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A NEW TEST PREPARATION FOR BIO-ASSAY OF FACTOR I AND GAMMA-AMINOBUTYRIC ACID

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During our recent attempts to characterize 'inhibitory' agents (Factor I) in crustacean nerve extracts, we have found that the responses of the isolated hind gut of crayfish can be successfully used for bio-assay of inhibitory actions of nerve extracts, eluates of chromatograms and drugs. Although some pharmacological characteristics of the crayfish intestine have already been described (Florey, 1954*a*, *b*) it appears now worth while to report on the technique of using the isolated intestine preparation for the bio-assay of Factor I activity, and on some newly discovered pharmacological properties. The new method is simpler and more sensitive than that using crayfish stretch receptors.

METHODS

Three species of crayfish have proved equally useful. These were *Procambarus clarkii*, *Orconectes virilis* and *Pacifastacus leniusculus*. The animals should have a minimum body length of 4 in. (10 cm). After the dorsal half shell of the abdomen is removed, the hind gut can be seen as a straight tube which runs along the mid line towards the last abdominal segment. The whole length of the abdominal intestine should be excised. It is advisable gently to empty it of its contents with the aid of a blunt instrument and to flush the lumen with saline medium. The organ should be mounted in a long and narrow muscle chamber. The later performance of the preparation is greatly improved if the gut is filled and slightly distended with saline medium before the second ligature is tied. The proximal end of the gut is then connected to a well balanced light lever so as to give a tenfold magnification of the contractions. The movements of the intestine are recorded on the smoked drum of a slow-moving kymograph. Only glossy kymograph paper should be used, in order to offer minimal frictional resistance to the writing stylus.

A saline medium of the following composition has been found suitable (g): NaCl 12·0, KCl 0·4, $CaCl_2$ (anhydrous) 1·5, $MgCl_2.6H_2O$ 0·5, H_2O to 1000 ml. No buffer is needed, but the muscle bath should be aerated with a constant stream of fine air bubbles to achieve oxygenation and rapid mixing of the bathing media. Organ and solutions are held at room temperature.

Before application of test samples, these have to be freed from all traces of organic solvents and any acid or base must be neutralized. Although it is advisable to make the samples isotonic with the saline medium used, by adding 1/9 volume of 10 times concentrated saline stock solution (K-free where necessary), this is not very important because the samples are

usually so greatly diluted in the bath that the change in the osmotic concentration of the bathing medium does not exceed 10%.

Before the addition of samples to the bath the volume of the latter is reduced to 9/10 of the standard level. An amount of the sample is then added to bring the bath up to the original level. In our tests we use a total bath volume of 2 ml., the sample volumes added are therefore 0·2 ml.

Before the test proper one has to find a concentration of acetylcholine which will cause a marked but submaximal increase in 'spontaneous' contractions and a definite rise of the base line. A concentration of acetylcholine at 10^{-7} g/ml. bathing medium was found adequate in most tests.

The test for inhibitory action of samples consists in the application of suitable concentrations of a test sample and subsequent addition of acetylcholine. Reduction of spontaneous contractions and depression of the response to acetylcholine are criteria for inhibitory actions.

RESULTS

The actions of Factor I, amino acids and picrotoxin

Factor I has originally been defined as that agent in nerve extracts which is responsible for inhibition of impulse generation in the slow-adapting neurone of the abdominal stretch receptor organs of crayfish (Florey, 1954*a*). It has later been shown that several substances occurring in nerve tissue can inhibit the stretch receptor neurone. Some of these are known: beta-alanine, guanido-acetic acid, gamma-aminobutyric acid, beta-hydroxy-gamma-aminobutyric acid, gamma-guanidinobutyric acid and gamma-aminobutyro-choline (Bazemore, Elliott & Florey, 1957; McLennan, 1959). Other components of Factor I preparations which have inhibitory activity on the stretch receptors are still unknown. One of them is referred to by McLennan as Fraction A of Factor I. A similar compound has been chromatographically isolated from crustacean nerve tissue by Florey & Chapman (1961) who refer to it as Substance I (Florey, 1960). Fraction A and Substance I seem to be identical.

