in the oxidized form but is dissociated when reduced.

The presence of free radicals in the reaction mixture was detected by paramagnetic resonance absorption at a wavelength of 3.2 cm. The sample (20 to 40 μl of solution) was placed in a glass tube of 1 mm bore that was centered in the cavity, and measured at room temperature. The best result was obtained in the following experiment. A solution of 2 mg of TPNH in 20 μl of phosphate buffer (*p*H 7.0) was added to 5 mg of old yellow enzyme crystals which had been centrifuged from the $(NH_4)_2SO_4$ mother liquor. This resulted in dissolution of most of the crystals. The sample was transferred at once to the capillary and placed in the resonance spectrometer. The amount of free radicals increased during the first 5 hours, remained essentially constant for about 20 hours, and thereafter decreased. Between the measurements, the sample was stored at 4°C. At 33 hours the sample was temporarily removed from the capillary to obtain an aliquot of the solution for determination of the enzyme concentration. The free radical signal disappeared following the exposure to air but returned in the succeeding hour to reach the level present upon withdrawal, then slowly decayed. The enzyme concentration was found to be 1.2m*M* (equivalents FMN). The derivative curve at maximum development of free radicals is seen in Fig. 2. A weak but still detectable signal was obtained at an enzyme concentration of 230 μ*M*.

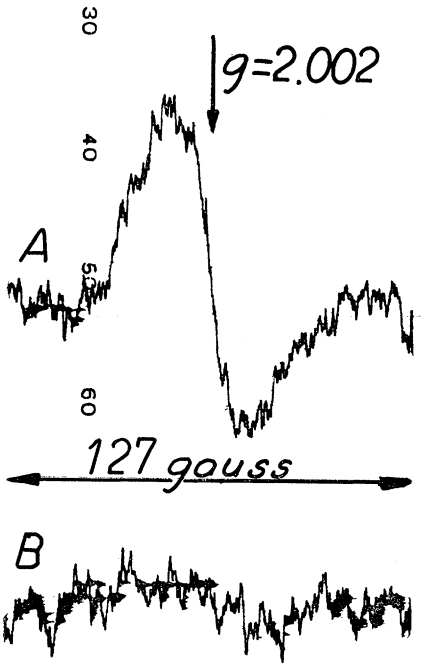

Fig. 2. Records of derivative of paramagnetic resonance absorption curves: (*A*) Old yellow enzyme (1.2m*M* equivalents FMN) + TPNH (50m*M*), 5 hours after mixing. The buffer was 0.05*M* phosphate (*p*H 7.0) and 30 percent $(NH_4)_2SO_4$. The effective volume in the glass capillary (of 1 mm inner bore) was 18 μl. (*B*) Blank experiment: old yellow enzyme (0.03m*M* equivalents FMN) + TPNH (50m*M*). Same buffer and capillary as in *A*. The remaining signal is due to the glass. Conditions: microwave frequency, 9360 Mcy/sec; Magnetic field sweep, 127 gauss increasing field, 12 minutes sweep time; peak-to-peak modulation at 70 cy/sec, 27 gauss; time constant of phase-sensitive detector, 40 seconds.

The derivative curves recorded were compared with those obtained from FMN reduced by zinc in 1*N* HCl. They could not be distinguished from each other with respect to shape, width, or *g*-value. In fact, the curve obtained for 500 μ*M* FMN coincides exactly with that of Fig. 1.

Attempts have been made to determine the free radical concentration by measuring the magnetic susceptibility of the FMN semiquinone and by comparison with the resonance absorption of diphenylpicrylhydrazyl after extrapolation of the data to the case of an infinitely narrow cylindrical sample. Both methods indicate a maximum yield of free radicals of about 15 percent in the solution containing 1.2m*M* old yellow enzyme.

