 10 min to inactivate the enzyme. The inhibitory potency of the drugs contained in the tissues was not altered by this treatment. The material thus prepared was mixed with an equal volume of human hemolysate (1 : 100) as an enzyme source, and 0·1 ml of the mixture was used for the measurement of the enzyme activity. Degree of inhibition of carbonic anhydrase by the drug contained in the tissues was calculated, and the concentration of the drugs was determined with a calibration curve previously prepared to indicate concentration–inhibition relationship.

In the brain, per cent inhibition of carbonic anhydrase obtained by the direct method was essentially equal to that obtained by the indirect method for every drug. In the erythrocytes, on the contrary, the degrees of inhibition of the enzyme obtained by direct and indirect methods were not always equal. This discrepancy could not be attributed to the difference between rat (for direct method) and human (for indirect method) erythrocyte carbonic anhydrase, because the material for the indirect method and the drugs themselves inhibited the erythrocyte enzyme prepared from rats and human to the same degree. It was postulated that the drugs might partially bind with some constituents in the erythrocytes, lose the activity to inhibit the enzyme *in vivo*, but recover the activity by being set free from the binding on heating. Thus the data obtained by the direct method are presented under Results for the degree of inhibition of carbonic anhydrase in the tissues *in vivo*.

Distribution of activity of carbonic anhydrase in the brain

Changes in the distribution pattern of the enzyme activity in different regions of the brain was studied after i.p. administration of the inhibitors. For this purpose, the animals receiving no electrostimulation were used. Before (control) and 2 hr after drug administration, the brain was dissected out after the removal of the blood. The brain was divided macroscopically into nine anatomical regions (cerebral gray and white matter, cerebellum, hippocampus, caudate nucleus, thalamus, hypothalamus, corpus quadrigemina, and pons–medulla oblongata), each region was homogenized with 49 volumes of distilled water, and 0·1 ml of the homogenate was utilized to determine the enzyme activity.

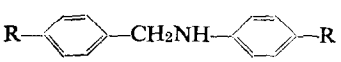
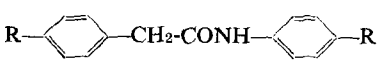
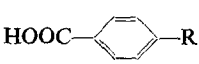
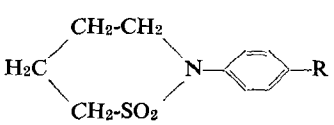
RESULTS

Relationship between inhibitory effect on carbonic anhydrase and anticonvulsant action of compounds

By introducing a sulfonamide grouping in the para-position of the benzene ring of two or three aromatic compounds, the compounds came to possess anticonvulsant

TABLE 1. COMPARISON BETWEEN AROMATIC COMPOUNDS, WITH AND WITHOUT SULFONAMIDE GROUPING, OF INHIBITORY EFFECT ON CARBONIC ANHYDRASE AND ANTICONVULSANT ACTION

Inhibitory effect was measured *in vitro* by the pH-changing method, with human hemolysate (1 : 100) as an enzyme source; the anticonvulsant effect was examined by electroshock-seizure method in rats injected i.p. with the compounds.

Compounds	R = H		R = SO_2NH_2	
	Inhibitory effect on carbonic anhydrase	Anticonvulsant effect	Inhibitory effect on carbonic anhydrase	Anticonvulsant effect
	none	none	strong	strong
	none	none	strong	strong
	none	none	weak	weak
	none	weak	strong	strong

properties in addition to an inhibitory effect on carbonic anhydrase *in vitro*, as shown in Table 1. Phenylbutansultam itself exhibited a weak anticonvulsant effect before modification but, after the substitution of a free sulfonamide grouping, it showed both a stronger anticonvulsant effect and an inhibitory effect on carbonic anhydrase.

A similar correlation was reported for sulfathiazole by Mann and Keilin[16] and for acetazolamide by Gray *et al.*[10] A close relationship is suggested between the inhibitory effect on carbonic anhydrase and the antiepileptic property of carbonic anhydrase inhibitors.

