Multiple Nature of Inhibitory Factor (Factor I) from Brain. (22974)

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(Introduced by H. P. Broquist)
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Florey(1) and Lissak(2) have described the inhibitory effects of a substance or substances present in mammalian brain and spinal cord, and the name Factor I(1) has been suggested for this principle. In preparing concentrates of Factor I, Florey used as assay for activity the stretch receptor preparation of the crayfish(3). He also indicated that Factor I imitated the action of the inhibitory neurons in crustaceans(1). Equipment was available to us to use the "slow" closer and opener systems(4) of the crayfish claw (*Cambarus virilis*)* as an assay. Although the preparations fatigue rapidly enough to make quantitative evaluation difficult, we found in preliminary experiments that dialyzable aqueous extracts of fresh rabbit and pig brain showed inhibitory activity in both these systems. The activity was not destroyed when extracts were heated at 100°C for 30 minutes in either 0.1 *N* hydrochloric acid or 0.1 *N* sodium hydroxide under nitrogen or oxygen. Activity was not removed from solution by Amberlite IR-45† (OH form) but was removed by Amberlite IR-120† (H form), from which it could be eluted with hydrochloric acid. Activity was lost when dried aqueous extract was treated with ethanolic hydrochloric acid, but was regenerated by heating with dilute aqueous hydrochloric acid. These results, which suggested that the activity was associated with a compound having both a basic group and an esterifiable acidic group, led us to prepare and study the dialyzable, amphoteric fraction of fresh brain.

Methods. Beef brains (85 kg) were collected as soon as possible after slaughter (*ca.* 15 to 30 minutes) and were frozen in dry ice and stored in freezer until use. The frozen brains were pulverized with dry ice, and the resulting powder was added to 100 l of boiling water at such a rate that boiling continued. After 10 minutes the mixture was filtered with the aid of 22.5 kg of Hyflo‡, and the filter cake was washed with 100 l of hot water. The combined extracts were concentrated *in vacuo* to 8 l which contained 2 kg of solids. This was placed in cellulose bags and dialyzed against 80 l of distilled water at room temperature for 44 hours. The dialysate was heated with 500 g of Norit A and filtered. The resulting solution, containing 0.98 kg of solids, was poured through a column of Amberlite IR-120† (H form), 23″ x 6″ diameter. The column was washed with water and eluted with 40 l of 3 *N* hydrochloric acid. The eluate was concentrated to 1 l, diluted with equal volume of alcohol, and the precipitated salts were filtered. The filtrate was concentrated, diluted with water, concentrated again to remove ethanol, diluted, and stirred with 1 kg of Amberlite IR-45† (OH form) to remove excess hydrochloric acid and increase the pH to 4-5. The resulting solution (4 l, 319 g of solids) was made basic (pH 9) with ammonium hydroxide, decolorized with Norit A, and poured through a column of Amberlite IRA-400† (Cl form), 29″ x 2½″ diameter, prewashed with 0.05 *N* ammonium hydroxide until effluent was basic (pH 9). The column was washed with water and eluted with 0.2 *N* hydrochloric acid until eluate was strongly acidic and no longer gave a precipitate with saturated phosphotungstic acid. The eluate was neutralized to pH 5 with Amberlite IR-45† (OH form) and concentrated for use in ion-exchange chromatography. The fraction contained 14 g of solids.

A 1.69-g. aliquot of this material was chromatographed on a 580 mm x 38 mm diameter column of Dowex 50-X12§, 200-400 mesh,

* Supplied by General Biological Supply House, Chicago, Ill.

† Rohm & Haas, Philadelphia, Pa.

‡ Filter aid of Johns Manville, Inc., New York City.

§ Dow Chemical Co., Midland, Mich.

using 1.5 *N* hydrochloric acid as developer. Fractions (10 ml) were collected with automatic fraction collector and were analyzed for nitrogen content by slight modification of the procedure of Johnson(5). Four well separated bands were observed with maximum nitrogen content in the tubes indicated: I, tube 106; II, tube 147; III, tube 160; and IV, tube 185. The distribution of nitrogen recovered in the 4 bands was 16.2, 0.9, 2.3, and 80.6% in bands I to IV respectively. When aliquots of the 4 bands were tested in the opener system of the crayfish claw, I and IV showed inhibition. Band I gave a strong ninhydrin test, and a preliminary paper chromatogram indicated that the substance might be aspartic or glutamic acid. Tests in the opener system showed that the unknown, aspartic acid, and glutamic acid each caused inhibition in the range 10 to 100 γ/ml depending on the particular claw, the frequency of stimulation and age of preparation. When compared in the same claw the unknown and glutamic acid usually appeared slightly more active than aspartic acid. All solutions were adjusted to pH 6.7 before testing.

Additional paper chromatograms confirmed that the unknown was not aspartic and was probably glutamic acid. Proof that the substance was glutamic acid was obtained by evaporation of band I to dryness and recrystallization of residue from 12 *N* hydrochloric acid to give colorless crystals, melting at 190.5 to 191.5°, $[\alpha]_D^{25} + 22.8°$, (*c*, 1.27, 6 *N* HCl), and neutralization equivalent, 90.9 (expected for *l*-glutamic hydrochloride: m.p., 189-191.5°(6): $[\alpha]_D^{22.4}$, 25.0° (free acid, 31.2°(7)): and neut. eq. 91.8).

