white inhibits the fungal proteinase but purified ovomucoid does not.

The new inhibitor was separated from egg white as follows. Egg white was diluted with an equal volume of 0.25 percent NaCl solution and brought to *p*H 6 with HCl. The precipitate of mucin was removed by centrifugation. The supernatant was half-saturated with ammonium sulfate, and the precipitate that formed was dissolved in a small volume of distilled water. When this was dialyzed against 0.25 percent NaCl solution, more of the mucin separated (*5*). The inhibitor was then precipitated by ammonium sulfate at 0.35 saturation, dissolved in distilled water, and dialyzed in a cellophane bag against distilled water until it was free from ammonium and sulfate ions. The globulins that settled in the bag were removed by centrifugation.

To the supernatant, which was a fairly clear solution, was added trichloroacetic acid to a final concentration of 0.15*M*; the *p*H was then adjusted to 3.5. After it had stood for about 10 minutes at 25°C, the precipitate was collected by centrifugation and was redissolved in distilled water. Adjustment of the solution to *p*H 4.8 (indicator paper) gave a considerable amount of precipitate of foreign protein which has no inhibitory action. After removal of the precipitate, the solution, in the presence of 1 percent NaCl, was brought to 40 percent acetone concentration at room temperature, and the precipitate that formed was discarded. The inhibitor was finally precipitated by acetone at a concentration of 60 percent. The white precipitate was washed with acetone followed by ether, and then dried in a vacuum desiccator. The yield of the inhibitor was about 0.07 g per 100 g of egg white, and the specific activity was about 63 times that of the original egg white. The proteolysis and its inhibition were measured according to the procedure of Anson (*6*); milk casein was used as substrate.

The new inhibitor, which would be called "ovoinhibitor," in contrast to ovomucoid, showed a single moving boundary distinctly different from that of ovomucoid in paper electrophoresis, and the ultraviolet absorption of an aqueous solution was typical of protein, showing absorption maximum at about 280 mμ. With the view of evaluating the potency of the new inhibitor as compared to ovomucoid, the effect of different concentrations of both inhibitors on trypsin was studied. The level of trypsin added to various levels of the inhibitors was 60 μg, and the enzyme was added to the mixture of substrate and inhibitor. Digestion was run at *p*H 7.6 for 10 minutes at 35°C. From the results presented in Fig. 1, it will be seen that the new inhibitor, like ovomucoid, inhibited trypsin

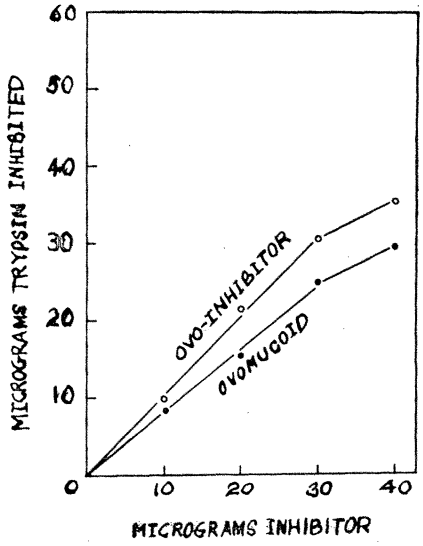

Fig. 1. Trypsin-inhibiting activity of inhibitors from egg white.

stoichiometrically and was more effective than ovomucoid. It resembles ovomucoid in that it is considerably stable in acid solution and also in that it is readily digested by pepsin but not by papain. Unlike ovomucoid, however, it gives a negative anthrone test, it was precipitated by 2.6 percent trichloroacetic acid, and it acted on proteinase of fungal and bacterial origin (Table 1).

Consequently, attention must be called to the fact that "Egg white trypsin inhibitor" does not consist only of ovomucoid; there also exists an additional inhibitor, "ovoinhibitor."