Relationship between anticonvulsant effect and inhibition of carbonic anhydrase in brain and erythrocytes, and diuresis

Eight known inhibitors of carbonic anhydrase were injected into rats i.p., and the anticonvulsant effects and degree of carbonic anhydrase inhibition in the brain and erythrocytes were examined 2 hr later. The results are summarized in Fig. 1. Ethox-

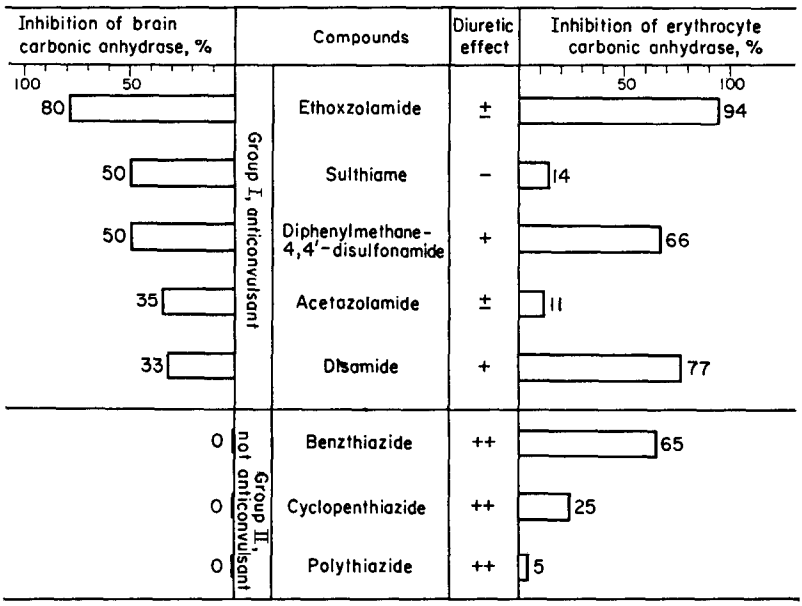


FIG. 1. Relationship between anticonvulsant effect and inhibition of carbonic anhydrase in brain and erythrocytes, and diuresis, by known carbonic anhydrase inhibitors. All data were obtained in rats 2 hr after the i.p. injection of $2{\cdot}5 \times 10^{-4}$ moles of the compounds per kg (except 1×10^{-3} moles disamide/kg). Anticonvulsant effect was judged by abolition of the tonic extensor phase of the hindlimbs of rats, induced by electrostimulation. Values of per cent inhibition of carbonic anhydrase were calculated from enzyme activity in the tissues measured with 6-fold dilution of 50-fold homogenate (for brain) or hemolysate (for erythrocytes). Diuretic effects are cited from the literature[10, 14]

zolamide, sulthiame, diphenylmethane-4,4'-disulfonamide, acetazolamide, and disamide suppressed the maximal electroshock seizure in rats completely, and were classified in Group I, to which potent anticonvulsants belong. On the other hand, benzthiazide, cyclopenthiazide, and polythiazide, which are thiazide derivatives, failed to show any anticonvulsant action, and were classified in Group II. Definite inhibition of carbonic anhydrase in the brain was found for all the drugs belonging to Group I, whereas no inhibition of the brain enzyme was found for any drug in Group II.

The degree of inhibition of carbonic anhydrase in the erythrocytes, on the contrary, differed from drug to drug independently of the anticonvulsant effect. Diuretic effects

were found to be rather stronger for thiazide derivatives in Group II than for the drugs in Group I.

These data seemed to indicate that the anticonvulsant property of the inhibitors of carbonic anhydrase was closely related to the inhibition of the *brain* carbonic anhydrase, and was independent of general acidosis following the inhibition of the erythrocyte carbonic anhydrase or diuresis by the drug action. Lack of anticonvulsant action of thiazide derivatives in Group II might be attributed to their inability to inhibit the brain carbonic anhydrase *in vivo*.

