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ELEVATION OF γ-AMINOBUTYRIC ACID IN BRAIN WITH AMINO-OXYACETIC ACID AND SUSCEPTIBILITY TO CONVULSIVE SEIZURES IN MICE: A QUANTITATIVE RE-EVALUATION*

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Abstract—The time courses of changes in brain content of γ-aminobutyric acid (γABA) and sensitivity to electroconvulsive shock were studied in mice after administration of amino-oxyacetic acid (AOAA), an inhibitor of γABA-transaminase. Some correlative observations were made of ATP and glutamate contents and of susceptibility to seizures induced by strychnine, pentylenetetrazole (Metrazol), and picrotoxin. The increase in γABA concentrations as a function of time after AOAA administration (25 mg/kg) was biphasic. A plateau in γABA level was attained between 3 and 4 hr, and a subsequent secondary rise took place between 4 and 6 hr. Elevated values were still observed at the 24-hr period. No changes in ATP content were found after AOAA. There was a remarkable decrease in electroshock seizure incidence (75 mA stimulus) during the first 1·5 hr after AOAA. Subsequently, the susceptibility to seizures began to return to normal, attaining the control values at 6 hr, at which time the γABA content was maximal. Only during the first 1·5-hr period after AOAA was there a good correlation of decrease in seizure susceptibility with increase in γABA content. Similar findings were made on a more limited scale with regard to the parameters measured when chemical convulsants were employed. One of several possibilities suggested by the findings is that the increases in γABA levels and changes in seizure susceptibility after AOAA administration are completely unrelated. Another way to view the results is that soon after γABA-transaminase blockade with AOAA the increased levels of γABA may, indeed, decrease neuronal excitability; however, compensatory increases in excitatory influences or decreases in inhibitory influences other than γABA, or both, may take place with the consequent restoration of normal neuronal sensitivity even during the time when a gross elevation of γABA exists. Glutamic acid, a potentially important excitatory factor in the central nervous system, did not increase after AOAA.

WORK in our laboratory and in many others is concerned with studies of the physiological and metabolic relationships of γ-aminobutyric acid (γABA), an easily extractable, simple substance with a unique occurrence in the central nervous system (CNS) of vertebrate organisms, in which it is found in extracts of brain, spinal cord, and retina (see Refs. 1–9 for pertinent reviews). In the CNS, there are enzymes both for the formation of γABA from glutamic acid and for its utilization by transamination and subsequent oxidation of the succinic semialdehyde formed via the

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tricarboxylic acid cycle. Presumptive evidence strongly favors the view that γABA may be an inhibitory transmitter in *Crustacea*, where it has been shown to increase the stability of both pre- and postsynaptic membranes.[10–12] Findings from a number of directions have suggested that γABA may be importantly involved in some aspects of the control of neuronal excitability in the vertebrate CNS.[1–9, 13–15]

Previous results have indicated clearly that a general depression of γABA levels is not a necessary requirement for the induction of seizures by thiosemicarbazide (TSC) or chemically related substances.[16–21] Elevations of γABA by γABA-transaminase blockade with NH_2OH and amino-oxyacetic acid have been correlated with the occurrence of a decrease in sensitivity to stimulation by several measurable criteria (see Refs. 2 and 3). However, in one instance, it was found that the γABA level in brains of rats attained maximal values at 6–8 hr after injection of amino-oxyacetic acid (AOAA), while the optimal protection against the lethality of TSC seizures was attained much earlier and had fallen off markedly at 6 hr.[20] In another study, although a marked elevation in γABA levels of rat brain was produced by the injection of hydrazine, no protection against electrically-induced seizures was found at 12 hr after hydrazine administration, the time at which maximal levels of γABA were observed.[21] In neither of the latter studies was an extensive series of observations made of both the levels of γABA and of some measure of neuronal excitability as a function of time at early times after administration of the γABA-elevating substance. In view of the possibility that, with time, compensatory mechanisms could come into play after production of a large change in content of normally occurring neuroactive substance, the present study was undertaken to obtain accurate quantitative data in mice of the time course of changes of γABA content and sensitivity to electroconvulsive shock after administration of AOAA. Some correlative observations also were made of γABA content in brain and sensitivity to seizures induced by strychnine, pentylenetetrazole, and picrotoxin.

METHODS

Handling of animals. Swiss albino male mice of an inbred stock, approximately 25 g, were used in all experiments. The animals were fasted for 18 hr prior to use, since preliminary results indicated that more uniform results were obtained in this manner. All injected drugs were dissolved in physiological saline and the pH adjusted to 7·0 immediately before use. Volumes of 0·1 ml of each drug solution were injected i.p., and control mice received the same amount of physiological saline. The doses of drugs employed are noted in the description of the individual experiment. Drug-treated or electrically-stimulated animals surviving an experiment were never used again.

Handling of brain-tissue samples. Mice were sacrificed by rapid freezing in liquid nitrogen or by decapitation. For γABA and adenosine triphosphate (ATP) assays in whole brain, the former technique was employed. The mice were placed in an especially designed wire-mesh cage which was dropped directly into a Dewar flask containing liquid nitrogen. The mice were placed in the cage at least 15 min prior to sacrifice in order to allow for a period of accommodation. After complete freezing of the whole mice, they were decapitated and whole brain minus the cerebellum and lower brain stem (medulla oblongata and pons) was extirpated in the cold room with cold

instruments. The tissue was weighed rapidly while completely frozen and was homogenized quickly in 10 volumes of 75% ethanol. In another group of experiments γABA assays were performed on brains of mice decapitated in the usual fashion. The heads were dropped immediately into an ice bath, and dissection of the brain into cerebral hemispheres and brain stem was performed quickly on ice. After dissection each piece of tissue was transferred immediately into an acetone–dry ice bath and frozen completely. The frozen tissue was weighed rapidly and homogenized with 10 volumes of 75% ethanol solution.