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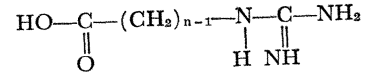
18 February 1958

Central Synaptic Effects of ω-Guanidino Acids and Amino Acid Derivatives

The synaptic effects of the aliphatic ω-amino acids (*1*) relate to the general problem of the nature of bioelectric activity and particularly to the special properties of synaptic electrogenesis. Furthermore, at least one of the substances, γ-amino butyric acid (GABA), occurs abundantly in mammalian brain, where it is enzymatically produced (*2*). By virtue of its potent synaptic effect—selective inactivation of depolarizing (excitatory) dendritic synapses (*1*)—GABA may play a role as an "inhibitory transmitter" of a special type (*3*). While all the tested ω-homologs of GABA were synapse inactivators, their effects varied not only in degree but also in the quality of action. The latter depended upon the length of the carbon chain. The 5-carbon δ-amino valeric acid, like GABA, but less strongly, blocks selectively the depolarizing synapses of the superficial dendrites of the cat cortex (*1*). The 6- and 8-carbon compounds, on the other hand, selectively inactivate the hyperpolarizing synapses. Both are convulsant agents, ω-amino caprylic acid (C_8) being about as powerful on topical application as strychnine, which also inactivates selectively the hyperpolarizing synapses (*4*).

Thus, the affinities of two classes of synapses can now be defined in terms of electrophysiological effects and of a relatively simple configurational change in synaptic drugs. The availability, in almost innumerable modifications and in various grades, of compounds derived from amino acids offers a new series of pharmacological tools for the analysis of molecular structures of excitable membrane. One homologous series related to the aliphatic ω-amino acids is that of the guanidino acids



This series is of particular interest because γ-guanidino butyric acid ($n=4$) normally occurs in brain (*5*), and by transamidation is a source of γ-amino butyric acid (*6*).

The method employed in the analysis of actions of the drugs on cortical synapses, detailed earlier (*1*), need be described only briefly here. Selective blockade of depolarizing dendritic synapses leads, in the cerebral cortex, to reversal of the evoked cortical response from negativity to positivity as the normally masked responses of hyperpolarizing synapses become evidenced. Because the cerebellar cortex is relatively devoid of hyperpolarizing synapses (*4*), there is no positivity to unmask by abolition of the surface negativity. Blockade of hyperpolarizing synapses in the cerebral cortex is denoted by augmentation of the surface negative response. The potential of the cerebellar cortex is not affected (*7*). These various actions are produced rapidly and reversibly, thus denoting their nature as effects of drugs upon synaptic surfaces, rather than interference with intracellular metabolic pathways. Onset occurs within 1 to 5 seconds after application of 2 to 3 drops of buffered 1 percent solution of the drug to the cortical

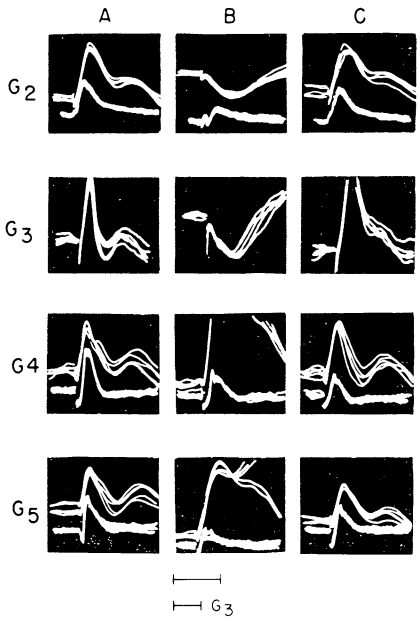


Fig. 1. Synaptic actions of ω-guanidino acids of carbon chain lengths $n = 2$ to $n = 5$ (G_2 to G_5). Five superimposed responses in each record before (*A*), and 20 to 30 seconds after application of three drops of the 1 percent solution of the compound (*B*), then, 10 minutes after the cortical surface is flushed with Ringer's solution (*C*). The dual trace records show simultaneous tests on the cerebral (upper) and cerebellar (lower) responses in one succinylcholine preparation. The records for G_3 (β-guanidino propionic acid) were obtained from a different preparation, and only for the cerebral cortex. The time base (20 msec in each case) is also different for these records.

