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THE INHIBITORY ACTION OF β-HYDROXY-γ-AMINOBUTYRIC ACID UPON THE SEIZURE FOLLOWING STIMULATION OF THE MOTOR CORTEX OF THE DOG

By TAKASHI HAYASHI

From the Department of Physiology, Medical School Keio University, Tokio, Japan

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Several years ago Florey (1953, 1956*a*) reported the presence in the mammalian brain of a certain factor, namely Factor I, which inhibits the generation of impulses by stretch receptor neurones of the crayfish; following this there were reports on the action of Factor I on the nervous system of marine organisms (Florey 1956*b*) and of mammals (Florey & McLennan, 1955, 1956).

An assay procedure for Factor I was developed by Elliott & Florey (1956, 1957) who noted that most of the Factor I present in the brain is held in an inactive form, from which it can be released by mild procedures. Basemore, Elliott & Florey (1956, 1957) purified it and obtained crystals which showed the highest activity yet obtained, and which were identified with γ-aminobutyric acid (GABA).

From a quite different angle, Hayashi has arrived at β-hydroxy-γ-aminobutyric acid (GABOB), which has a stronger inhibitory action on mammalian motor activity than GABA; and Hayashi & Nagai (1956) reported this at the 20th International Physiological Congress at Brussels.

The presence of GABA in the brain was reported by Awapara Landua, Fuerst & Seale (1950), Roberts & Frankel (1950) and Udenfriend (1950), but we have not yet learned of any evidence for the presence of GABOB in the central nervous system in higher animals. In the present studies I have obtained at least one piece of evidence which shows that it can exist in dog's brain. The effect of synthetic GABOB and GABA upon the generalized seizure following electrical as well as chemical stimulation of the motor cortex of dogs has been observed.

METHODS

The first experiment to determine the ammonia content of the brain was carried out on six rats each of 70–80 g weight. The animals were killed by decapitation and thrown into liquid air within 1–2 sec. The brains were homogenized and were deproteinized with trichloroacetic acid. The clear

supernatant was used for the determination of free ammonia according to Conway's micro-diffusion method (1950). Serine, threonine and GABOB were determined after the development by paper chromatography, the eluates being estimated according to Winnick's (1942) micro-adaptation of the method of Shinn & Nicolet (1941); GABA was estimated by the method of Awapara *et al.* (1950), glutamic acid as well as aspartic acid by that of Yemm & Cocking (1955).

The second experiment was carried out on six dogs which weighed 8–12 kg. The fluid drained from the ventricular or cisternal spaces was collected from the dogs, and the content of free ammonia was measured by the method of Shinn & Nicolet (1941). All experiments on dogs were performed under anaesthesia by sodium *N*-methylcyclohexanyl-methyl-barbiturate (Ouropan-soda, Shionogi laboratories; 60–80 mg/kg intraperitoneally). This dose was repeated every 50 min until after the completion of exposure of the cerebral cortex when no further doses of anaesthetic were needed.

The third experiment was carried out on twelve dogs weighing each 8–13 kg, to determine the inhibitory effect of GABOB on the seizure following electrical as well as chemical stimulation of the exposed motor cortex. Electrical stimulation was affected by an ordinary inductorium, faradization of an appropriate strength being applied to the motor cortex of the dogs with ordinary platinum electrodes. Chemical stimulation was carried out by the application of a concentrated solution of sodium glutamate by ventricular injection. All the chemicals used were of a guaranteed grade of commercial origin and were used without further purification.

γ-Aminobutyric acid (GABA) was obtained commercially, as were serine and threonine. β-Hydroxy-γ-aminobutyric acid (GABOB) was prepared by the method of Tomita (1923), at first as DL-GABOB and afterwards fractionated by the brucine method to yield L-GABOB.

RESULTS

Existence of GABOB in rats' brains

The free ammonia content of the specimens from rats' brains was first measured. The specimens were then treated with periodic acid and the content of the released ammonia was measured. As is shown in the first column of Table 1,

TABLE 1. The identification of GABOB in the normal rat's brain with reference to the content of serine and glutamic acid

Content of free ammonia of the whole brain of rat

Before treatment with periodate (μmoles/g)	After treatment with periodate (μmoles/g)	Difference (μmoles/g)
0·37	1·54	1·17

Content of GABOB and other amino acids in rat's brain

Amino acid	Mean ± S.D. (μmoles/g)	Number of experiments
Serine	0·29 ± 0·06	(4)
GABOB	0·48 ± 0·02	(7)
GABA	2·72 ± 0·92	(4)
Glutamic acid	10·10 ± 2·50	(4)
Aspartic acid	3·56 ± 1·30	(4)

the difference between the above two measurements was 1·17 μmoles/g, which would be released from serine, threonine and GABOB if they were present. Besides these three, hydroxylysine must be referred to, but as this has been said to exist only in the collagen tissue (Rogers, Weidmann & Parkinson, 1952), it may be disregarded.

