line decreased slightly. Total tissue nitrogen parallels these changes. 2) Above 330 g however, the relative hexosamine concentration increases with body weight. The effect of body weight upon the ratio of hexosamine to hydroxyproline closely parallels the response of relative concentration of hexosamine to rat age.

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Seizure Patterns in Young Animals. Significance of Brain Carbonic Anhydrase. II.*† (23821)

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Previous work has demonstrated a direct relation between the activity of brain carbonic anhydrase and the susceptibility of animals to experimental seizures. In mice, acetazolamide has been shown to be approximately twice as potent as sulfanilamide as an inhibitor of brain carbonic anhydrase and as an anticonvulsant; the maximum degree of inhibition of brain enzyme corresponded with the time of peak anticonvulsant effect of each drug(1). In addition, it has been shown recently in newborn animals of different species that the development of susceptibility to various experimental seizure patterns is directly related to the level of carbonic anhydrase in the brain(2). In rats between 20 and 35 days of age, in which a high level of brain carbonic anhydrase was observed, it was possible to induce both major and minor seizure patterns by variation of the strength of an electroshock stimulus. A current of 50 m. amps. applied for 0.2 sec. was the threshold stimulus required for induction of the maximal seizure pattern, which consists principally of tonic extension of the hind-limbs (grade 5); tonic flexion or extension of the forelimbs with clonus (grade 4) was induced by a stimulus of 40 m. amps.; and symmetrical clonic seizures with loss of posture (grade 3) occurred in response to a threshold current of 30 m. amps. Stimuli of 20 and 10 m. amps. respectively were required for the induction of catatonic (grade 2) and hyperkinetic (grade 1) minor seizure patterns. In rats younger than 20 days, the grade of seizure pattern obtained in response to an electroshock stimulus of 50 m. amps. was less severe than that observed in older rats between 20 and 35 days of age, and the level of brain carbonic anhydrase was correspondingly lower. In the newborn, a hyperkinetic grade 1 seizure response was associated with a minimal amount of carbonic anhydrase in the brain. With a rapid increase in enzyme activity between 10 and 20 days, seizure patterns of grades 2, 3 and 4 were obtained.

In the present investigation, designed to test further this apparent relation between carbonic anhydrase activity in the brain and various patterns of seizure activity, the anticonvulsant effect of acetazolamide has been observed in parallel with estimations of the degree of inhibition of the enzyme.

Methods. Effect of acetazolamide on seizure patterns. The effect of acetazolamide on seizure patterns induced by electroshock was determined in albino rats (Holtzman strain) between 28 and 32 days of age and weighing between 75 and 100 g. Acetazolamide sodium was dissolved in distilled water and administered by subcutaneous injection. Since tolerance develops on repeated injection(3,4), in-

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dividual animals were tested only once. The susceptibility of treated animals to grade 5 and grades 3 and 4 seizure patterns was tested by electroshock stimuli of 100 m. amps. and 60 m. amps. respectively, applied for 0.2 sec. by corneal electrodes. These stimuli were approximately twice threshold intensity. A current of 60 m. amps. was used also to test for grades 1 and 2 minor seizure responses in treated animals. With these stimuli, a seizure response of a grade not less than that under investigation would be obtained in 99% of control animals untreated with acetazolamide. In a preliminary experiment, the anticonvulsant activity of acetazolamide in doses between one and 1000 mg/kg was observed in five groups of animals tested at an interval of one and one-half hours after administration of the drug. Since the approximate doses required to protect rats against the various grades of seizure patterns varied widely and since it was necessary to give solutions of different concentration, the time of peak anticonvulsant effect was determined separately at two dose levels of 5 and 400 mg/kg. In each instance, 8 groups of 4 animals were tested at intervals of one half-hour for a period of 4 hours after receiving the drug.

To compare the relative potency of acetazolamide against the various seizure patterns, the effects of graded doses were determined in a total of 65 animals at the times of peak effect, until points were established between complete and no protection. For each seizure pattern of grades 2 to 5, results obtained were plotted on logarithmic probability paper, and a regression line fitted to the plotted points by eye. From these plotted data, values for ED_{50} were determined and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon(5). The effect of acetazolamide on seizure patterns of rats aged 12 to 14 days and weighing between 15 and 20 g was studied also. Five groups of 4 animals were given acetazolamide sodium by intraperitoneal injection in doses of 10, 50, 100, 200, and 500 mg/kg in a volume of 0.2 to 0.5 ml. A control group received an equal volume of 0.85% sodium chloride solution. All animals were tested with an electroshock stimulus of 50 m. amps. for 0.2 sec. at an interval of one and one-half hours after receiving the drug.

