J. Mar. biol. Ass. U.K. (1956) 35, 193–201
Printed in Great Britain

NEUROHORMONES OF INVERTEBRATES

I. CARDIO-REGULATORS OF *CYPRINA* AND *BUCCINUM*

By John H. Welsh

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts, U.S.A.

(Text-figs. 1–5)

The present study had two aims: first to seek among the molluscs of the Plymouth region, one with a heart suitable for the bioassay of acetylcholine and 5-hydroxytryptamine; and secondly, to examine molluscan nervous tissue for the presence of cardio-regulator substances. Brief reports of some of the results have appeared elsewhere (Welsh, 1953*a*, 1954*a*).

Material and Methods

Following a preliminary study of the hearts of *Pecten maximus* (L.), *Modiolus modiolus* (L.), *Mytilus edulis* L., *Cyprina islandica* (L.), and *Buccinum undatum* L., the last two were considered most promising for purposes of bioassay. The common whelk, *Buccinum*, was available in adequate numbers but the supply of the bivalve, *Cyprina*, was limited.

The *Cyprina* heart was isolated and perfused in the same manner as previously described for the heart of *Venus mercenaria* (Welsh & Taub, 1948). The *Buccinum* heart was exposed and a glass cannula inserted into the ventricle either by way of the auricle or the aorta. When the cannula was inserted via the auricle, two or three separate ligatures of rather coarse thread were used to secure the cannula. This usually prevented the tearing of the delicate auricle from the thick-walled ventricle. The *Buccinum* ventricle was perfused in a manner similar to that used by Alexandrowicz & Carlisle (1953) in perfusing hearts of decapod crustaceans. Sea water was the perfusion fluid.

When examining a tissue for the presence of acetylcholine (or acetylcholine-like substance) an extract was made by grinding with sand in the presence of eserine, 1:10,000, or grinding after heating the tissue at 100° C for a few minutes. When looking for an opposing, heart-exciting substance, a tissue was ground and allowed to stand at room temperature for 30 min before filtering, or, the extract that had been made with eserinized sea water was brought to pH 9–10 with NaOH and boiled for 10 min to hydrolyse the acetylcholine.

Several drugs were used to help in the identification of the suspected cardio-regulator substances. Among them were lysergic acid diethylamide or LSD (supplied by Sandoz Products Ltd., London), 5-hydroxytryptamine (supplied

as serotonin creatinine sulphate by the Abbott Laboratories), and 2:5 bis-(3-diethylaminopropylamino)-benzoquinone bis-benzylchloride or mytolon (supplied by Sterling-Winthrop Research Institute).

Concentrations of drugs are expressed in grams per millilitre of fluid surrounding the heart (*Cyprina*) or passing through the heart (*Buccinum*); thus: 10^{-9} ACh = 10^{-9} g acetylcholine chloride per millilitre of perfusing fluid or 0·001 μg/ml.

Results

The Cyprina *heart*

This heart was tested for its usefulness in the bioassay of acetylcholine and 5-hydroxytryptamine. The threshold to acetylcholine lay between 10^{-10} and 10^{-9} g/ml. (see Fig. 1). Unlike the *Venus* heart, which usually shows a graded slowing and a graded decrease in amplitude of the beat with gradually increasing concentrations of acetylcholine, the *Cyprina* hearts which were tested showed only a slowing. One heart with a normal frequency of 12 beats/min was unaffected by 10^{-10} acetylcholine; at 10^{-9} acetylcholine the frequency was 9·5 beats/min; at $2{\cdot}5 \times 10^{-9}$ acetylcholine, 8 beats/min; at 5×10^{-9} acetylcholine, 6 beats/min; while at 10^{-8} acetylcholine the heart stopped.