Determination of γABA content. All analyses were performed on individual mouse brains or portions thereof. After homogenizing in 75% ethanol, these samples were centrifuged at 3,500 rpm for 15 min at 0° in a refrigerated centrifuge. The pellet was washed twice with 75% ethanol. All the supernatants from a particular sample were combined in a beaker and evaporated to dryness under an infrared lamp in a steady stream of dry air. The residue was suspended completely in distilled water and mixed with one fifth its volume of chloroform. After complete emulsification was achieved wih vigorous mixing, the mixture was centrifuged at 3,500 rpm for 15 min and clarification achieved. These supernatants were used directly for γABA assay. The γABA content was determined enzymatically as previously described.[22] This procedure always gave recoveries of 95–100% of known amounts of γABA added to brain homogenates.

Glutamic acid determinations. The brain samples frozen in liquid nitrogen were homogenized in 0·02 N HCl, rapidly inactivated by placing the homogenizer in a boiling water bath for 5 min, and centrifuged at 100,000 *g* for 30 min in a Spinco model L centrifuge. Half the supernatant was adjusted to pH 8·3, and the γABA content was determined as previously described after clarification by centrifugation at 3,500 rpm for 15 min. The other half was adjusted to pH 4·25, similarly centrifuged, and employed for glutamic acid determination by the enzymatic decarboxylase method (*Escherichia coli* bacterial powder Type 1; Sigma). The bacterial enzyme was homogenized in 0·02 M acetate buffer, pH 4·25, so that the final suspension contained 1 mg of the bacterial powder/ml. One-ml aliquots of the enzyme suspension and of the above extract were mixed and incubated at 37° for 1 hr anaerobically. The mixture was inactivated in a boiling water bath for 5 min and then centrifuged at 3,500 rpm for 15 min. The supernatant was poured off, the pH adjusted to 8·3, and the total γABA content measured by the usual method. The glutamic acid content of the extracts was calculated from the difference between the γABA content of the original extract and that found after the conversion of the glutamic acid to γABA by the decarboxylase. The recoveries of added glutamic acid by this method averaged about 95% (range 93–97%). This was the same as that achieved with the bacterial enzyme by the less sensitive Warburg technique.

Determination of ATP content. Brain tissue frozen by the liquid nitrogen procedure was immersed in 2 ml of ice cold 10% trichloroacetic acid and homogenized quickly. The homogenate was centrifuged in the cold and the supernatant treated three times with 6 ml of cold, water-saturated ether. After the final ether extraction, the remaining ether was bubbled off by gently shaking the test tube in a water bath at 75–80°. The final solution was used directly for ATP determination. ATP was measured

enzymatically as described in Boehringer article no. 15979 based on an unpublished method of Bucher and Schuart; 0·2-ml aliquots of the samples were added to a reaction mixture containing 2·4 ml of solution with triethanolamine buffer (pH 7·6, 4×10^{-3} M $MgSO_4$ and 6×10^{-3} M 3-phospho-D-glycerate to which had been added 0·04 ml of $1{\cdot}2 \times 10^{-2}$ M NADH. To the cuvette was added 0·04 ml of an enzyme mixture containing 4 mg glyceraldehyde-3-phosphate dehydrogenase/ml and 1 mg of 3-phosphoglycerate kinase/ml. Readings were taken in the Zeiss spectrophotometer at 340 mμ. Under these conditions there is a total decrease in NADH content which is isomolar with the content of ATP in the extract according to the following total equation:

$$\text{3-phospho-D-glycerate} + \text{ATP} + \text{NADH} + \text{H}^+ \longrightarrow \text{glyceraldehyde-3-phosphate} + \text{ADP} + \text{NAD}^+$$

Quantitative recoveries of known amounts of ATP were achieved by this method.

Electroconvulsive seizures. A pulse train consisting of 12 pulses at a frequency of 60 cycles/sec (60 cycles/sec for 0·2-sec duration) was employed as stimulus. The pulses were generated by a Grass S-4 type stimulator. Current measurements were performed with a dual beam cathode ray oscilloscope (type 502, Tektronix, Inc.) with which the current was measured by the minute voltage drop across a low ohm resistor connected in series with the electrode. Bipolar point silver electrodes (polar distance 3·5 mm) were applied to the skull (parietal median, pre-auricular line). Anticonvulsant efficacy of a treatment was measured by the supramaximal electrical seizure threshold technique[23] with the modification for mice introduced by Swinyard *et al.*[24] By the above method, the maximal electrical shock threshold for 16 control mice was found to be $61{\cdot}5 \pm 6$ mA. Current intensities are specified for each experiment.

Separation of nerve-ending particles and determination of glutamic decarboxylase (GAD) activity. Nerve-ending particles were prepared according to a procedure developed by Whittaker *et al.*[25] The P_2-B fraction (containing synaptosomes and some microsomes) was diluted with an equal volume of water and centrifuged at 78,500 *g* for 20 min. The contents of the pellet were liberated by suspension in water containing 0·01 % Triton X-100 and the GAD activity was measured by the isotopic procedure of Roberts and Simonsen.[26] Activity was expressed in micromoles γABA produced per hr per g protein.