Extracts of vertebrate and crustacean nerve tissue which have inhibitory actions on crayfish stretch receptors depress the spontaneous activity of the isolated crayfish intestine and block the stimulatory action of acetylcholine on this organ. The sensitivity of the intestine to inhibitory agents is greater than that of the stretch receptors, but it can be shown that the inhibitory substances which are effective on the two organs are the same.

Aqueous extracts of brains of rats, rabbits and cattle were found to depress spontaneous and acetylcholine-induced contractions of the crayfish intestine in concentrations up to five times lower than those which show liminal effectiveness on the stretch-receptor preparation. Aqueous and methanol extracts of lobster (*Homarus americanus*) and crab (*Cancer magister*) nerves were found to be 20–25 times as active if tested on the intestine, compared to their action on stretch receptors. By means of paper chromatography (see Florey & Chapman, 1961) it could be demon-

strated that the compounds which inhibit the stretch receptors are the same as those which inhibit the intestine of the crayfish. This is true for components of extracts from nerve tissue of both crabs and mammals. It is difficult to express the activities of the inhibitory components in terms of absolute concentrations of the compounds, since their nature and amount in nerve tissue has not been established (with the exception of certain amino acids which will be dealt with below). It can, however, be stated that the lowest effective concentrations of extracts of mammalian brain (rat, rabbit, cattle) are 10–25 mg fresh tissue/ml. for stretch receptors and 5–10 mg/ml. for isolated intestines. For extracts of crab nerve extracts (*Cancer magister*) the concentrations are 10–25 and 0·5–1 mg/ml. respectively. Details of the experiments involving paper chromatography will be published later.

Gamma-aminobutyric acid was found to inhibit spontaneous and acetylcholine-induced contractions of the crayfish intestine in liminal concentrations of 2×10^{-6} g/ml. Inhibitory actions are produced by a number of other compounds of similar chemical nature. These substances and their liminal concentrations (g/ml.) are: guanido-acetic acid 10^{-6}; glutamic acid, 5×10^{-5}; monosodium glutamate, 5×10^{-5}; beta-hydroxy-gamma-aminobutyric acid, $2 \cdot \times 10^{-5}$; gamma-aminobutyrylcholine, 5×10^{-5}; beta-alanine, 10^{-4}; aspartic acid, 2×10^{-5}; taurine 10^{-4}.

The following compounds were found to have no detectable action in concentrations as high as 10^{-4} g/ml.; gamma-guanidinobutyric acid; succinylcholine chloride; L-alanine; pyroglutamic acid; acetylglutamic acid; DL-methylglutamic acid; L-histidine; acetylhistidine; DL-phenylalanine; DL-alpha-phenyl-alpha-alanine; L-leucine; DL-acetylleucine; DL-tyrosine; L-cysteine; S-carbamyl-l-cystein; DL-proline; DL-acetylproline; creatine; creatinine; L-tryptophan; cadavarine HCl; canavanine sulphate; sarcosine; carnitine HCl and carnosine.

The following compounds were found to have a stimulatory action, causing an increase in strength and frequency of the contractions at the following liminal concentrations (g/ml.): acetylcholine, 10^{-8}; acetyl-beta-methylcholine, 2×10^{-6}; butyrylcholine, 2×10^{-6}; adrenaline, 10^{-6}; noradrenaline, 10^{-6}; 5-hydroxytryptamine, 2×10^{-6}; picrotoxin, 5×10^{-5}. Atropin in a concentration of 5×10^{-6}–5×10^{-7} g/ml. is an effective blocking agent for acetylcholine, acetyl-beta-methylcholine and butyrylcholine. It does not interfere with the action of the other excitatory compounds. Picrotoxin in concentrations from 10^{-5} to 10^{-6} g/ml. effectively reduces or blocks the action of all inhibitory compounds mentioned, including the chromatographically isolated inhibitory components of nerve extracts. It does not interfere with the action of the excitatory compounds mentioned. In somewhat higher concentrations, such as 5×10^{-5} g/ml.,

picrotoxin has a slight stimulatory action. Atropin if applied in higher concentrations, such as 10^{-5} g/ml., depresses the spontaneous activity of the intestine. Representative responses of the isolated crayfish intestine to nerve extracts and drugs are shown in Fig. 1.