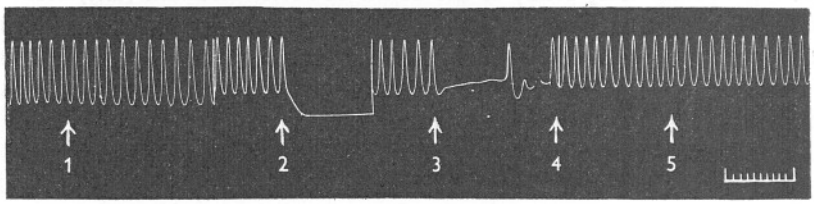

Fig. 1. Records of the action of acetylcholine ACh on the isolated heart of *Cyprina islandica*. (1) ACh 10^{-9} g/ml.; (2) ACh 10^{-8} g/ml.; (3) ACh 5×10^{-9} g/ml.; (4) Mytolon 10^{-6} g/ml. for 10 min, drum stopped; (5) ACh 10^{-8} g/ml. Kymograph stopped and heart washed between each test. Time units = 6 sec and 1 min.

Mytolon, a neuromuscular blocking agent in vertebrates, is the most effective acetylcholine antagonist known for the *Venus* heart (Luduena & Brown, 1952; Welsh & Taub, 1953). It is also highly effective as an antagonist to acetylcholine on the *Cyprina* heart. In Fig. 1 the action of 10^{-8} acetylcholine is recorded before mytolon was added to the bath and again after the heart had been exposed to 10^{-6} mytolon for 5 min. This concentration of mytolon completely antagonized a dose of acetylcholine that earlier had caused diastolic stoppage. Treatment of a heart with mytolon makes it possible to estimate the amount of excitor substance in an extract when acetylcholine is also present.

The *Venus* heart responds to 5-hydroxytryptamine with an increase in both frequency and amplitude (Welsh, 1953*b*). The same is true for the *Cyprina* heart (Fig. 2). Threshold is near 10^{-10} 5-hydroxytryptamine, but in contrast

to acetylcholine action there is a graded response over a wide concentration range. Certain of the ergot alkaloids have a remarkable excitatory action on the *Venus* heart (Welsh & Taub, 1948; Welsh, 1953*b*). LSD is a synthetic derivative of the ergot alkaloids. Since LSD had been shown by Gaddum (1953) to be an effective antagonist for 5-hydroxytryptamine on certain vertebrate smooth muscles it was tested on the *Cyprina* heart. It proved to be a very satisfactory antagonist, as may be seen in Fig. 2.

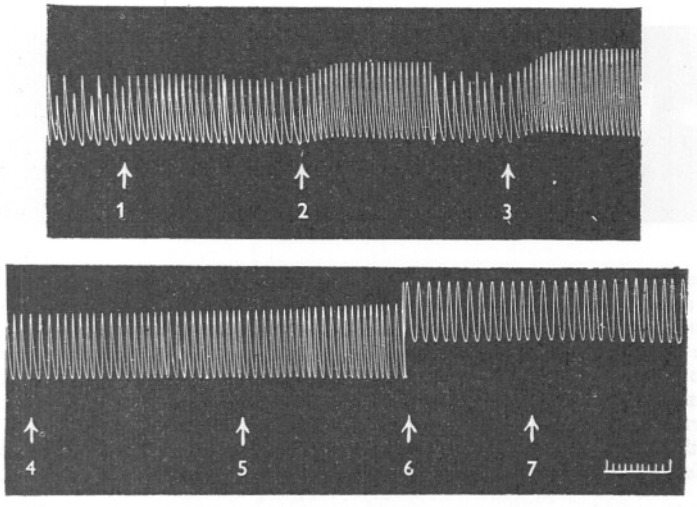

Fig. 2. Records of the action of 5-hydroxytryptamine (5-HT) on the isolated heart of *Cyprina*. (1) 5-HT 5×10^{-9} g/ml.; (2) 5-HT 5×10^{-8} g/ml.; (3) 5-HT 5×10^{-7} g/ml.; (4) LSD 10^{-6} g/ml.; (5) 5-HT 5×10^{-8} g/ml.; (6) LSD 10^{-6} g/ml.; (7) 5-HT 5×10^{-7} g/ml. Heart washed after each test with 5-HT. Time units = 6 sec and 1 min.