The identification of GABOB was carried out as follows. First, serine, threonine, GABOB and GABA were developed by paper chromatography, and their positions on the paper determined. Secondly, the specimens of rats' brains were developed by paper chromatography and each part of the paper corresponding to the above four positions, sometimes with an indifferent one, was cut into blocks of the same size: the aqueous ethanolic extracts of these blocks were treated by periodic acid and the content of free ammonia released by each was ascertained. The results are shown in Table 1.

The indifferent block, or the block with GABA, released a certain amount of ammonia and this was taken as a control value. The difference between the ammonia released from the block of serine and from the control was taken to be the content of serine in the rat's brain, and it was 0·29 μmoles/g. According to the same procedure the content of GABOB was 0·48 μmoles/g. The content of glutamic acid and aspartic acid as well as of GABA is shown in the table.

Concentration of GABOB in the drained saline solution after the cessation of salt discharge of the nerve cells

A small metal syringe was inserted into a lateral ventricle of the dog and isotonic saline solution (NaCl 0·9%) was administered into the ventricular spaces and then drained from the cerebellomedullary cisterna. As the cerebrospinal space of a dog is calculated to be 15–18 ml. (in a 10 kg dog), the first 50 ml. is enough to wash out most of the cerebrospinal fluid and to replace it with saline solution. Then the cerebrospinal space was certainly perfused with 200–250 ml. of the isotonic saline solution, into the ventricle and out from the cisterna, to be introduced again into the ventricle repeatedly. When the cerebrospinal fluid was replaced with saline, a generalized seizure was induced and continued. This was called a 'salt discharge of nerve cells' (SDNC) and it ceased on its own accord after about 4 hr (Hayashi, 1958). After it ceased, when the fluid was replaced by fresh saline solution the convulsions were set up again.

The 'salt discharge' of the nerve cells could be stopped by adding a small quantity of Ca ion, but the solution drained from the brain did not contain more than one-twentieth of the concentration of calcium which was sufficient to inhibit the salt discharge.

In the examination of drained fluid, its content of NH_3 was first measured and compared with the NH_3 released after the treatment by periodic acid. These substances were tested to determine whether they had an inhibitory effect upon the seizure.

The NH_3 content of the fluid was 1·47 μmoles/ml. Next the fluid was treated with periodic acid and the released NH_3 was determined. It was 2·11 μmoles/ml. The difference between these two measurements is 0·64 μmoles/ml. The drained saline solution thus contained a certain substance which

released NH_3 on treatment with periodic acid. Its concentration was about 0·0071%, if it is estimated as GABOB. The replacement of the cerebrospinal fluid of a dog by an isotonic saline solution with 0·0071% of synthesized DL-GABOB confirmed that this substance prevented the salt discharge.

Besides GABOB there would be also serine and threonine in the fluid, and a test was made to determine whether these acids could inhibit the seizure following electrical stimulation of the motor cortex, but neither of them had an inhibitory action.

The blood–brain barrier in the case of a rapid injection of GABOB and GABA through the carotid artery, and the fluid–brain barrier in the case of ventricular injection

To determine the inhibitory action of the substances against a generalized seizure two methods were employed. One method was to introduce the substance into the carotid artery at a rapid rate, or into the cerebrospinal space of a dog, during a seizure which was evoked by electrical or chemical stimulation. For chemical stimulation metrazol or sodium glutamate was used. When 1·0 ml. of an appropriate concentration of one of these substances was introduced into the ventricles of a dog, a generalized seizure was produced within 13–20 sec, and continued for 60–180 sec. After the seizure began the test substances were introduced into the carotid artery. When the inhibitory action was sufficient, the seizure would cease. In this case the dose and concentration were both to be calculated by the following equation.

In the case of rapid injection into the carotid artery, C_E, the critical concentration to inhibit the seizure would be:

$$C_E = \frac{C_o \times V}{\frac{1}{2}(15\% \times Q \times t) + V} \tag{1}$$

where Q = rate of flow (ml./sec) of blood in the carotid artery,

C_o = concentration of inhibitory substance,

V = volume (ml.) of solution introduced in time t (sec).