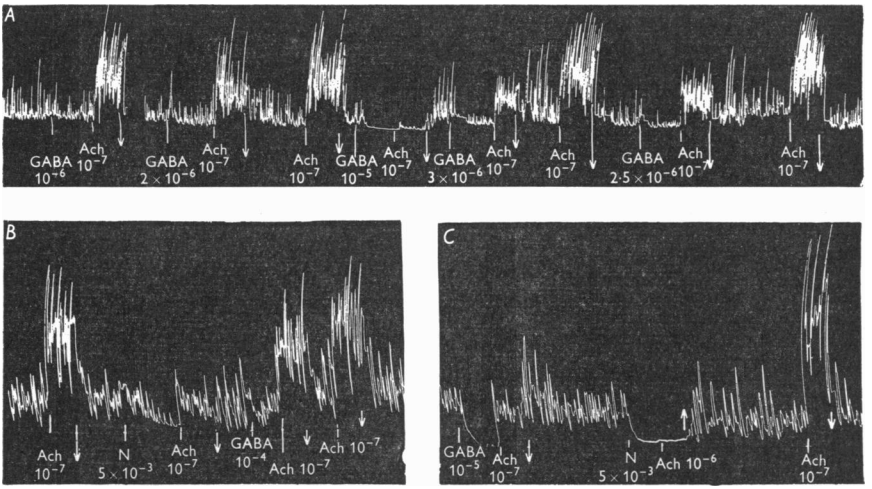

Fig. 1. Responses of an isolated intestine of *Pacifastacus leniusculus* to applied drugs and nerve extract (N). *A*: interaction of gamma-aminobutyric acid (GABA) and acetylcholine (ACh); note the graded response. *B*: onset of the action of picrotoxin which is maintained at a concentration of 10^{-5}g/ml; note that GABA even in high concentration is unable to prevent the action of ACh; the inhibitory action of a nerve extract (N) is partially blocked. *C*: after 15 min washing the inhibitory actions of GABA and N are restored. The record *A* was obtained from a different organ from those of *B* and *C*. The arrows indicate washing. In *B* the washing solution contained picrotoxin.

The method of bio-assay

The isolated crayfish intestine can be used for bio-assay of Factor I activity in the following manner: after a concentration of acetylcholine has been found which causes marked but submaximal contractions, 1/10 volume of the unknown sample is added to 9/10 volume of the bath. Thirty seconds later 1/10 volume of fluid is removed from the bath and 1/10 volume of the appropriate acetylcholine solution is added. One minute later the bath is emptied and the preparation is washed twice with pure saline medium. After an interval of 2 min the volume of the bath is reduced to the 9/10 level and 1/10 volume of the acetylcholine solution, as used before, is added. After 1 min this is followed by a double washing. Two minutes later another dilution of the same unknown can be applied, to be followed 30 sec later by addition of acetylcholine as described above.

Inhibitory action is characterized by the depression of spontaneous

contractions and depression of the acetylcholine action. The inhibitory activity of an unknown sample can be standardized against known concentrations of gamma-aminobutyric acid. For this purpose it is necessary to find a concentration of gamma-aminobutyric acid which will reduce the action of acetylcholine by an amount equal to that achieved with a certain concentration of the unknown. The most economic way to do this is first to find the lowest concentration of the unknown which will still definitely depress the acetylcholine action and then to find a dilution of gamma-aminobutyric acid which will match the effect of the unknown. Once such a concentration of gamma-aminobutyric acid is found, the unknown should be applied once more to make certain that the level of sensitivity of the test organ has remained the same.

Differences in inhibitory activity of plus or minus 20% are readily detectable with this test. The inhibitory activity can be expressed in terms of μg gamma-aminobutyric acid/g fresh nerve tissue. For instance, if an extract diluted to 1:1000 (original nerve tissue 1 mg/1 ml. bath) causes an inhibition comparable to that produced by the application of 10 μg of gamma-aminobutyric acid/ml., 1 g of the original nerve tissue from which the extract was prepared must have contained (at least) as much inhibitory activity as 1000×10 μg of gamma-aminobutyric acid.

