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Marine Invertebrate Preparations useful in the Bioassay of Acetylcholine and 5-Hydroxytryptamine

CERTAIN isolated muscle and heart preparations of marine invertebrates are highly sensitive to acetylcholine, to 5-hydroxytryptamine (enteramine, serotonin), or to both of these substances. Bacq[1] was the first and Ambache and Sawaya[2] among the more recent to demonstrate the sensitivity to acetylcholine of muscle strips from holothurians (Echinodermata). Inhibition of heart-beat of the bivalve mollusc, *Venus mercenaria*, was first used by Welsh[3] for the quantitative estimation of acetylcholine in tissue extracts. Recently, the same heart has been found to respond by increases in amplitude and frequency to low concentrations of 5-hydroxytryptamine[4]. Earlier, it had been reported[5,6] that certain molluscan hearts had a low threshold to 5-hydroxytryptamine or tissue extracts containing this substance. At the Marine Biological Laboratory, during the summer of 1953, the need arose for the quantitative estimation of acetylcholine and 5-hydroxytryptamine in certain tissues. Several species of echinoderms and molluscs from the Plymouth region were secured, and isolated muscle and heart preparations were screened for their suitability in bioassay of these substances. Available holothurians were *Holothuria nigra*, *Cucumaria normani*, *C. elongata*, and a single specimen of *Thyone* sp. Pieces or entire lengths of the longitudinal muscle bands were dissected free from the inner body wall and set up in the manner commonly used for muscle strips during recording of contractions. In the case of *C. normani* the peristomal (pharyngeal) retractor muscles were also tried. While all these preparations contracted in low concentrations of acetylcholine, especially after eserinization, the difficulties that were encountered by earlier workers[1,7] who had used other species of holothurians were obvious. Chief among these were spontaneous contractions, tendency to contract and remain contracted after eserine, and sensitivity to mechanical stimulation after eserine. This last was especially marked in the muscles from the single specimen of *Thyone* which, otherwise, were most promising since they were not spontaneously active.

Of these holothurians from the Plymouth region, *H. nigra* was considered most suitable. Its longitudinal body-wall muscles responded to as little as 5×10^{-9} gm./ml. acetylcholine chloride before eserine and often were not spontaneously active. The response to acetylcholine was nearly maximal at 10^{-7} gm./ml. Although a low concentration of eserine (10^{-6} gm./ml.) produced or increased spontaneous activity, it lowered the threshold to acetylcholine about a hundred-fold. When an acetylcholine contraction was superimposed on minimal spontaneous activity, a satisfactory quantitative measure of action was obtained. Isolated muscles of *H. nigra* were kept for twenty-four hours in aerated sea-water and there was little or no change in sensitivity to acetylcholine. The responses of holothurian muscle to 5-hydroxytryptamine were not observed.

Hearts from *Pecten maximus*, *Modiolus modiolus*, *Cyprina islandica* and *Buccinum undatum* were tested for their sensitivity to acetylcholine and 5-hydroxytryptamine. The heart of *Pecten* was cannulated and perfused *in situ* and also isolated and perfused. Much difficulty was encountered in maintaining a heart with a regular beat for an adequate period of time, and the sensitivity to acetylcholine and 5-hydroxytryptamine was low. The hearts of *Modiolus* and *Cyprina* were isolated and suspended in a bath, without cannulation, after the method employed with the heart of *Venus mercenaria*[3]. The *Modiolus* heart had a regular beat that held for many hours, but it was extremely insensitive both to acetylcholine and 5-hydroxytryptamine. In one instance no inhibition with acetylcholine was obtained at a concentration of 10^{-6} gm./ml. and no measurable excitation with 5-hydroxytryptamine at 10^{-7} gm./ml. Higher concentrations were not tested.

Three different hearts of *Cyprina* were tested. All beat at a slow rate but with a regular rhythm. The threshold dose of acetylcholine was about 5×10^{-10} gm./ml. With increasing doses the heart-rate was slowed in a graded manner; but there was no graded decrease in amplitude. At about 10^{-8} gm./ml. the heart stopped beating. The beat was quickly restored on washing. Eserine failed to potentiate the action of acetylcholine significantly. The *Cyprina* heart responded to 5-hydroxytryptamine with an increase in amplitude and frequency. Threshold was near 10^{-10} gm./ml. and a graded action was obtained up to 10^{-6} gm./ml.