Evaporation of an aliquot of band IV gave a crystalline residue which was shown to be mostly ammonium chloride by tests with Nessler's reagent and with chloroplatinic acid (8). The inhibitory activity of this material was much greater than that of ammonium chloride, and paper chromatograms indicated a second component which gave a purple color with ninhydrin. It seemed likely that the activity of band IV was due primarily to the presence of this other amino acid which was neither glutamic nor aspartic acid. The recent disclosure(9) that γ-aminobutyric acid has strong Factor I activity in the stretch receptor test, prompted us to make a paper chromatographic comparison of this amino acid with the amino acid in band IV. Mobilities of the substances were the same in 2 solvent systems. Therefore, the activity of band IV is undoubtedly due to the presence of γ-aminobutyric acid.

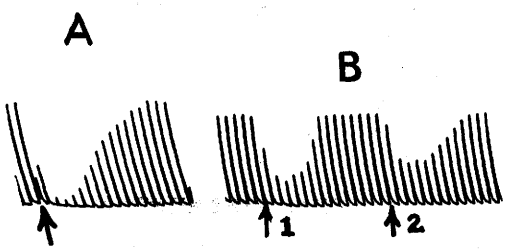

FIG. 1. Movement of dactylopodite of crayfish claws perfused at .5-.6 ml/min. on stimulation of motor axon for 3 sec. every 30 sec. with Grass Stimulator Model S4C giving square wave of .9 mSec. duration at frequency indicated below. At arrows the perfusion tube was momentarily squeezed shut and .5 ml of test solution was inj.: (A) Opener axon, 90 impulses/sec., glutamic acid at 100 γ/ml; (B) Slow closer axon, 30 impulses/sec.; 1, γ-aminobutyric acid at 100 γ/ml; 2, glutamic acid at 500 γ/ml.

The slow closer system of the crayfish claw was less sensitive than the opener system to glutamic and aspartic acids which gave inhibition at 100 to 1000 γ/ml depending on the particular claw, the frequency of stimulation, and age of preparation. Tests with γ-aminobutyric acid indicated that it was 5 to 10 times as active as glutamic acid. Typical kymograph tracings of the activities are shown in Fig. 1. A few experiments were conducted with the crayfish heart perfused with aerated crayfish solution(10). In the presence of 20 to 40 γ/ml of glutamic acid the heart rate became irregular and somewhat slower, and the contractions decreased considerably in amplitude, with the heart tending to remain relaxed. In the presence of *ca.* 100 γ/ml of aspartic acid the heart rate remained regular but became more rapid, and contractions decreased in amplitude, with the heart tending to remain in the contracted state. Neither glutamic acid (10 or 20 mg) nor γ-aminobutyric acid (10 or 20 mg) injected intraperitoneally into 20-g. Webster Swiss mice protected the animals from a lethal dose of strychnine (40 γ) injected 5 min-

utes later. It has been reported that the convulsive and lethal effects of strychnine in mice are prevented by injection of Factor I(11).

In view of the above results it is apparent that there is present in brain more than one substance having some of the activities ascribed to Factor I.

Summary. Extracts of fresh mammalian brain contained substances which inhibited the "slow" closer and opener systems of the crayfish claw. Glutamic acid was one of the substances. Aspartic acid and γ-aminobutyric acid were also active. Glutamic acid had an inhibitory effect and aspartic acid had a stimulatory effect on the crayfish heart.

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Production of Hypercholesteremia in the Rabbit by Infusion of Phosphatide or Neutral Fat.* (22975)

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Recently we reported(1,2) that a rapid rise occurred in plasma cholesterol concentration of the rat, if a *sustained* rise in either its plasma phospholipid or triglyceride was induced initially by intravenous infusion of phosphatide or triglyceride. The mechanism responsible for the hypercholesteremic effect of a sustained hyperphospholipidemia or hypertriglyceridemia has not been determined but it has been observed(3) that a hypercholesteremia occurring after infusion of phosphatide takes place, surprisingly enough, in complete absence of the liver.

To determine whether this hypercholesteremic effect of experimentally induced and maintained hyperphospholipidemia or hypertriglyceridemia was applicable to other species, studies were made upon the rabbit.

Methods. Healthy male rabbits weighing approximately 1500-1800 g and 6 to 8 weeks of age were used. A sustained hyperphospholipidemia was induced in six rabbits for 12 and in 5 rabbits for 24 hours by rapid intravenous administration of 20 ml of 5% phosphatides,† w/v, in 5% dextrose solution

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† The phosphatide mixture was furnished by Chemurgy Division of Glidden Co., labeled Soya Lecithin, oil free, "RG" brand. Approximate composition as furnished by Glidden Co. as follows: Chemical lecithin, 29.5%; chemical cephalin, 29.5%; inositol phosphatides, 31.6%; sugar, sterol glucosides, etc., 5.3%; soybean oil, 3.1%; ether and insolubles and moisture, 1%. $CaCO_3$ is added in manufacture. Sterol content of this substance as measured by Liebermann-Burchard color reaction was 1.1%. Actual amount injected as 5% solution in our experiments therefore contained a negligible quantity of sterol (i.e. $<$ .6 mg/ml). Before use phosphatides were freed from $CaCO_3$.