At the end of the assay procedure picrotoxin should be added to the saline medium in the bath, to give a final concentration of 2×10^{-5} g/ml. After the picrotoxin solution has been in contact with the preparation for 10 min, gamma-aminobutyric acid and unknowns are applied once more to ascertain the Factor I nature of the unknown. Gamma-aminobutyric acid serves as a control. Both inhibitory actions should be prevented or greatly reduced by the presence of picrotoxin in the bath. The effects of acetylcholine-blocking agents such as atropine are not changed by picrotoxin.

DISCUSSION

Although the bio-assay for Factor I activity by means of the crayfish stretch receptor preparation, as described by Elliott & Florey (1957) and Florey & Elliott (1961), is more precise, the new bio-assay method has the advantage of greater sensitivity and simplicity. Not only is the dissection the easiest possible, but the equipment needed for the tests is more readily available and the assay can be carried out by persons who are not familiar with electronic apparatus.

The physiology of the test organ is, unfortunately, little understood. The intestine is innervated by two large lateral nerve strands, which originate in the sixth abdominal ganglion. The nerves usually remain attached to the intestine when this is removed from the animal. The responses of the intestine are, however, not altered if they are dissected

off. Alexandrowicz (1909) has shown that there are numerous nerve cells within the wall of the intestine. We know that stimulation of the lateral nerves induces a contraction and peristaltic wave, but it is uncertain whether this action is a direct one on the muscle or whether it is mediated by the nerve cells. It is likely that the action of acetylcholine is through stimulation of the nervous elements of the intestine, for the following reasons: The musculature of the intestine is of the striated type and none of the muscles of the crayfish responds to acetylcholine. Crustacean nerve cells are stimulated by acetylcholine and 5-hydroxytryptamine (Wiersma, Furshpan & Florey, 1953; Florey, 1954*c*). Adrenaline and noradrenaline stimulate the intestine. Atropine blocks the action of acetylcholine but not that of adrenaline or noradrenaline. Factor I and gamma-aminobutyric acid, on the other hand, block the actions both of acetylcholine and of adrenaline and noradrenaline. This means that during atropine action the intestinal musculature is still excitable (by adrenergic agents), while during Factor I action it is not. One could thus conclude that acetylcholine activates non-cholinergic nerve cells which are responsible for the 'spontaneous' activity of the intestinal musculature, and that Factor I as well as gamma-aminobutyric acid acts at the neuromuscular junctions and—quite likely—on the nerve cells as well. Since Factor I and gamma-aminobutyric acid prevent the excitatory action of adrenaline and noradrenaline, it is very unlikely that their mechanism of action consists in (competitive) inhibition of cholinoceptive receptor molecules.

The relative insensitivity of the intestine towards common amino acids which tend to obscure bio-assays using the crayfish stretch receptor preparation is of great advantage to those interested in the chemical isolation and identification of 'inhibitory' substances. The extraordinary inhibitory action of crustacean nerve extracts, as described in the preceding section, points once more to the fact that gamma-aminobutyric acid is not likely to be the transmitter substance (see Florey, 1960; Florey & Biederman, 1960; Florey & Chapman, 1961).

SUMMARY

1. The spontaneous contractions of the isolated hind gut of crayfish are greatly enhanced by acetylcholine and depressed by certain inhibitory substances that can be isolated from mammalian and crustacean nerve extracts.

2. The agents responsible for the inhibitory actions are identical with those which inhibit impulse generation in the crayfish stretch receptor preparation. The crayfish gut can, therefore, be used to detect compounds with Factor I activity. It is very much more sensitive than the stretch receptor.

3. Substances with Factor I activity (in order of decreasing activity) are gamma-aminobutyric acid, guanido-acetic acid, beta-hydroxy-gamma-aminobutyric acid, aspartic acid, glutamic acid, monosodium glutamate, gamma-aminobutyrylcholine, beta-alanine and taurine. All these block spontaneous contractions and the action of acetylcholine. The inhibitory action of these compounds is prevented by picrotoxin.

4. Compounds are listed which are excitatory and others which are without effect on the crayfish gut.

5. Crustacean nerve extracts contain inhibitory material which appears to be very much more inhibitory than gamma-aminobutyric acid.

6. A method of bio-assay is described which permits quantitative evaluation of Factor I activity using the action of known concentrations of gamma-aminobutyric acid as standards.

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