From these results, obtained with a limited number of hearts, it is suggested that the *Cyprina* heart (ventricle) should be a useful object for the quantitative estimation of small amounts of acetylcholine and 5-hydroxytryptamine in tissue extracts and biological fluids. Also, it appears probable that the *Cyprina* heart, like the *Venus* heart, is doubly innervated, with the inhibitor nerves releasing an acetylcholine-like substance and the excitor nerves a 5-hydroxytryptamine-like substance.

The Buccinum *heart*

Acetylcholine produces a decrease in amplitude of beat of the *Buccinum* heart with little effect on frequency (Fig. 3). The threshold is below 10^{-9}, and complete inhibition occurs at about 10^{-8} acetylcholine. Mytolon is an effective acetylcholine antagonist (no record of its action is illustrated).

The threshold to 5-hydroxytryptamine is between 10^{-10} and 10^{-9}. A graded response occurs over a wide range of concentrations. The response may be an increase in amplitude with little change in frequency or rise in base-line (Fig. 4), or there may be a marked rise in base-line and increase in frequency as seen in Fig. 5. This variation in response is largely due to the manner of perfusion. A heart from which the perfusion fluid escapes readily is less likely to shorten and beat in a fast, irregular manner, even when strongly excited by 5-hydroxytryptamine.

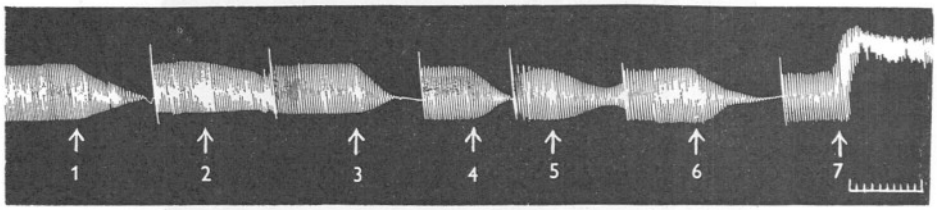

Fig. 3. Records of the actions of extracts of *Buccinum* ganglia and salivary glands compared with acetylcholine (ACh) on the isolated heart (ventricle) of *Buccinum*. (1) Extract of *Buccinum* ganglia made with eserinized sea water. Active material from one milligram of tissue per millilitre of perfusion fluid; (2) ACh 10^{-9} g/ml.; (3) ACh 10^{-8} g/ml.; (4) ACh 5×10^{-9} g/ml.; (5) ACh 2×10^{-9} g/ml.; (6) *Buccinum* ganglion extract as in record 1; (7) extract of salivary glands of *Buccinum* made with sea water (no eserine). Active material from 1 mg tissue/ml. perfusion fluid. Time units = 6 sec and 1 min. (From these records, if the action of the ganglion extract is taken to be equal to 5×10^{-9} AChCl, *Buccinum* ganglia may be considered to contain the equivalent of 5 μg AChCl/g tissue.)

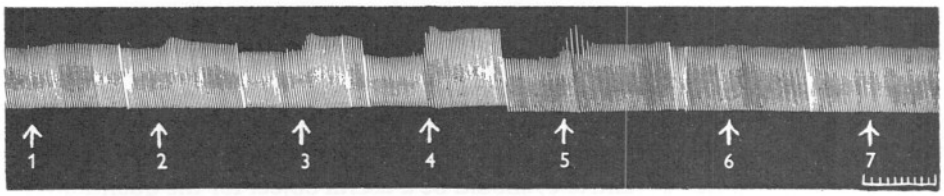

Fig. 4. Records of the action of 5-hydroxytryptamine (5-HT) on the isolated *Buccinum* heart and the blocking action of lysergic acid diethylamide (LSD). (1) 5-HT 5×10^{-9} g/ml.; (2) 5-HT 5×10^{-8} g/ml.; (3) 5-HT 5×10^{-7} g/ml.; (4) 5-HT 5×10^{-6} g/ml.; (5) LSD 10^{-5} g/ml.; (6) 5-HT 5×10^{-7} g/ml.; (7) 5-HT 5×10^{-6} g/ml. Time units = 6 sec and 1 min.