RESULTS AND DISCUSSION

Comparison of γABA contents in brain samples prepared by different methods. Prior to determining the γABA contents of mouse brains under various experimental conditions it was of interest to determine whether or not significant differences would be obtained when the experimental mice were treated in different ways prior to removal of the brain. The data in Table 1 show that the lowest values for the γABA content were obtained in brains of animals frozen whole in liquid nitrogen prior to removal of the brain in the frozen state. Somewhat higher values were found in the brains of animals which were decapitated prior to freezing the head in liquid nitrogen or in those which were decapitated and the dissection performed in ice prior to the freezing of the brain in acetone–dry ice mixture. However, the values in the immediately frozen animals were not significantly different from those of the other two groups. Other

TABLE 1. INFLUENCE OF METHOD OF PREPARATION ON γABA CONTENT OF MOUSE BRAIN

Method of preparation	Number of mice	γABA Content (μmoles/g ± S.D.)
Whole mouse frozen	12	2·60 ± 0 27
Head frozen after decapitation	7	2·91 ± 0·30
Brain frozen after decapitation and dissection	12	3·01 ± 0·43

workers reported increases in γABA to have taken place when animals were not frozen in liquid nitrogen.[27–29] In one such study no further increase, beyond the initial rapid one, was noted when brains were maintained subsequently at 0°.[29] It has been reported that by employing a near-freezing method, results were obtained for the content of acetylcholine in brain which were similar to those found by complete freezing in liquid nitrogen.[30] The results for γABA content of mouse brain found in the present study are similar to those previously reported for mouse brain.[31] With the exception of the study in which brain stem and cerebral hemisphere were analyzed separately, all results to be reported subsequently were obtained on brains from whole frozen mice. In the case where separate analyses of brain stem and hemisphere were performed, the dissection was done prior to freezing the brain in acetone–dry ice mixture.

γABA and ATP contents in brains of mice at various times after administration of AOAA. In Fig. 1. are shown the values of γABA and ATP contents of whole brains of mice as a function of time after the i.p. injection of 25 mg AOAA/kg. Results also are shown for an experiment in which the brain stems and cerebral hemispheres of mice were analyzed separately (Fig. 2). Control values were obtained in saline-injected mice at several times after the injection of saline. This was necessary since the animals had been starved for 18 hr prior to the beginning of the experiment and were not fed subsequently. The period of inanition did not result in significant changes in the variables measured in the controls. In both the whole brains and dissected brain samples the increase in γABA content as a function of time after AOAA administration was biphasic. A plateau had been attained between 3 and 4 hr, and a subsequent secondary rise took place between 4 and 6 hr. Maximal γABA values were reached at 6 hr. The levels of γABA then returned at 8 hr to those found at the 3–4 hr intervals, and this high content of γABA was maintained for a long period, only a small decrease being noted at 14 hr. The γABA level of whole brain was still elevated at the 24-hr period, a finding consistent with the previously reported prolonged elevation of γABA[31, 32] and depression of γABA transaminase activity[32] found after administration of AOAA. However, none of the preceding studies was sufficiently detailed to show the dual nature of the γABA rise after the injection of AOAA. Although the control levels of γABA in the brain stem (3·54 ± 0·35 μmoles/g) were significantly ($P < 0{\cdot}01$) higher than those of the cerebral hemispheres (2·37 ± 0·30 μmoles/g) (Fig. 2), it is interesting that after the administration of AOAA the γABA contents changed in a parallel manner, and at no time were the differences between the mean values for the two brain areas statistically significant.

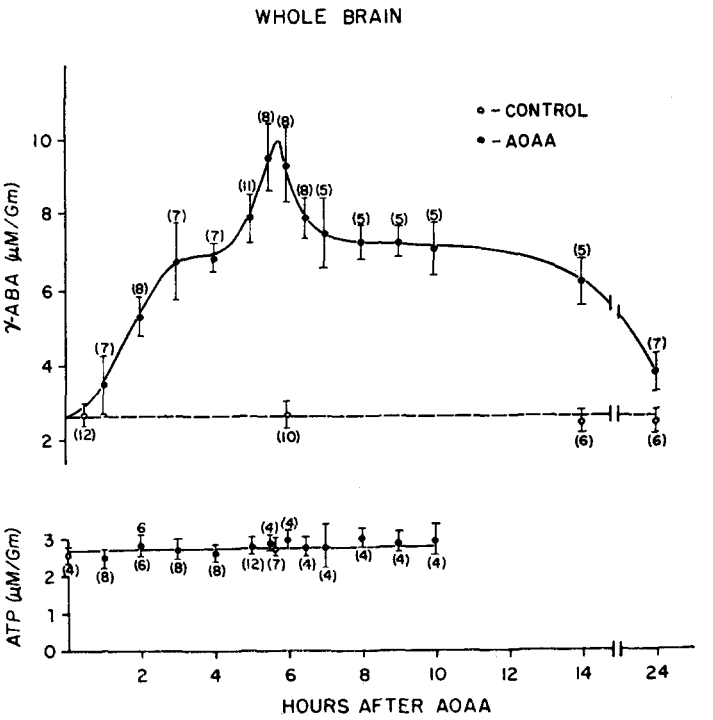

FIG. 1. γABA and ATP contents of whole brains of mice as a function of time after the i.p. injection of 25 mg amino-oxyacetic acid (AOAA) per kg.