Estimation of degree of inhibition of brain carbonic anhydrase. Rats of the same Holtzman strain, aged 29 days and weighing between 70 and 85 g, were given acetazolamide sodium by subcutaneous injection in doses of 2.5, 10, 100, and 1000 mg/kg. At times of peak anticonvulsant effect, as determined previously for both small and large dose ranges, the rats were killed by decapitation. Larger blood vessels and macroscopic blood were removed from the cerebral cortex and white matter, and the brain tissue was weighed and then homogenized with 0.85% sodium chloride solution in a dilution of 1:10. In previous experiments, enzyme activity in contaminating red cells had been estimated and found to be less than one per cent of that in the specimen of brain tissue prepared in this manner. After centrifugation, the carbonic anhydrase activity of the supernatant saline extract was estimated by the manometric method of Meldrum and Roughton(6). All observations were made at atmospheric pressure and at 0°C. The rate of evolution of carbon dioxide was corrected for the limiting effect of diffusion through the gas-liquid interface(7) and the concentration of active enzyme was calculated from the corrected rate. Three animals were tested at each dose level of acetazolamide, and the amount of free enzyme [E] in the brain extracts prepared from these animals was compared with the total enzyme activity [ΣE] in brain extracts prepared from 6 control untreated animals. The concentrations of inhibitor in the reaction vessel and in the undiluted brain tissue and the degree of enzyme inhibition at body temperature were then calculated, the dissociation constants for acetazolamide at 0°C and at 38°C being employed(1,8). The concentration of inhibitor was referred to brain water which, in rats aged 28-32 days, is 80% of the weight of the whole brain(2). The inhibition of carbonic anhydrase by some sulfonamide inhibitors has been studied by Davenport(9). The mass action expression for the reaction between carbonic anhydrase and its inhibitors is $\frac{[E]\,([\Sigma I]-[EI])}{[EI]} = K$ where [E] is the enzyme concentration in presence of inhibi-

tor, [ΣI] the total concentration of inhibitor in the reaction mixture, [EI] the concentration of enzyme-inhibitor complex, and K the dissociation constant. In calculations of the concentration of inhibitor in the brain sample, the assumption that the mass action equation is valid in the body tissues is implicit. [E] and [EI] were measured in terms of enzyme activity; since purified carbonic anhydrase was unobtainable, the absolute value of the enzyme unit could not be defined, and it was necessary to regard the expression ([ΣI] – [EI]) as equal to [ΣI]. However, with acetazolamide, a strong inhibitor, the amount bound to the enzyme may not be negligible with respect to the total inhibitor concentration and, by this indirect method of calculation, a degree of error may be unavoidable. For this reason, the concentrations of inhibitor in the brain extracts were estimated directly by a biological assay for comparison with the values calculated indirectly. The brain extracts were heated to 100°C in a boiling water bath for 5 min. to destroy the enzyme, and a known volume of the extract which contained inhibitor was added to a known amount of brain carbonic anhydrase in the reaction vessel. From the percentage inhibition of enzyme observed at 0°C, the concentration of acetazolamide in the vessel was read from the data in Fig. 1, in which percentage enzyme inhibition is plotted against total inhibitor concentration. The concentration of inhibitor in the brain was calculated and, by use of the appropriate constant for acetazolamide, the percentage inhibition of brain carbonic anhydrase at body temperature was then estimated. Thus, 2 independent measurements of inhibitor concentration in the brain were utilized in the determination of percentage inhibition of the enzyme at 4 different dose levels of acetazolamide.

Results. Anticonvulsant effects of acetazolamide. In rats aged 28 to 32 days, the time of peak anticonvulsant effect of acetazolamide sodium varied with the dose. At 5 and 400 mg/kg, the times of peak effect were one hour and 2 hours respectively. The values for ED_{50} of acetazolamide against the various grades of electroshock seizure patterns are shown in Table I, with 95% confidence limits in parentheses. The seizure response of each individual animal treated with acetazolamide is recorded in Fig. 2.