LSD blocks the action of 5-hydroxytryptamine on the *Buccinum* heart. While 5-hydroxytryptamine is readily washed out of the heart, LSD is not. From a concentration of 10^{-5} LSD in the bath, the amount adsorbed by the heart in a period of a few minutes is such that after prolonged washing the excitor action of added 5-hydroxytryptamine is effectively abolished (Fig. 4).

Cardio-excitor and inhibitor substances in molluscan tissues

When the paired, visceral ganglia of *Venus mercenaria* are stimulated electrically, the heart beat is depressed or the heart stops, depending on the voltage and frequency of the stimuli. Prosser (1940) presented evidence that this inhibition of the *Venus* heart resulted from the release of acetylcholine from endings of cardio-inhibitor neurons that had their cell bodies in the visceral ganglia. The results from stimulating isolated hearts led Welsh & Slocombe (1952) to a similar conclusion.

TABLE I. ACETYLCHOLINE CONTENT OF GANGLIA AND HEARTS OF MOLLUSCS

Class of Mollusc	Species	Tissue	ACh μg/g	Reference
Gastropoda	*Helix pomatia*	Ganglion 'cérébroides'	12	Corteggiani (1938)
	Haliotis tuberculata	Ganglion 'cérébroides'	20	Corteggiani (1938)
	Aplysia depilans	Peri-oesophageal	2–3	Bacq (1935)
	Busycon canaliculatum	Pooled ganglia, except visceral	3–9, av. 5·5	Welsh (unpublished)
Lamellibranchiata	*Venus mercenaria*	Pooled ganglia	1–5, av. 2	Welsh (unpublished)
Cephalopoda	*Octopus vulgaris*	Cerebral ganglia	77	Bacq (1935)
	O. vulgaris	Cerebral ganglia	90	Corteggiani (1938)
	Sepia officinalis	Cerebral ganglia	80	Corteggiani (1938)
Gastropoda	*Aplysia depilans*	Heart	0·2*	Vincent & Jullien (1938)
	Helix pomatia	Heart	2·5–5	Vincent & Jullien (1938)
	Limnaea stagnalis	Heart	5·3–5·5	Vincent & Jullien (1938)
	Murex brandaris	Heart	21	Vincent & Jullien (1938)
	M. trunculus	Heart	23–35	Vincent & Jullien (1938)
Lamellibranchiata	*Anodonta cygnea*	Heart	0·3–0·4	Vincent & Jullien (1938)
	Ostrea edulis	Heart	0·7	Vincent & Jullien (1938)
	Mytilus galloprovincialis	Heart	0·12	Vincent & Jullien (1938)
	Venus mercenaria	Heart	0·1	Welsh (unpublished)
Cephalopoda	*Octopus vulgaris*	Heart	0·1	Vincent & Jullien (1938)
	O. vulgaris	Heart	0·2	Bacq (1935)
	Sepia officinalis	Heart	0·1	Vincent & Jullien (1938)

* See Vincent & Jullien (1938) for values from other species and by a different method of extraction.

Nerve tissue from a variety of molluscs has been shown to contain acetylcholine (or a closely related substance). If the mollusc heart is innervated by cholinergic neurons it, too, should yield acetylcholine. This appears to be true. In Table I will be seen some of the published values for acetylcholine equivalents in molluscan nerve tissue and hearts as well as some unpublished values obtained by the author.

When the cerebral ganglionic mass of *Buccinum* was extracted in eserinized sea water, a heart-depressing substance was found to be present (Record 1, Fig. 5). This substance had an action on the *Buccinum* heart resembling that of acetylcholine. When a portion of this same extract was brought to pH 9–10 with NaOH and boiled for a few minutes, its effect on the heart was excitatory (Record 2, Fig. 5). The excitor action of the extract closely resembled that of 5-hydroxytryptamine.