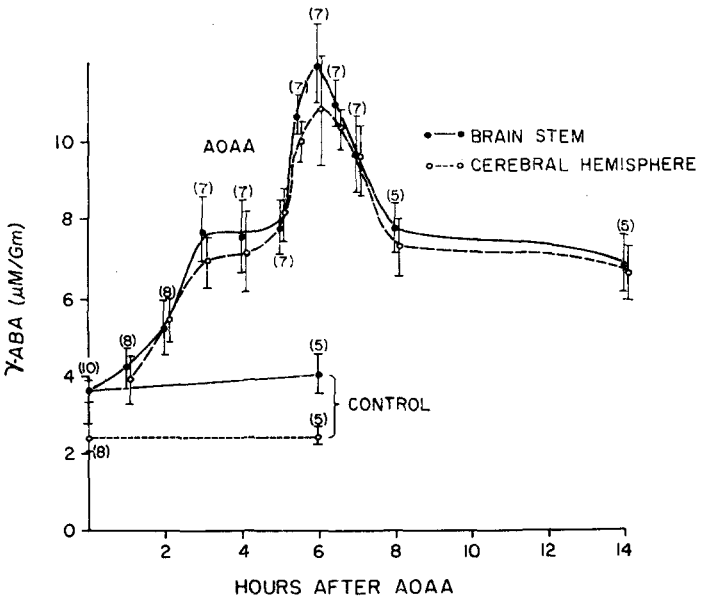

FIG. 2. γABA content of brain stem and cerebral hemispheres as a function of time after the i.p. injection of 25 mg AOAA/kg.

Contents of ATP in brain were determined at various times of injection of AOAA in a separate experiment (Fig. 1). At no time after the injection of AOAA were significant changes noted in the ATP levels. This is particularly interesting in view of the possibility suggested that the physiological effects observed on blocking the operation of the γABA shunt by inhibiting the γABA transaminase might be intimately related to the decreased contribution of this pathway to energy metabolism. It has been estimated that the γABA shunt might account for as much as 40% of the oxidative metabolism of brain and suggested that the energy thus derived might be important for neuronal function.[33] If this were the case, it might have been expected that extensive blockade of this pathway, as indicated by the marked elevation of γABA, would have resulted in a decrease in ATP content of the brain at some time during the course of the measurements. The negative findings suggest either that *in vivo* the γABA pathway might be relatively insignificant quantitatively with regard to maintenance of the ATP level in whole brain or, that when this pathway is blocked, compensatory changes can take place in other ATP-generating or utilizing pathways so that the ATP levels in brain remain constant.

Time course of susceptibility to electroconvulsive seizures after AOAA administration. In order to choose a suitable electrical stimulus for testing seizure susceptibility as a function of time after administration of AOAA, experiments were performed with control animals in which the occurrence of tonic convulsions was observed at various current intensities. Measurements were made at 25, 50, 60, 75, 100, and 125 mA, respectively. The control curve (starved, saline-injected mice) shown in Fig. 3 is a typical dose–response curve. The stimulus intensity chosen for routine testing was

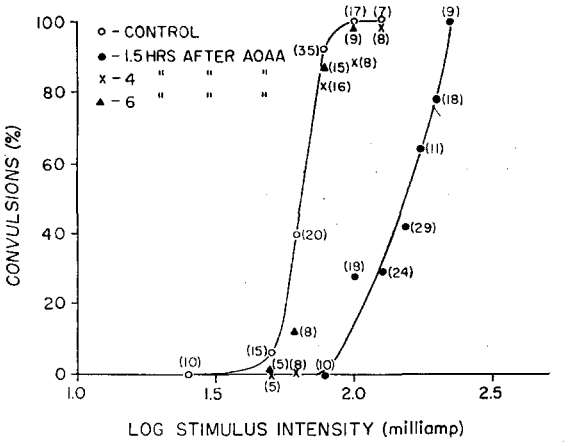

FIG. 3. Seizure susceptibility as a function of current before and at several times after AOAA administration.

75 mA, which in further tests with approximately 100 mice gave an incidence of tonic seizures of 96%. An experiment was then performed in which the incidence of seizures with the 75-mA stimulus was observed at different times after the administration of 25 mg AOAA/kg (Fig. 4). There was a remarkable decrease in seizure incidence during the first 1·5 hr, complete protection being observed at 1·25 hr. Subsequently, the

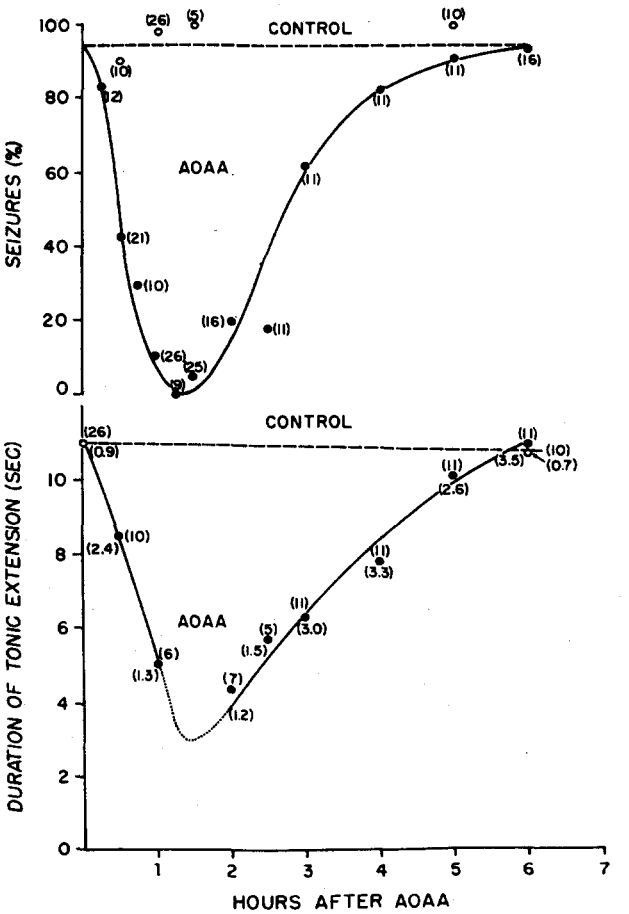

FIG. 4. Incidence of seizures with a 75 mA stimulus at different times after the administration of 25 mg AOAA/kg. The lower curve shows the duration of the tonic extensions in the animals which had convulsions at the various time intervals; the upper numbers associated with the points on the curve show the numbers of animals, and the lower numbers are the standard deviations of the means.