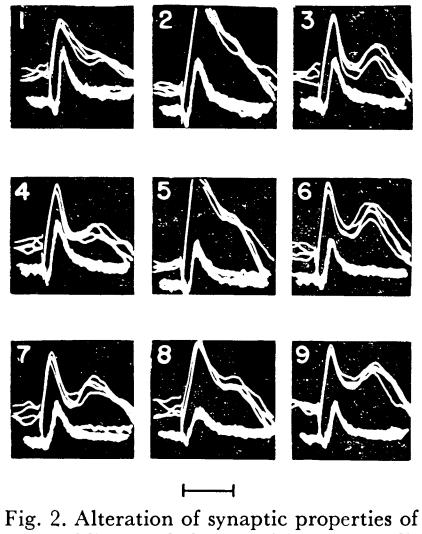
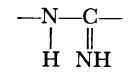


Fig. 2. Alteration of synaptic properties of ω-guanidino acids by substitutions. Modification of guanidino acetic acid (G_2) to creatine (1–3) or creatinine (4–6) produces substances that are selective blockaders of inhibitory synapses. Arginine (7–9), derived from δ-guanidino valeric acid, is a weakly acting agent. Dual traces in all records show the effects on cerebral (upper) and cerebellar (lower) dendritic activity. Four superimposed responses in each record. Time, 20 msec.

surface. Reversal begins within 10 to 20 seconds after the surface is flushed with Ringer's solution. Although intravenous injections of the amino acids produce no electrophysiological effects, local destruction of the blood-brain barrier permits the access of these substances to the synapses only in the altered cortical region (*8*). Effects similar to those produced by topical application are then seen about 15 seconds after as little as 8 mg/kg of the synaptically active amino acid is injected into the femoral vein.

Like the ω-amino acids, the ω-guanidino acids tested are synaptic agents, and all inactivate dendritic synapses. Their mode of action also depends upon the length of the chain (Fig. 1). However, the $n = 2$ compound (guanidino acetic acid) is a strong inactivator of depolarizing synapses like GABA (C_4) and not like glycine (C_2). On the other hand, the $n = 4$ compound, γ-guanidino butyric acid, unlike GABA, blocks not the depolarizing but the hyperpolarizing synapses, resembling in this respect the C_6 amino acid.

These differences indicate that the ω-guanidino acids of carbon lengths n behave like the ω-amino acids of carbon lengths $n + 2$. Thus it would seem that the



coupling between the aliphatic chain and the terminal NH_2 of the guanidino compounds is equivalent to two carbons of an aliphatic ω-amino acid. However, the substitution is not a fully equivalent one. Whereas the ω-amino acids act rather selectively upon one or the other variety of synapses, the guanidino acids are less selective. While the butyric and valeric guanidino compounds markedly augment the evoked potential of the cerebral cortex, an indication of their blockade of the hyperpolarizing synapses, the substances also depress somewhat the cerebellar response. This indicates that the $n = 4$ and $n = 5$ guanidino acids also block depolarizing synapses to some extent (*9*).

The relative nonselectivity of the guanidino acids can account satisfactorily for the finding (cited in *6*) that γ-guanidino butyric acid "inhibits" the crayfish stretch receptor, but somewhat less effectively than dose GABA. A more elaborate test object, such as is available in the cortex because of the presence of both depolarizing and hyperpolarizing synapses, provides information not only upon this common aspect of the drugs, but also furnishes details about their differences.

Instructive examples of the modification of synaptic actions can be derived from compounds related to the guanidino series. The monomethyl substitution of guanidino acetic acid, without or with condensation, which yields creatine and creatinine, respectively, markedly changes the synaptic effects. The powerful inactivator of depolarizing synapses (Fig. 1) is converted into blockaders of hyperpolarizing synapses (Fig. 2). On the other hand, insertion of an $—NH_2$ group in the α-position of δ-guanidino valeric acid converts this powerful blockader of hyperpolarizing synapses into one (arginine) that acts weakly in this manner. It has been noted earlier (*1*) that the introduction of an α-amino group into the ω-amino acids diminished, or abolished, synaptic potency.