A more detailed study was performed to solve the problem of the dependency of anticonvulsant action of the drugs on the inhibition of carbonic anhydrase in the brain. In this study, six newly synthesized sulfonamide derivatives were used as inhibitors of carbonic anhydrase. As clearly shown in Fig. 2, the degree of anticonvulsant effect, which was indicated by the shortening ratio (%) of the duration of the maximal

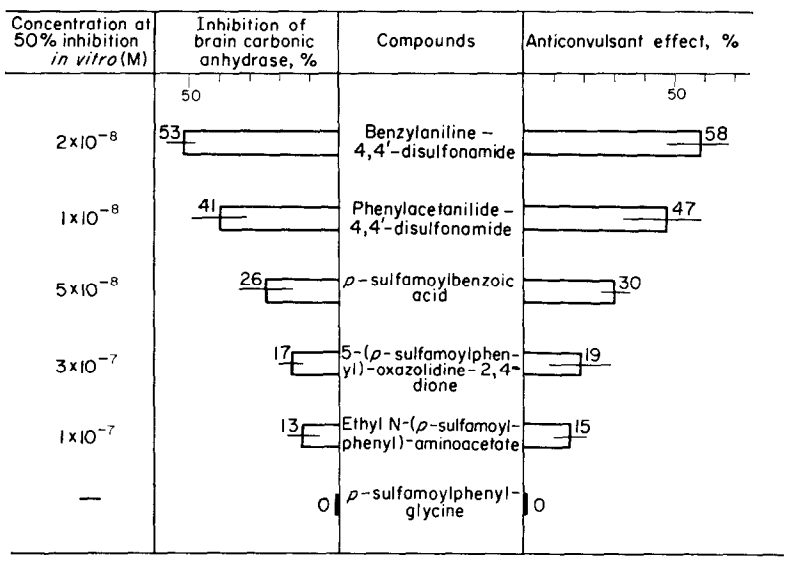


FIG. 2. Relationship between inhibition of brain carbonic anhydrase and anticonvulsant effect by newly synthesized carbonic anhydrase inhibitors. Data were obtained in rats 2 hr after the i.p. injection of $2{\cdot}5 \times 10^{-4}$ moles of the compounds per kg. Anticonvulsant effect was expressed by shortening ratio (%) of the duration of the maximal electroshock seizures. Values of per cent inhibition of carbonic anhydrase were calculated from enzyme activity measured with 6-fold dilution of 25-fold homogenates of the brains taken from the animals with or without injection of the compounds. Concentration of the compounds to inhibit the enzyme by 50% was determined *in vitro* by pH-changing method, with human hemolysate (1 : 100) as an enzyme source.

electroshock seizure, was in direct proportion to the degree of inhibition of the brain carbonic anhydrase *in vivo* and independent of the inhibitory potency of the compounds on carbonic anhydrase *in vitro*.

Intraventricular administration of inhibitors of carbonic anhydrase

If the anticonvulsant effect of the inhibitors is a result of the inhibition of the brain carbonic anhydrase, thiazide derivatives in Group II ought also to display an

anticonvulsant effect when they are given directly into the brain. This postulation was verified by an experiment with the intraventricular administration of the drugs. Benzthiazide was used as a compound representative of Group II because it was the most soluble of the three thiazide derivatives; since these, as a rule, are difficult to dissolve in water, 0·04 N sodium hydroxide was used as a solvent. Sodium hydroxide, when given intraventricularly, antagonized the anticonvulsant effect of carbonic anhydrase inhibitors, probably because of its alkaline nature, although sodium hydroxide itself in that dose seemed to act as an anticonvulsant when administered into the brain (Fig. 3).