susceptibility to seizures began to return to normal along an S-shaped curve, attaining the control value at 6 hr. In animals having seizures, measurements were made in some instances of the duration of the tonic extension. There were reductions in the duration of the tonic extensions in animals having seizures at the times when the total incidence of seizures was decreased (Fig. 4), the curves showing the same general trends. The P-values for the differences from the controls were less than 0·03 for the 0·5- and 4-hr intervals and less than 0·01 for the intervals of 1, 1·5, 2, 2·5 and 3 hr. Thus, the tests revealed not only that the susceptibility to seizures among the treated mice was decreased during the first 4 hr after administration of AOAA but also that the severity of the seizures in the susceptible mice was reduced.

A complete dose–response curve was then derived (Fig. 3) for mice at 1·5 hr after AOAA, the period of minimal seizure susceptibility. The stimulus intensities employed were 75, 100, 125, 150, 175, 200, and 225 mA. Seizures were induced in all animals tested only at the highest stimulus intensity employed The shape of the dose–response

curve was not the same as that of the control curve. While a plot (not shown) of the distribution of the values for the control animals showed the perfect symmetry of a normal distribution curve, that of the curve for the 1·5-hr values did not. Measurements made at 4 hr after AOAA still gave values for seizure incidence below the control levels at stimulus intensities of 50, 60, 75, and 100 mA (Fig. 3), while those made at 6 hr were closer to those of the controls. Thus, not only did the response of the AOAA-treated animals to a 75-mA stimulus return to normal in 6 hr but the responsivity to a range of stimuli, from completely ineffective to completely effective, also returned essentially to normal in that period.

It is not possible at this time to interpret with certainty the change in shape from the normal of the dose–response curve at the 1·5-hr period. When a stimulus reaches sufficient intensity to cause a seizure, there has been a destruction of the effectiveness of the systems of checks and balances that exist between inhibitory and excitatory phenomena at synapses in key neural circuits, thus allowing uncoordinated excitation to take place. Since in the control animals the susceptibility to seizures is distributed strictly along a normal distribution curve, it is likely that one property (neuronal, glial, or circulatory; membrane structural or metabolic) becomes limiting when seizures occur. If the same property were to remain limiting after a protective treatment, then a complete dose–response curve should still give the same shape as the control curve but should be displaced toward higher stimulus values. One possibility that the present data suggest is that during maximal protection after AOAA administration, a different property (or properties) becomes rate-limiting in the response to the electrical stimulus.

Correlation of γABA content with seizure susceptibility for the first 1·5 *hr after AOAA; lack of correlation thereafter.* In Fig. 5 is shown a three-dimensional plot in which seizure incidence and whole-brain γABA contents (as per cent of control values) are plotted as a function of time for 6 hr after administration of AOAA. Only during the first 1·5-hr period was there a correlation of decrease in seizure susceptibility with increase in γABA content. Thereafter, the seizure susceptibility increased while the γABA content continued to rise. One of the possibilities suggested by the above data is that the increases in γABA levels and changes in seizure susceptibility after administration of AOAA are completely unrelated. Another possibility is that a relationship does exist but that it is localized to a particular key brain area. A number of studies[34–36] point to the midbrain reticular activating system as playing a key role in seizures of various types. However, our analyses of grossly dissected portions of the midbrain region gave the same type of result that obtained for whole brain or cerebral hemispheres. It is also possible to suggest that determinations of contents of neuroactive substances in whole brain or portions thereof may have little physiological meaning, since such determinations may give different results from those performed on neural regions from which such substances are liberated during function in the CNS. This latter point is now being further approached in our laboratory by study of isolated nerve endings from normal and AOAA-treated mice, with regard to γABA content and the activity of the enzymes involved in its metabolism.

Another way to view the problem is that γABA may, indeed, be importantly involved in decreasing neuronal excitability soon after γABA transaminase blockade with AOAA and that total-brain γABA is directly related to the physiologically