The synapic effects of the amino, or guanidino acids and related compounds, including some of the B vitamin group (*10*), and the occurrence of many of these substances in the body, indicate that synaptic as well as metabolic actions must be considered when the role of the substances in the bodily economy is described. Thus, for example, the trophic, neurological, and psychological changes that are frequently noted in the syndromes of various B vitamin deficiencies may be associated more with the synaptic effects of the substances than with their metabolic roles. In states of abnormal amino acid metabolism similar manifestations are also commonly observed. Direct synaptic actions may account for the "toxic" central nervous system effects attributed to amino acids and their derivatives (*11, 12*).

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7. The paramedian lobule of the cerebellum appears to have a synaptic organization resembling that of the cerebral cortex (unpublished data). Among other consequences is the augmentation of evoked negative surface responses by topical application of strychnine [J. Jansen, Jr., *Acta Physiol. Scand.* **41**, Suppl. 143 (1957)].
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Electrode and Cannulae Implantation in the Brain by a Simple Percutaneous Method

In an investigation of the psychologically active motivational (reward and punishment) systems of the brain stimulated by electrical means, we have utilized "roving" electrodes implanted in the unanesthetized monkey's brain (*1–4*). The technique of implantation by hammering sleeve-shaped guides into the skull for these movable electrodes apparently has not been used before and simplifies the problem in chronic preparations (*2, 3, 5, 6*). The stereotaxic instrument (*7*) can be used to place the guides. During times when experiments are not being carried out on the animal, all electrodes and cannulae can be removed, leaving inconspicuous self-closing and self-healing skin lesions.

The method consists of implanting in the skull (but beneath the skin and outside the dura) a hollow tube (sleeve) which guides the electrode (or electrode array or cannula)through the skull in a definite direction into the brain. An electrode (*8*) is pushed through the skin and subcutaneous tissues, into the outer end of the sleeve in the skull, through the barrel, and thence into the brain.

The sleeves are made from stainless steel (type No. 316) hypodermic needle tubing (No. 20, 0.90 mm outside diameter, 0.57 mm inside diameter, in one case—a macaque implantation) as is shown in Fig. 1c.

In the spot desired for the implantation, a small indentation is made in the soft tissues and bone with a hardened steel spear-shaped tool (Fig. 1*a*), which is guided through a long tube-shaped rigid bearing in a director used in place of the electrode carrier in the stereotaxic instrument. The director has a cone-shaped lower end which is pressed into the skin; the spear is lightly pounded into the bone (for a distance of about ½ mm) and then is withdrawn. The sleeve is placed on the mandrel (as in Fig. 1*b*); the mandrel is inserted in the director; the mandrel and the sleeve are driven into the bone by light hammering on the outer end of the mandrel. After each one-half millimeter or so of the guide is driven into the bone, the mandrel is manually tugged lightly upwards; if it comes out of the sleeve easily, the lower end of the guide (Fig. 1*c'*) has passed the inner table of the skull (but not the dura).

After the sleeve is in place in the skull, the skin and the subcutaneous tissues are allowed to pull together over the upper end and to heal. The sleeves are placed in definite patterns in the skull by means of the stereotaxic instrument and allowed to protrude above the skull about 2 mm. The operator palpates these ends through the soft tissues and finds the opening in the sleeve with the spear's sharp tip. By pressing the cone end of the spear into the guide's outer end, the skin and subcutaneous tissues are pierced. The skin is held in place with a forceps, the spear is withdrawn, and a sharp needle is inserted far enough to puncture the dura. The needle is withdrawn, and the electrode or cannula is inserted into the sleeve and lowered into the brain. To measure the depth of penetration of electrode or cannula, a pointed scale is used to measure the distance from the outer end of the sleeve to the outer end of the inserted cylinder of the electrode or cannula. The length of the sleeve varies with the animal and the loci in the skull. For the top of the skull of a macaque of 6 kg (13.2 lb) weight, for example, suitable lengths are 3½ to 4½ mm; for the skull of a porpoise, 20 to 50 mm.