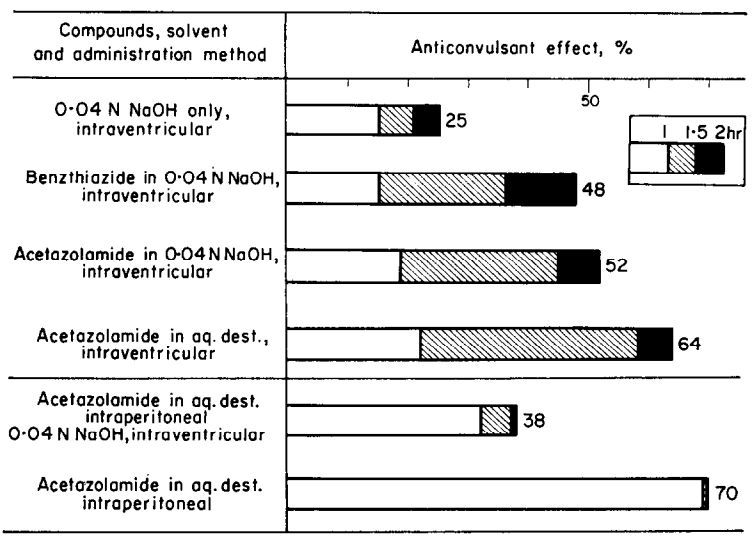


FIG. 3. Anticonvulsant effect of intraventricular and intraperitoneal administration of acetazolamide and benzthiazide. Both compounds were used with $2{\cdot}5 \times 10^{-7}$ moles/g. Anticonvulsant effect was examined 1, 1·5 and 2 hr after the injection of the compounds and is expressed by shortening ratio (%) of the duration of the maximal electroshock seizures. In control animals, physiological saline was injected. Value presented is the mean of 5–10 experiments.

In spite of the antagonizing effect of the solvent, benzthiazide ($2{\cdot}5 \times 10^{-7}$ moles/g brain weight) by intraventricular administration displayed an anticonvulsant effect of the same degree as that displayed by equal concentrations of acetazolamide dissolved in sodium hydroxide (Fig. 3). At this time the brain carbonic anhydrase was highly inhibited. This result clearly indicates that the anticonvulsant action of the drugs is dependent upon the inhibition of carbonic anhydrase in the brain.

Distribution patterns of carbonic anhydrase activity in the brain after drug administration

It is known that the various antiepileptics have varying indications for the clinical types of epileptic seizures: ethoxzolamide is especially effective for *grand mal*,[3] acetazolamide[1, 2] and disamide[7, 8] for *petit mal*, and sulthiame[4–6] is almost selectively effective for psychomotor attacks, although carbonic anhydrase inhibitors are in general effective for all types of epileptic seizures. Because a different anatomical epileptogenic focus is indicated for each type of seizure, the clinical effectiveness of an

antiepileptic might be regulated by the site of the drug action; in other words, the difference in the distribution pattern of carbonic anhydrase activity in the brain associated with different inhibitors of carbonic anhydrase might result in the difference in the clinical effect. Distribution patterns of carbonic anhydrase activity in the brain were examined 2 hr after the i.p. administration of the inhibitors belonging to Group I, and three different patterns of distribution were obtained, corresponding to three different clinical indications of the drugs, as is shown in Fig. 4.