While the hind-limb tonic extensor component of the grade 5 seizure was abolished by very small doses of acetazolamide, large doses were required to protect animals from tonic flexion or extension of the fore-limbs (grade 4 seizures), from generalized severe clonus and loss of posture (grade 3), and

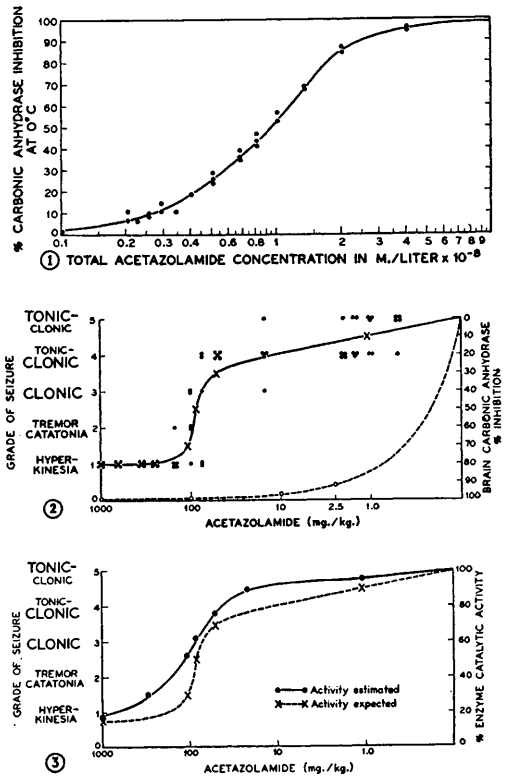

FIG. 1. Inhibition of carbonic anhydrase by acetazolamide at 0°C.

FIG. 2. Seizure patterns of rats aged 29 days and percentage inhibition of brain carbonic anhydrase in relation to dose of acetazolamide sodium. Points represent the seizure responses of individual test animals, and crosses, the values of the ED_{50} of acetazolamide for various seizure patterns.

FIG. 3. Susceptibility to seizure patterns in relation to functional activity of carbonic anhydrase in the brain of young rats. ○——○, mean values calculated from percent fractional enzyme activity observed in presence of acetazolamide and from the data of Davenport, H. W.(9). ×---×, mean values determined from enzyme activity observed in relation to development of susceptibility to seizure patterns.

TABLE I. Percentage Inhibition of Brain Carbonic Anhydrase and of the Reaction Catalyzed by the Enzyme in Rats Protected from Seizure Patterns by Acetazolamide.

100% susceptibility to seizures				50% protection from seizures			
Electroshock seizure pattern		Brain carbonic anhydrase		Inhibition of enzyme expected,*	ED_{50} acetazolamide in rats with 125 units enzyme,	Inhibition of enzyme estimated,	Inhibition of catalyzed reaction calculated,†
Grade	Type	Units	%	%	mg/kg	%	%
5	TONIC-Clonic	125	100	10	1.1(0.7– 1.8)	86.0 (80 –90)	5 (4– 6)
4	Tonic-CLONIC	100	80	30	51 (32 – 81)	99.0 (98.6–99.4)	24 (16–36)
3	CLONIC	75	60	50	86 (61 –120)	99.4 (99.2–99.6)	38 (28–52)
2	Tremor-Catatonia	50	40	70	107 (79 –145)	99.6 (99.4–99.7)	48 (36–58)
1	Hyperkinesia‡	20	16	84		99.98	83

* The reciprocal of the level of enzyme activity observed in relation to development of susceptibility to seizure patterns. (Confidence limits not calculated owing to small No. of samples at individual levels of seizure activity.)

† Calculated from percent enzyme inhibition observed in presence of acetazolamide and from the data of Davenport, H. W.(9). Mean values, with 95% confidence limits in parentheses.

‡ Not modified by acetazolamide, 1000 mg/kg, and 99.98% inhibition of enzyme.

from the tremor and catatonia of grade 2 seizures. Grade 1 electroshock seizure patterns, which consist of violent hopping and running movements but without loss of posture, were refractory to acetazolamide, even in doses up to 1 g/kg.