Q is calculated from the total blood volume of a dog (9·8% of its weight) and the circulation time, assumed to be 20 sec. The blood volume which flows into the brain through four arteries, the two carotids and the two vertebrals, is 15% of the total blood of the animal, but the vertebrals perfuse mainly the brain stem, and the carotids the cortex.

The critical concentration of GABOB as well as that of GABA required to prevent a seizure was measured by the rapid introduction of the solution through a carotid artery. Both substances had a strong inhibitory action against a sodium glutamate seizure, as is shown in Table 2. As is also shown in Table 2, the ventricular administration of 1·0 ml. of 0·1 M sodium glutamate

was sufficient to produce a long continued seizure, and 1·0 ml. of 0·005 M GABOB abolished the seizure within 10–20 sec. As for GABA, the critical dosage was between 1·0 ml. of 0·1 M and 1·0 ml. of 0·01 M. Judging by the latent periods for both substances to inhibit the seizure, the blood–brain barrier was not important. The critical concentration was 0·0006 M for GABOB 0·0012 M for GABA.

TABLE 2. Comparison of the critical concentration of GABOB and GABA to inhibit sodium glutamate seizure

Sodium glutamate in c.s.f.	Inhibitory agent injected into carotid artery (1 ml.)	Effect*	C_E (calculated from eqn. 1) and $C_1 \sim C_2$ (calculated from eqns. 2 and 3) (M)
1 ml. × 0·1 M	GABOB 1 ml. × 0·01 M	−	0·0012
	GABOB 1 ml. × 0·005 M	−	0·0006
	GABA 1 ml. × 0·1 M	−	0·012
	GABA 1 ml. × 0·01 M	+	0·0012
Comparison of the inhibitory action of GABOB and GABA when applied at the same time with convulsants via c.s.f.			
1 ml. × 0·2 M	GABOB 1 ml. × 0·005 M	−	0·00027 ~
	GABOB 1 ml. × 0·001 M	+	0·000078
	GABA 1 ml. × 0·1 M	−	0·0027 ~
	GABA 1 ml. × 0·01 M	+	0·00078

* + Convulsion; − convulsion arrested or prevented.

When the convulsant sodium glutamate was introduced into the ventricle of a dog, it produced a generalized seizure with a latent period of 15–30 sec. If GABOB or GABA was introduced with the sodium glutamate, it inhibited the seizure, as is shown in Table 2.

In this case, the concentration of GABOB producing its inhibiting effect could be calculated as either:

$$C_1 = \frac{C \times V}{1{\cdot}6\,W}, \tag{2}$$

or

$$C_2 = \frac{C \times V}{6{\cdot}4\,W}, \tag{3}$$

where C = concn. of substance introduced,

V = volume introduced (ml.),

C_1 = concn. when diluted by volume of c.s.f. (1·6 ml./kg body wt.),

C_2 = concn. when diluted by volume of brain (6·4 ml./kg body wt.),

W = weight of dog (kg).

The critical concentration of GABOB to stop the seizure was 0·00027–0·000078 M; for GABA it was 0·0027–0·00078 M.

Comparison of the inhibitory action of L-*GABOB and* DL-*GABOB*

The GABOB which was used in the above experiments was all of the racemic (DL) form. But recently L-GABOB has been separated, and a comparison was made between it and the DL form.

Electrical stimulation was applied to the exposed motor cortex of a dog, and the strength required to produce a generalized seizure of maximal duration was ascertained. For example, the threshold strength to evoke a seizure was 180 mm coil distance in the ordinary inductorium with 40 pulses/sec. The duration of the seizure became longer with increasing strength, and it reached a maximum of 60–150 sec, as shown in Table 3, at a coil distance of 110 mm.