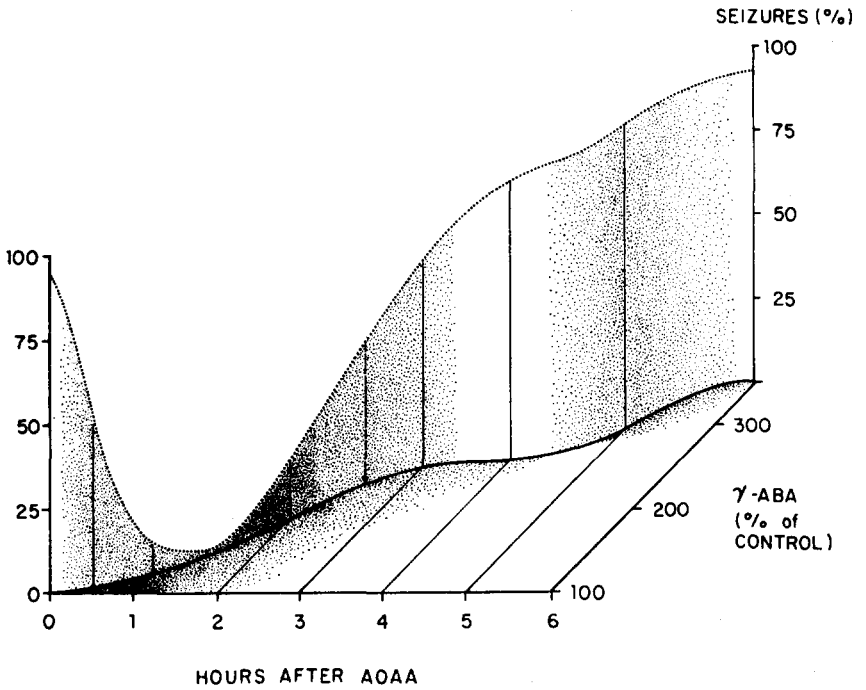

FIG. 5. Three-dimensional plot of seizure susceptibility and γABA content of brains of mice as a function of time after administration of 25 mg AOAA/kg. Arrows point away from the inflection points of the curves from which they are drawn.

effective amounts of the substance; but compensatory increases in excitatory factors or decreases in inhibitory factors other than γABA, or both, may take place with the consequent restoration of normal sensitivity to electroshock in spite of the persistence of elevations in γABA content. With this in mind it was of interest to examine the type of relationship between γABA content and protection against seizures during the first 1·5 hr after AOAA (Fig. 6). At the lower levels of γABA a linear relationship was found to exist between seizure susceptibility and brain γABA content, with the curve approaching the point of complete protection asymptotically, as would be expected to occur if some saturable inhibitory neuronal site were involved. A further look at Figs. 1, 2, 4, and 5 reveals the interesting fact that the curves depicting the fall and subsequent rise in seizure susceptibility and the various portions of the γABA curves all have the shape of typical logistic functions. The inflection point of the first portion of the γABA curve occurred at the time when the curve of seizure susceptibility began to rise toward normal, and the inflection point of the latter curve just preceded the beginning of the second rise in γABA content. This would be understandable in terms of a biochemical servomechanism in which a progressive, self-limited increase in amount of effective excitatory transmitter (increased rate of formation, decreased rate of destruction, increased sensitivity of receptor) or a progressive, self-limited decrease in effective amount of one or more inhibitory substances other than γABA would be triggered at the time when the increase in γABA is occurring at the maximal rate (inflection point); likewise, at the time of maximal rate of change of the compensatory

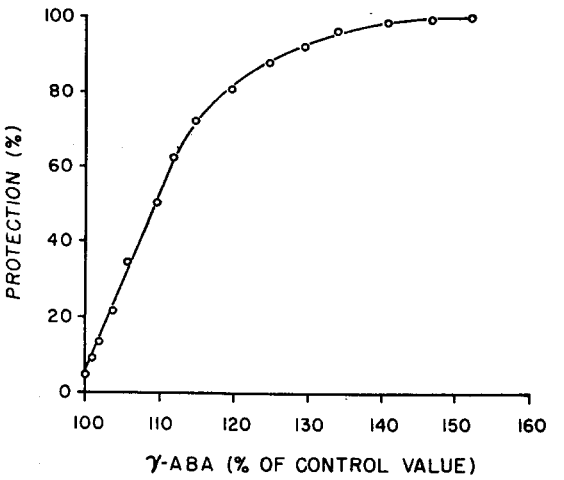

FIG. 6. Relationships between brain content of γABA and protection against seizures (75 mA current during the first 1·5-hr period after AOAA (25 mg/kg).

change a secondary rise in γABA could be induced through an increase in the rate of its formation or a decrease in the rate of utilization. At present, the most likely excitatory substances to measure would be acetylcholine and glutamic acid.

Glutamic acid content in whole brain after AOAA; effect of high concentrations of γABA on glutamic decarboxylase. It has been shown that glutamic acid has a potent excitatory effect on neurons in both invertebrate and vertebrate preparations.[13, 37, 38] The possibility is still open that glutamic acid may be an excitatory transmitter.[13, 38] It appeared worthwhile to determine the changes in glutamic acid contents in relation to those of γABA at several times after the administration to mice of AOAA (25 mg/kg). The results in Table 2 show a decrease in glutamate content from the control value at 1·5 hr ($P < 0.05$), but that the values at 4 and 6 hr were not significantly different from those of the controls. The progressive increases in γABA levels resulted in decreasing glutamic acid/γABA ratios. Thus, from the whole-brain analyses it would appear that the return to normal electroshock seizure susceptibility after

TABLE 2. γABA AND GLUTAMIC ACID CONTENTS OF MOUSE BRAIN AFTER ADMINISTRATION OF AMINO-OXYACETIC ACID (AOAA)*

Time after AOAA (hr)	No. of animals	γABA (μmoles/g ± S.D.)	Glutamic acid (μmoles/g ± S.D.)	Glutamic/γABA
0†	5	3·05 ± 0·66	15·07 ± 0·94	4·9
1·5	6	4·73 ± 0·63‡	12·82 ± 1·47§	2·7
4·0	6	7·14 ± 0·41‡	13·42 ± 1·53	1·9
6·0	6	12·7 ± 2·00§	13·46 ± 2·00	1·1

* 25 mg/kg i.p.
† Physiological saline 1·5 hr before sacrifice.
‡ $P < 0.01$ (difference from control values).
§ $P < 0.05$ (difference from controlvalues).