After 5 to 6 weeks, a thin plate of bone grows over the ends of guides which are flush with the skull's surface. This is easily drilled out with two beveled hypodermic needles—first with one smaller than and then with one the same size as the electrode. Five months after an implantation the bone has not grown over the outer ends of guides which protrude 1.0 to 2.0 mm above the periosteum.

From 20 to 60 zones with 1 to 2 mm resolution have been explored along each track, running from pial cortex through the brain to the base of the skull. Previously, with a stereotaxic button and roving electrodes (*4*), we explored about 500 zones in two monkeys, with no problems assignable to either intracranial bleeding or infection. Currently, two monkeys are being investigated, with sleeves in their skulls at interguide intervals of 2 mm (one with four and one with 20 sleeves, to date). Figure 2 shows an x-ray of the skull of the animal with 20 implanted sleeves (3½ to 4½ mm long) and one electrode in place.

The animal is restrained to avoid pulling out the electrodes or cannulae (*9*). Self-limited amounts of bleeding from penetration of veins does occur but does not cause detectable signs in an upright monkey nor in a floating porpoise with a closed calvarium. Using roving electrodes, we have not yet seen (in exploration of about 30 tracks, in four animals, over a period of 18 months) any

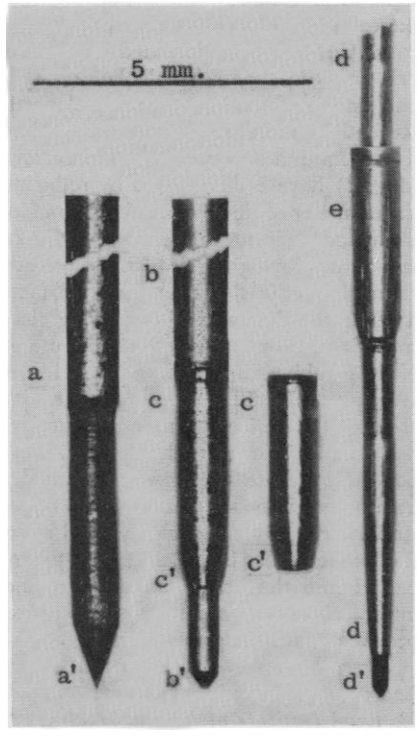

Fig. 1. Parts used in method of electrode implantation described in this report: *a,a'*, the lower end of the spear-shaped hardened steel tool (41 mm over-all length) used for starting the hole in the bone; *b,b'*, the lower part of the mandrel (41 mm over-all length), with a sleeve on the small cylindrical lower end (made of tungsten wire, 0.56 mm in diameter) (*b'*); *c,c'*, sleeves (one on mandrel and one by itself); *d,d'*, electrode; *e*, a sleeve guide on the electrode, showing a tight fit at the tapered inner end.

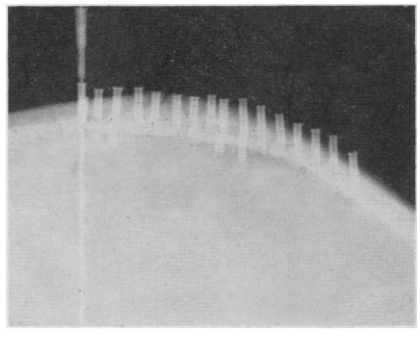

Fig. 2. X-ray photograph of monkey's skull (No. 230857, Horatio) containing 20 sleeves and one electrode; 16 sleeves are in the midplane and two are on each side, 10 mm lateral to the midplane. The latter four sleeves are displaced downward because of skull curvature, not because of deeper penetration. Careful inspection of stereo x-ray pairs shows that none of the sleeves penetrates more than a small fraction of a millimeter beneath the inside surface of the skull. Some angulation of those sleeves which were started on sloping parts of the skull can be seen; this "angulation error" has been reduced by modifications in the size of the sleeve and in the fit of the director on the sleeve (see text).