TABLE 3. The latent period to arrest by L-GABOB and DL-GABOB of seizure following electrical stimulation

Maximum duration of the seizure after faradization for 5 sec (sec)	Concentration of GABOB 1 ml. introduced into c.s.f. (M)	Latent period for inhibiting the seizure (sec)	Calculated concentration of L-GABOB and DL-GABOB from eqns. 2 and 3 $C_1 \sim C_2$ (M)
	L-β-hydroxy-γ-aminobutyric acid		
98	0·084	20	0·0053 ~ 0·0013
64	0·042	24	0·0026 ~ 0·00065
60	0·042	23	
130	0·016	25	0·0010 ~ 0·00025
90	0·016	25	
67	0·008	32	0·00050 ~ 0·00013
75	0·008	25	
75	0·004	13	0·00025 ~ 0·000065
110	0·004	40*	
70	0·0016	130†	
150	0·0016	145†	
	DL-β-hydroxy-γ-aminobutyric acid		
98	0·084	22	0·0053 ~ 0·0013
55	0·042	25	0·0026 ~ 0·00065
66	0·016	55*	0·0010 ~ 0·00025
70	0·016	40*	

* Slight inhibition; † no inhibition.

At 5–10 sec after the beginning of the induced seizure, 1·0 ml. of L-GABOB solution, of an appropriate concentration, was introduced into either a lateral ventricle or the cisterna cerebellomedullaris of the dog. For example, in experiment No. 8 in Table 3, the duration of the generalized seizure in response to 5 sec stimulation was 75 sec. Five seconds after the beginning of the seizure, L-GABOB was injected. When the concentration was 0·00025–0·000065 M, according to calculation from equations 2 and 3, the seizure ceased within 13 sec, this time representing the latent period in the action of L-GABOB. The inhibitory effect of GABOB persisted for 5 min, after which the excitability returned.

In contrast, when DL-GABOB was injected the critical concentration to stop the seizure was below 0·0010–0·00025 M, as is shown in Table 3. The inhibitory action of L-GABOB was stronger than that of DL-GABOB.

Neither serine nor threonine could stop the seizure following electrical stimulation of the motor cortex, up to a dose 1·0 ml. of 0·2 M.

DISCUSSION

Rat's brain contained GABOB as a normal constituent in a concentration of 0·48 μmoles/g (wet weight) when the content of serine was 0·29 μmoles/g.

After the cerebrospinal spaces of a dog were continuously perfused with isotonic saline, a generalized seizure occurred, which later ceased as the solution continuously reperfused the spaces. The saline solution emerging from the cisterna after a seizure contained an inhibitory principle, which is considered to be the inhibitory mediator. It was an amino acid which released NH_3 on treatment with periodic acid, and it was identified as GABOB. Elliott & Florey (1956) believed GABA to be the real inhibitory principle in the brain, and there have appeared several related papers (Kuffler & Eyzaguirre, 1955; Iwama & Jasper 1957), among them one by McLennan (1957*b*) who compared the inhibitory actions of GABA and GABOB. McLennan confirmed that they had almost the same action, but appeared to believe that GABA was more likely to be the inhibitory principle in the brain of higher animals.

It appears, however, that stress should be laid on GABOB, especially L-GABOB, as the inhibiting substance in the brain of higher animals for the following reasons. (1) The critical concentrations required to inhibit sodium glutamate seizures are for GABA 0·012 M, and for GABOB 0·0006 M. (2) L-GABOB has a stronger inhibitory action on the generalized seizure following electrical stimulation: it has a critical concentration of 0·00025–0·000065 M, against 0·0010–0·00025 M for DL-GABOB. (3) GABOB can be considered to be derivative of GABA, of which the content in the brain is normally higher, being estimated by several authors as 0·031 %. The concentration of GABOB which was found in the solution emerging from the cisterna after a seizure was 0·0071 %, and this concentration sufficed to stop the 'salt discharge' when applied in saline solution.

SUMMARY

1. Rat's brains contained β-hydroxy-γ-aminobutyric acid (GABOB) in addition to γ-aminobutyric acid (GABA), in the ratio 1:5·6.

2. After the cerebrospinal spaces of a dog were continuously perfused with isotonic saline solution (NaCl 0·9 %), a generalized seizure occurred, which later ceased as the solution continuously reperfused the spaces. When the seizure stopped the saline solution contained some inhibitory substance

derived from the brain which was identified as β-hydroxy-γ-aminobutyric acid.

3. The generalized seizure due to the injection of convulsants into the lateral ventricle was abolished by the introduction of GABOB or GABA through the carotid artery. The inhibitory action of GABOB was stronger than that of GABA.

4. The generalized seizure which was evoked by an electric current was abolished by L-GABOB as well as by DL-GABOB within 30 sec after introducing it into the cerebrospinal fluid of the dog. The critical concentrations were calculated to be 0·00025–0·000065 M for the former and 0·0010–0·00025 M for the latter.

5. The real inhibiting factor in the brain is presumed to be L-GABOB.

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