1·5 hr could not be attributable to increases in glutamic acid content. A uniform distribution of GAD and glutamate assumed, it was calculated from previous data[26] that the rate of γABA formation from glutamic acid at the concentration observed would be approximately 75% of the maximal potential rate attainable. It is well known that glutamic acid is compartmented in brain,[39] and recent data in our laboratory suggest that the same may be true for γABA. Thus, changes in contents of these substances in whole brain may or may not reflect changes in physiologically active loci.

The possibility was suggested that cessation of increase in γABA content (Figs. 1 and 2) in spite of prolonged inhibition of the γABA transaminase (see Ref. 32; also, unpublished data) might be a result of feedback inhibition of the GAD by γABA. The maximal levels of γABA in brains gave a concentration of 0·0127 M (Table 2), if it is assumed that the substance is free and is uniformly distributed throughout the tissue. The data in Table 3 show that significant inhibition (25%) of GAD activity

TABLE 3. INFLUENCE OF γABA ON GLUTAMIC DECARBOXYLASE ACTIVITY OF MOUSE BRAIN NERVE-ENDING PARTICLES

γABA conc.* (M)	Decarboxylase activity (μmole/hr/g protein)
	474
0·1	357
0·01	486
0·001	479
0·0001	470

* Concentration of added γABA; some γABA is associated with the isolated nerve-ending particles.

occurred at 0·1 M γABA, but that no inhibition was found at the lower concentrations employed. These results would make it unlikely that γABA, even at the maximal levels observed in brain after AOAA, would exert feedback inhibition of its own formation. However, this interpretation must be made with caution since it has been reported that efferent inhibitory axons of the lobster leg contain approximately 0·1 M γABA while the motor axons contain only 0·001 M γABA. The cell bodies of the inhibitory and motor nerves were found to have γABA contents comparable to the fibers.[40] If in the vertebrate CNS only a fraction of the total cell population were to consist of "γABA neurons," then the possibility would still be open that after AOAA the levels of γABA could reach levels high enough in specific neurons to exert significant inhibition of GAD contained in these neurons.

Effects of administration of AOAA on seizures induced by pentylenetetrazole, strychnine, or picrotoxin. Suitable doses of these three convulsant drugs were chosen to produce their typical convulsogenic effects. The same doses of drugs were tested at 1·5 and 6 hr after injection of saline or AOAA (25 mg/kg).

With pentylenetetrazole, closely similar results were obtained in the measured parameters when the drug was administered either at 1·5 or 6 hr after the injection of saline (Table 4). At 1·5 hr after AOAA there were far fewer convulsions than in

TABLE 4. EFFECT OF ADMINISTRATION OF AOAA ON METRAZOL-INDUCED SEIZURES

Treatment	Time after saline or AOAA (hr)	Convulsions		Duration of seizure components*			Deaths
		Clonic	Tonic	Latency† (sec ± S.D.)	Clonic (sec ± S.D.)	Tonic (sec ± S.D.)	
Saline							
Metrazol‡	1·5	22/24	20/24	94 ± 25 (22)	7.2 ± 3·2 (22)	6·3 ± 1·4 (20)	7/24
Metrazol	6·0	24/24	21/24	102 ± 28 (24)	7·9 ± 2·0 (24)	7·0 ± 1·2 (21)	9/24
AOAA§							
Metrazol	1·5	14/24	2/24	238 ± 32¶ (14)	3·8 ± 1·0¶(14)	2·7 ± 0·6¶ (2)	2/24
Metrazol	6·0	18/24	8/24	139 ± 51**(18)	8·9 ± 2·3 (18)	8·0 ± 1·8 (8)	6/24

* Numbers in parentheses in Tables 4–6 indicate number of animals in which observation were made.
† Time between Metrazol injection and onset of clonic convulsion.
‡ 65 mg/kg i.p.
§ 25 mg/kg i.p.
¶ $P < 0·01$ (difference from saline controls).
** $P < 0·02$ (difference from saline controls).

the saline controls. In those animals which showed convulsions there was a significantly longer time between the pentylenetetrazole injection and the onset of clonic convulsions, and the duration of both the clonic and tonic phases of the convulsions was significantly shorter. Also, fewer deaths occurred in the AOAA-treated group. Although at 6 hr after AOAA some protection was still evident in that fewer animals had seizures in the control groups and the latency to convulsions was prolonged, the other measurements showed that the protective effects of AOAA administration were far less than at 1·5 hr after the injection of the latter substance.

Strychnine was tested at three dose levels (Table 5) given at 1·5 and 6 hr, respectively, either after saline or AOAA injection. At the lowest dose of strychnine (2·7 mg/kg) there were fewer seizures and deaths in the animals given AOAA 1·5 hr before the

TABLE 5. EFFECT OF ADMINISTRATION OF AOAA ON STRYCHNINE-INDUCED SEIZURES

Treat-ment	Strychnine dose (mg/hg)	Time after saline or AOAA (hr)	Incidence of opisthotonic convulsions	Latency to convulsion* min ± S.D.)	Time before death† (min ± S.D.)	Deaths
Saline	2·7	1·5	18/18	4·42 ± 1·40 (18)	2·98 ± 1·21 (12)	12/18
AOAA‡	2·7	1·5	12/21	5·37 ± 0·66 (12)	6·67 ± 1·51§ (6)	6/21
Saline	2·7	6	18/18	3·62 ± 1·21 (18)	2·72 ± 0·80 (10)	10/18
AOAA	2·7	6	18/18	3·98 ± 1·37 (18)	3·97 ± 1·31 (12)	12/18
Saline	4·0	1·5	18/20	3·73 ± 0·61 (18)	0·44 ± 0·33 (18)	18/20
AOAA	4·0	1·5	14/18	3·93 ± 0·29 (14)	1·22 ± 0·41§(14)	14/18
Saline	4·0	6	20/20	3·52 ± 0·72 (20)	0·41 ± 0·29 (19)	19/20
AOAA	4·0	6	19/20	3·30 ± 0·83 (19)	0 40 ± 0 30 (18)	18/20
Saline	20 0	1·5	20/20	1·15 ± 0·16 (20)	0·30 ± 0·23 (20)	20/20
AOAA	20·0	1·5	20/20	1 10 ± 0 15 (20)	1·02 ± 0·28§(20)	20/20
Saline	20·0	6	20/20	1·08 ± 0·13 (20)	0·42 ± 0·29 (20)	20/20
AOAA	20·0	6	21/21	0·92 ± 0·11 (21)	0·51 ± 0·40 (21)	21/21