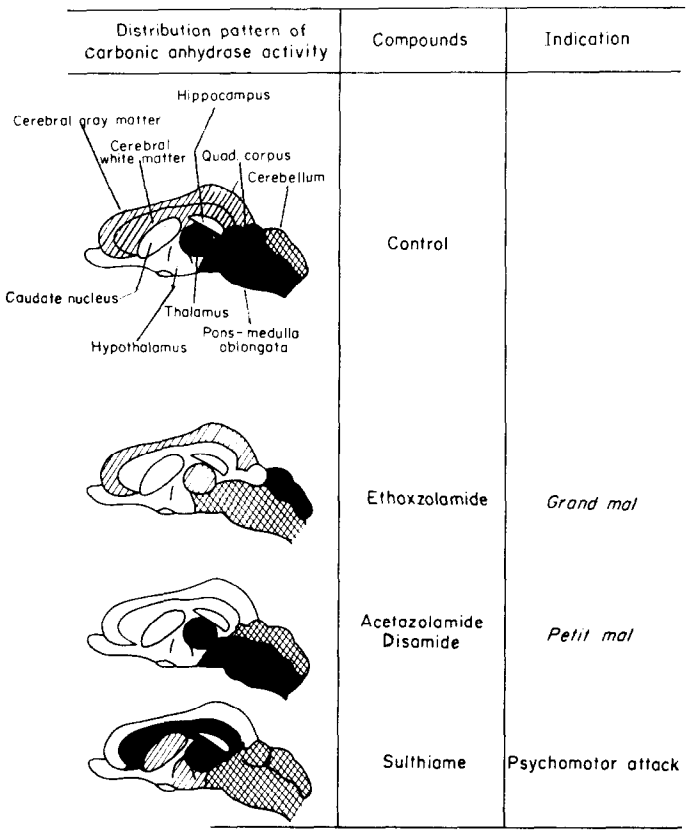


FIG. 4. Distribution pattern of carbonic anyhdrase activity in the brain after intraperitoneal administration of carbonic anhydrase inhibitors. The schema are drawn to show the relative relationships of the distribution of carbonic anhydrase activity in the brain 2 hr after drug administration. The density of the shadings of the brain does not represent the absolute activity of the enzyme but the relative values of the enzyme activity for each compound; the denser the shading, the higher the activity.

In the control animal, the black areas indicate higher than 35 units/100 mg; cross-hatched, 31–26 u/100 mg; hatched, 25–20 u/100 mg; white, lower than 18 u/100 mg.

DISCUSSION

A hypothesis that the antiepileptic effect of the inhibitors of carbonic anhydrase is attributable to their inhibitory action on the brain enzyme has been proposed by some authors.[9–11] This concept is based mainly on an experimental coincidence in the

time course of the anticonvulsant effect with that of inhibition of the brain carbonic anhydrase. It is considered to lack clear-cut evidence, however, because of the limited number of inhibitors examined and because of a lack of evaluation of the compounds without anticonvulsant action. In the present studies, the validity of the above concept was clearly demonstrated. The data presented in Fig. 1 showed that anticonvulsant action of the inhibitors of carbonic anhydrase was correlated with the inhibition of the brain carbonic anhydrase and was independent of the inhibition of the erythrocyte enzyme or diuresis.

This relationship was more distinctly demonstrated by the experiment with intraventricular injection of the inhibitors (Fig. 3). Benzthiazide was not an anticonvulsant at all by the intraperitoneal route, but it displayed a considerable anticonvulsant effect, comparable to that of acetazolamide, when given intraventricularly; it then inhibited the brain carbonic anhydrase. Thus the anticonvulsant effect of the inhibitors of carbonic anhydrase undoubtedly appears only when the brain enzyme is inhibited.

The parallelism between the potency of anticonvulsant effect of the drugs and the degrees of inhibition of the brain carbonic anhydrase was suggested by the data shown in Fig. 2, which were obtained by using newly synthesized compounds. Gray *et al.*[10] reported for acetazolamide and methazolamide that maximal concentration of inhibitors in brain preceded maximal anticonvulsant effect by both drugs, but their data for concentration of inhibitors in brain could not be considered valid because they determined it without washing out the cerebrospinal fluid through which acetazolamide and related compounds entered into the brain tissue.[11] In the present study much attention was paid to this aspect, so that the data presented are believed to be more valid.