In rats aged 14 days, the electroshock seizure patterns of acetazolamide-treated animals were not significantly different from those of control animals. The animals in all groups responded with a grade 1 hyperkinetic seizure pattern.

Relation of seizure pattern to brain carbonic anhydrase. The percentage inhibition of brain carbonic anhydrase at body temperature, shown in Table II, was calculated from the concentration of acetazolamide in the brain, estimated by the two methods described.

By direct assay, the degree of enzyme inhibition at 0°C was lower than that observed *in vitro* by the indirect determination. As possible explanations for this discrepancy of results, either the acetazolamide had been destroyed partially by boiling or, in the absence of enzyme, the inhibitor had become adsorbed on the deposit of the centrifuged brain extract. The higher values calculated by the indirect method were therefore accepted as more nearly correct than those obtained by direct bioassay.

In Fig. 2, the degree of inhibition of brain carbonic anhydrase and the modified seizure responses of 29-day-old rats are plotted in re-

TABLE II. Inhibition of Brain Carbonic Anhydrase in Relation to Dose and Brain Concentration of Acetazolamide.

	Indirect assay				Direct assay			
Dose of acetazolamide, mg/kg	Enzyme inhibition *(in vitro)* % at 0°C	Conc. of acetazolamide, M/l × 10^{-7}	Enzyme inhibition *(in vivo)* % at 38°C		Enzyme inhibition *(in vitro)* % at 0°C	Conc. of acetazolamide, M/l × 10^{-7}	Enzyme inhibition *(in vivo)* % at 38°C	
2.5	33	13	92.4		12	8	88.5	
	32	12	92.0	92.4	6	6	84.1	87.0
	34	13	92.8		12	8	88.5	
10	49	25	95.8		27	14	93.2	
	70	60	98.3	97.1	19	11	91.6	92.3
	59	38	97.2		22	12	92.3	
100	89	204	99.5		82	50	97.9	
	90	239	99.6	99.5	81	45	97.8	97.8
	90	226	99.5		76	45	97.7	
1000	99.3	3444	99.97		99.7	>275	99.6	
	99.5	6690	99.98	99.98	99.5	"	99.6	>99.6
	99.9	25190	99.99		99.2	"	99.6	

lation to acetazolamide at various dose levels. Table I shows the percentage inhibition of carbonic anhydrase in the brain in relation to the grade of seizure pattern observed in animals treated with acetazolamide. When 86% of the brain enzyme was inhibited, grade 5 tonic seizures were prevented in 50% of animals tested; with 99.4% inhibition, grade 3 clonic seizures were abolished in a similar proportion of animals. The grade 1 hyperkinetic seizure pattern was not controlled despite the inhibition of brain enzyme by 99.98%.

Discussion. In a previous study(2), the development of susceptibility to various seizure patterns has been correlated with the concentration of carbonic anhydrase in the brain of rats between 1 and 35 days of age. In this age group, rats of 25 to 35 days have the maximal degree of enzyme activity, equivalent to a mean value of 125 units/g/wet weight of brain; and this 100% enzyme activity is correlated with complete susceptibility to the grade 5 maximal seizure pattern. The lower levels of enzyme activity which correspond with susceptibility to less severe grades of seizure patterns are recorded in Table I. From these data, the percentage inhibition of enzyme activity expected to correspond with 50% protection from each seizure pattern has been calculated. For example, when 50% of animals are protected from the grade 5 tonic seizure, the expected degree of enzyme inhibition would be 10%. However, in response to the ED_{50} of acetazolamide for this seizure pattern, the degree of enzyme inhibition observed was approximately 86%.

While the percentage inhibition of carbonic anhydrase was inversely related to the severity of the modified seizure patterns, the degree of inhibition of the enzyme necessary to afford protection from seizures was much greater than that expected from estimations based on developmental studies of enzyme activity and seizure susceptibility. In addition, in rats treated with acetazolamide in doses up to 1 g/kg, the grade 1 hyperkinetic seizure pattern was completely refractory, despite the inhibition of brain carbonic anhydrase to a level of 0.02% of normal.