* Time between strychnine injection and onset of convulsions.
† Time between onset of convulsions and death.
‡ 25 mg/kg.
§ $P < 0·01$ (difference from saline controls).

strychnine; and in the case of those animals who died, the deaths occurred at a significantly later time than in the controls. No significant protection was found in the 6 hr AOAA-treated group. In the groups receiving the higher doses of the convulsant agent the only significant effect attributable to treatment with AOAA was a prolongation of time till death observed in the 1·5-hr animals.

The results obtained with picrotoxin (Table 6) showed that at the dose of convulsant employed AOAA was minimally protective. The only significant difference from the saline-injected controls was an increased latency to convulsions noted in the animals receiving AOAA at the 1·5-hr period after the drug.

TABLE 6. EFFECT OF ADMINISTRATION OF AOAA ON PICROTOXIN-INDUCED SEIZURES

Treatment	Time after saline or AOAA (hr)	Convulsions		Latency to convulsions* (min ± S.D.)	Deaths
		Clonic	Tonic		
Saline					
Picrotoxin†	1·5	19/20	6/20	9·58 ± 1·67 (19)	7/20
Picrotoxin	6·0	18/20	9/20	10·51 ± 1·80 (18)	9/20
AOAA‡					
Picrotoxin	1·5	16/21	9/21	14·65 ± 2·31§(16)	10/21
Picrotoxin	6·0	18/21	9/21	11·33 ± 2·81 (18)	9/21

* Time between injection of picrotoxin and onset of convulsions.
† 10 mg/kg i.p.
‡ 25 mg/kg i.p.
§ $P < 0·02$ (difference from saline controls).

In all three of the above drugs the protective action noted was greater at 1·5 hr after AOAA than at 6 hr. This corresponds to the findings obtained with the electroconvulsive seizures which were discussed previously and suggests that the time of maximal depressant action of AOAA may be the same regardless of the means of producing convulsions. The above results were similar to those previously obtained with hydroxylamine, an agent which, like AOAA, raises γABA levels by γABA transaminase inhibition, in that greater protection was given by it against the pentylenetetrazole effect than against strychnine or picrotoxin.[41] Likewise, Roa *et al.*[17] reported that AOAA raised the convulsion threshold of pentylenetetrazole, but showed little antagonism to picrotoxin.

Comment. Paper chromatographic examination of rat brain extracts after AOAA and hydroxylamine revealed no detectable change in ninhydrin-reactive constituents other than an increase in γABA content.[32, 42, 43] In dogs,[17] AOAA administration, in addition to the expected increase in brain γABA content, resulted in a decrease in asparate and increases in alanine, glutamine, lysine, tyrosine, lactate, and ammonia contents. AOAA also was found in experiments *in vitro* to inhibit glutamate–pyruvate,[44] aspartate–pyruvate,[45] and aspartate–oxalacetate[45] transaminases, and 5-hydroxytryptophan[45] and glutamic[26] decarboxylases. Thus, in addition to possible direct action on constituents of neuronal membranes, AOAA and other carbonyl reagents could produce complex changes in the relative amounts of various substances, which may be important in regulating synaptic reactivity (see Ref. 3 for detailed discussion).

When a large variety of excitatory substances is given in large amounts, paroxysmal discharges and seizures are often produced. However, it is certain that the modes of action of these substances at a molecular level are widely different, ranging from alterations produced in the physical state of the neural membranes by direct combination with some component in them to the inhibition of specific enzymes. Whatever their mechanism of action, these substances destroy the effectiveness of the coordination between inhibitory and excitatory phenomena at synapses and allow uncoordinated excitation to take place. Similarly, many inhibitory substances, probably acting by a variety of mechanisms, can cause anesthesia and finally death when given in excess. Indeed, little about detailed mechanism of action can be inferred only from the study of the effects on intact animals of substances which either cause convulsive seizures or prevent them.

Problems have been brought up in the present study which must be faced in any attempt to determine whether or not there is a causal relationship between the alteration of a particular biochemical variable in the CNS and a measurable behavioral change in the organism in which the change is induced. An early suggestion was made[46] that the *balance* between the γABA system (inhibitory) and the acetylcholine system (excitatory) rather than the absolute amount of any single substance or enzyme activity might be important in the regulation of activity in given regions of the CNS. At present it would appear that other inhibitory systems, in addition to the γABA system, must be assessed in a realistic evaluation of endogenous chemical inhibitory influences. Unfortunately, although the acetycholine system is believed by many not to be the sole, or perhaps even major, excitatory system in the CNS, no other system has yet been clearly defined. A further evaluation of brain changes with time after AOAA is being planned, in which the quantities of the various neuroactive amines and acetycholine will be measured in order to see whether changes can be indentified that will be correlated with the beginnning return to normal excitability at 1·5 hr after AOAA, while the cerebral levels of γABA are still increasing at a rapid rate.

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