Our experiments suggest that the orange-red compound is a complex containing TPN in addition to FMN, as assumed by Haas (*1*). However, this complex is not identical with the radical structure. The low yield of free radicals and their sensitivity to exposure to air suggest that they accumulate until a steady state is reached.

Since no TPN or DPN radicals have as yet been described, it would be premature to consider the similarity between the resonance absorption spectrum of the system old yellow enzyme plus TPNH and that of the FMN semiquinone as evidence conclusively showing that the radicals belong to the FMN moiety. On the basis of the minimal extinction of 3.3×10^6 $cm^2 \times mole^{-1}$ (*7*) at 565 mμ that Beinert (*5*) has calculated for the FMN radical and our finding that the maximal radical concentration amounts to 15 percent of the enzyme concentration, an absorbance increase of about 0.045 can be calculated in case of FMN radical formation in an old yellow enzyme solution with original unit absorbance at 465 mμ. This is the same order of magnitude observed by Beinert (*5*) and emphasizes the difficulty of observing these radicals by spectrophotometric methods.

While this work (*8*) was in progress, Commoner *et al.* (*9*) presented data, obtained by paramagnetic resonance absorption technique, which indicated free radical formation in a number of other enzyme systems. However, without kinetic data, neither the finding of Commoner *et al.* nor that reported here proves that the free radicals detected represent active intermediates in the enzyme reactions.

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7. The value 3.3×10^3 given by Beinert is evidently a misprint.
8. We wish to acknowledge the support and the stimulating suggestions of Professor Hugo Theorell during the course of this work.
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12 December 1957

An Undescribed Trypsin Inhibitor in Egg White

The existence of a powerful trypsin inhibitor in egg white has been noticed by some workers (*1*) since Delezenne and Pozerski (*2*) observed at the beginning of this century that fresh egg white inhibits trypsin. Meyer *et al.* (*3*) found that the active component of egg white shows the properties and composition of an ovomucoid, and recently this component was definitely identified as a native ovomucoid by Lineweaver and Murray (*4*). As a result of an investigation of the naturally occurring inhibitor in egg white against *Aspergillus* proteinase, I have reached the conclusion that egg white contains an undescribed trypsin inhibitor which is more effective than ovomucoid. This finding was derived from the observation that native egg

Table 1. Difference in properties of the trypsin inhibitors in egg white.

Test	Ovomucoid	Ovoinhibitor
Ammonium sulfate, at half saturation	No ppt.	Ppt.
Trichloroacetic acid, 2.6 %	No ppt.	Ppt.
Aspergillus proteinase	No inhibition	Inhibition
Bacterial proteinase*	No inhibition	Inhibition
Anthrone's reaction	Positive	Negative
μg of trypsin inactivated by μg of inhibitor†	0.79	0.99

* Crystalline Bioprase, prepared from culture filtrate of *Bacillus subtilis* var. *biotecus* by Nagase & Co., Ltd., Japan.
† Taken from data of Fig. 1.

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

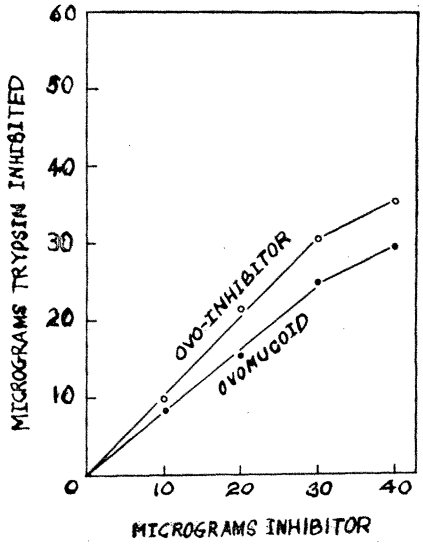

Fig. 1. Trypsin-inhibiting activity of inhibitors from egg white.

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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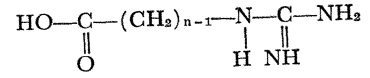
18 February 1958

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