Observations by Davenport(9) have shown that the inhibition of the hydration or dehydration of carbon dioxide and of the catalyzed reaction is not proportional to the inhibition of carbonic anhydrase. The rate of uptake of carbon dioxide by blood was studied in the presence of thiophene-2-sulfonamide and sulfanilamide. It was reported that in order to reduce the rate of the catalyzed reaction by 10% at 0°C the carbonic anhydrase was inhibited by more than 98%; and at body temperature, the concentration of inhibitor required to effect this degree of inhibition would be much higher. From a plot of these data and the percentage inhibition of brain carbonic anhydrase observed in response to acetazolamide, the corresponding values for the degree of inhibition of the reaction catalyzed by the enzyme were calculated and recorded in Table I. The reciprocals of these values, or the levels of functional activity of the enzyme which are correlated with susceptibility to the various grades of seizure patterns, are shown in Fig. 3.

That the functional activity of the enzyme observed in animals treated with acetazolamide is of a similar order of magnitude to that expected from estimations based on developmental studies of enzyme activity is suggested by the comparable distributions of the mean values obtained. A more exact correlation may have been observed had the estimations of the rate of the catalyzed reaction been possible in rats of all age groups between 1 and 35 days.

Thus, it is possible to reconcile the apparent discrepancy between the degree of enzyme inhibition observed in relation to suppression of various seizure patterns by acetazolamide and that expected from the known levels of brain enzyme which are correlated with development of seizure susceptibility. In addition, the failure of acetazolamide and a high degree of enzyme inhibition to control the grade 1 hyperkinetic seizure pattern may be explained by the persistence of the reaction catalyzed by brain carbonic anhydrase at a rate sufficient for the propagation of a minor seizure discharge. The functional significance of brain carbonic anhydrase in the mechanism of the seizure process and in the development of susceptibility to seizure patterns in young animals is strongly suggested by the data pre-

sented.

Summary. The relation between activity of brain carbonic anhydrase and susceptibility to various grades of seizure patterns has been studied in young rats by observations of the effects of acetazolamide. The percentage inhibition of carbonic anhydrase and of the reaction catalyzed by the enzyme is related to the suppression of tonic and clonic seizure patterns. It is suggested that brain carbonic anhydrase and its specific catalytic activity may be essential for the propagation of the seizure discharge. Low levels of enzyme activity may be sufficient for focal discharge and the induction of minor seizure patterns while a relatively high degree of activity may be required for the propagation of a generalized seizure discharge and the induction of a major tonic seizure.

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Tetramethylrhodamine as Immunohistochemical Fluorescent Label in the Study of Chronic Thyroiditis.* (23822)

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Since development by Coons and his coworkers(1) of the fluorescent immunohistochemical technic using fluorescein groups as the fluorescent label, there has been interest in developing fluorescent labels of a color other than the fluorescein apple green. Thus, Clayton(2) reported labelling antibodies with 1-dimethylaminonaphthalene-5-sulfanyl chloride and nuclear fast red to produce yellow and red fluorescence, respectively. Silverstein(3) recently reported that tetraethylrhodamine can be coupled through ureido groups to protein to yield a label which fluoresces orange in ultraviolet light. We had been working on the fluorescent label problem also and had prepared the tetramethylrhodamine isocyanate which when coupled to protein fluoresces orange. We are reporting the use of this label in a study of the serum components associated specifically with chronic thyroiditis (Hashimoto's disease). Roitt, Doniach, Campbell and Hudson(4) reported that serum of patients with Hashimoto's disease contains material capable of precipitating with human thyroid extract. Witebsky, Rose, Terplan, Paine and Egan(5) have shown that serum of patients with chronic thyroiditis agglutinates tanned sheep red cells coated with human thyroid extract. Previously, Witebsky and Rose(6,7) had shown that thyroiditis similar to that in humans can be produced in rabbits by injecting animals with thyroid extract, either from same species, or from a portion of individual's own thyroid.† From these observations, it has been postulated that chronic thyroiditis in humans may be the result of an auto-immunization process in which an individual produces antibodies against tissue components in his own thyroid. The se-

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† Beutner, Witebsky, Rose and Gerbasi(8) have shown that rabbit thyroid is stained by fluorescein labelled sera from rabbits injected with rabbit thyroid.