On the *Venus* heart 'Mytolon' (supplied through kindness of Sterling–Winthrop Research Institute) is the most effective known acetylcholine antagonist[9,10]. Likewise, lysergic acid diethamide (*LSD*-25) (supplied through kindness of Sandoz Products, Ltd.) is the most effective antagonist for 5-hydroxytryptamine yet found for the *Venus* heart[11]. 'Mytolon' was found to be a highly effective antagonist for acetylcholine and *LSD*-25 for 5-hydroxytryptamine on the heart of *Cyprina*.

Considering relative abundance, hardiness of the animals and regularity and responsiveness of the heart to the substances to be assayed, the whelk, *Buccinum*, proved most satisfactory. Hence it was used in the identification and estimation of acetylcholine and an excitor substance, probably 5-hydroxytryptamine, in molluscan tissues. A detailed account of the results will appear elsewhere. The *Buccinum* heart was removed and a cannula inserted into the ventricle, usually by way of the auricle, but sometimes by way of the aorta if the auricle was accidentally torn from the ventricle. The attachment of the thin-walled auricle to the thick-walled ventricle is delicate, and two or three separate ligatures of rather coarse thread avoided the cutting and tearing that otherwise sometimes occurred. The *Buccinum* ventricle was perfused with sea-water, as were the other molluscan hearts and holothurian muscles. It was necessary to distend the ventricle with a small head of pressure to start it beating and to introduce drugs and extracts under the same pressure used in perfusing and washing.

The *Buccinum* ventricle had thresholds to acetylcholine and 5-hydroxytryptamine near those of *Cyprina*. The response to acetylcholine, however, is a decrease in amplitude with little change in frequency. The frequency increase produced by 5-hydroxytryptamine is often accompanied by an increase in tonus. 'Mytolon' blocked acetylcholine and *LSD*-25 blocked 5-hydroxytryptamine, as on the *Cyprina* heart. These highly effective antagonists were used in obtaining estimates of the amounts of acetylcholine and 5-hydroxytryptamine occurring together in a given tissue. Preliminary evidence that the heart of *Venus mercenaria* has an excitor innervation mediated by 5-hydroxytryptamine was recently presented[4]. Nerve ganglia and certain other tissues of molluscs from the Plymouth region were found to contain mixtures of heart-exciting and -inhibiting substances. Reliable quantitative estimates could be made only when the test heart was treated with 'Mytolon' or with *LSD*-25 to block one or the other substance. Since leaving Plymouth, we have found by chromatography and biological tests that ganglia of *Venus mercenaria* and the whelk, *Busycon canaliculatum*, contain 5-hydroxytryptamine and acetylcholine. It is likely that these were the substances present in the tissues studied at Plymouth.

Other large molluscs were not available at Plymouth; but Prof. Brodie Hughes, at the University of Birmingham, has kindly permitted me to say that he has tested the sensitivity of the isolated heart of *Mya arenaria* to acetylcholine and has used it in the bioassay of acetylcholine in cerebrospinal fluid. A record sent me by Prof. Hughes was made in February. The record shows a decrease in amplitude of about 30 per cent when 0·005 μgm./ml. acetylcholine was present in the bath. The decrease in amplitude with 0·006 μgm./ml. was nearly 50 per cent and with 0·01 μgm./ml. the amplitude was reduced to zero. Prof. Hughes has noted that *Mya* hearts become much more sensitive from the months of April to June. A similar seasonal shift in responsiveness of the *Venus* heart to acetylcholine was first noted by Prosser[12], who found them most sensitive in late winter and spring. Whether this is generally true of marine molluscs from northern waters is not known.

It is apparent from this brief survey that there are several species of marine molluscs in British waters the hearts of which may be useful in the determination and estimation of small amounts of acetylcholine and 5-hydroxytryptamine. It is also clear that there are holothurian species the muscles of which respond to low concentrations of acetylcholine.

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