Carbonic anhydrase is considered to play a role in the brain in hastening the removal of excess carbon dioxide which inhibits or slows nerve conduction, and in maintaining the sensitivity of a neuron to stimulation.[17] Therefore, inhibition of this enzyme in the brain might result in accumulation of carbon dioxide in the tissue and thus inhibit propagation of the nervous activity. This may be a possible mechanism of the anticonvulsant effect of the inhibitors of carbonic anhydrase, but a more detailed mechanism remains to be evaluated because anticonvulsant action of the inhibitors has been reported to be dependent also upon the concentration of catecholamines in the brain.[18]

It is known clinically that different anticonvulsants in the group of the inhibitors of carbonic anhydrase have different indications for the seizure patterns of epilepsies, notwithstanding the view that all the drugs are considered to act universally by inhibiting brain carbonic anhydrase. Since it was assumed that the differences in clinical effectiveness were due to some differences in the site of drug action, we investigated the distribution pattern of the activity of carbonic anhydrase in the brain for the reason stated above, and the results are shown in Fig. 4. The enzyme activity was not inhibited maximally in the possible focus of the seizures. This, however, does not necessarily mean that the previous postulation was in error, since the carbonic anhydrase inhibitors may act as antiepileptics by inhibiting propagation of the nerve impulses and because the site of action is not considered to be at an epileptogenic focus but at the spreading pathway of the seizure discharges. As shown in Fig. 4, three characteristic patterns of the distribution of carbonic anhydrase in the brain

were distinguishable with three types of drugs, each having a different clinical indication. This observation seems to be suggestive for understanding the difference in the clinical effectiveness of the drugs, although the authors at present would not state positively that such a correlation exists.

REFERENCES

1. W. H. BERGSTROM, R. G. GARZOLI, C. T. LOMBROSO, D. T. DAVIDSON and W. M. WALLACE, *Amer. J. Dis. Child.* **84**, 771 (1952).
2. C. T. LOMBROSO and I. FORSYTHE, *Epilepsia* (*Amst.*) **1**, 493 (1960); Abst.in *Zbl. ges. Neurol. Physchiat.* **160**, 181 (1961).
3. S. SOLOMON and A. HIRANO, *Neurology* **9**, 167 (1959).
4. F. FLÜGHER, D. BENTE and T. ITIL, *Dtsch. med. Wschr.* **85**, 2199 (1960).
5. H. J. RAFFAUF, *Dtsch. med. Wschr.* **85**, 2203 (1960).
6. M. P. ENGELMEIER, *Dtsch. med. Wschr.* **85**, 2207 (1960).
7. I. EZIMA and T. IMAI, *Brain, Nerve, Tokyo* **13**, 139 (1961).
8. T. WADA and H. FUKUSHIMA, *Clinical Psychiatry* (*Tokyo*) **4**, 653 (1962).
9. J. G. MILLICHAP, D. M. WOODBURY and L. S. GOODMAN, *J. Pharmacol. exp. Ther.* **115**, 251 (1955).
10. W. D. GRAY, T. H. MAREN, G. M. SISSON and F. H. SMITH, *J. Pharmacol. exp. Ther.* **121**, 160 (1957).
11. H. TANIMUKAI, T. NISHIMURA and I. SANO, *Klin. Wschr.* **42**, 918 (1964).
12. E. A. SPIEGEL, *J. Lab. clin. Med.* **22**, 1274 (1937).
13. L. A. WOODBURY and V. D. DAVENPORT, *Arch. int. Pharmacodyn.* **92**, 97 (1952).
14. T. NISHIMURA, *Psychiat. Neurol. jap.* **65**, 423 (1963).
15. T. H. MAREN, *J. Pharmacol. exp. Ther.* **130**, 26 (1960).
16. T. MANN and D. KEILIN, *Nature* (*Lond.*) **146**, 164 (1940).
17. T. NISHIMURA, H. TANIMUKAI and K. NISHINUMA, *J. Neurochem.* **10**, 257 (1963).
18. W. D. GRAY, C. E. RAUCH and R. W. SHANAHAN, *J. Pharmacol. exp. Ther.* **139**, 350 (1963),