If there are two sets of cardio-regulator nerves innervating the molluscan heart, demonstration of excitor and inhibitor substances in extracts of heart tissue might be possible. Extracts of hearts (ventricles and auricles) of *Buccinum*, *Pecten* and *Mytilus* were found to have excitor or inhibitor actions on the *Buccinum* heart depending on the method of preparing the extract.

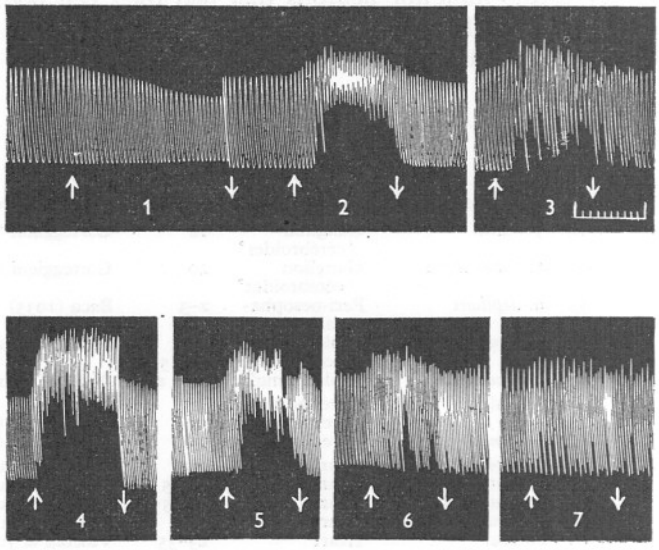

Fig. 5. Records of the action of extracts of *Buccinum* ganglia and salivary glands compared with 5-hydroxytryptamine (5-HT) on the isolated *Buccinum* heart. (1) Extract of ganglia made with eserinized sea water; active material from 1 mg tissue per ml. of perfusion fluid. (2) Part of same extract as in record 1 after bringing to pH 9–10 and boiling. (3) Extract of salivary glands made with sea water (no eserine); active material from 0·1 mg tissue per ml. perfusion fluid. (4) 5-HT 5×10^{-7} g/ml. (5) 5-HT 5×10^{-8} g/ml. (6) 5-HT 5×10^{-9} g/ml. (7) 5-HT 5×10^{-10} g/ml. Time units = 6 sec and 1 min.

5-Hydroxytryptamine occurs in considerable amounts in the posterior salivary glands of *Octopus vulgaris* and *Eledone moschata* (see Erspamer, 1954, for references). When the salivary glands of *Buccinum* were extracted in such a manner that acetylcholine would be destroyed by cholinesterase or by alkaline hydrolysis, the extracts were found to have a marked excitor action on the *Buccinum* heart (Record 3, Fig. 5). The action resembled that of 5-hydroxytryptamine. The excitor activity of a given weight of salivary gland tissue was nearly ten times as great as that of an equal weight of ganglion tissue. Further experiments would be necessary to determine the true nature of the heart-exciting material from the salivary glands of *Buccinum*.

Discussion

It has long been recognized (e.g. Carlson, 1905*a*, *b*) that molluscan hearts are innervated by cardio-regulator nerves. Carlson concluded that the innervation could be single and either excitatory or inhibitory, or, in some species, double with both sets of fibres apparently present. If opposing sets of fibres ran together in the same nerve, as they may in molluscs, it would be difficult to demonstrate double innervation by stimulation experiments. For example, it has long been held that the heart of *Venus mercenaria* is innervated only by inhibitor nerves (Budington, 1904). Actually, following stoppage of the *Venus* heart by electrical stimulation of the visceral ganglia, there is often a much increased frequency and amplitude of heart beat during the recovery period. If an agent is used to block the action of the inhibitor nerve, one then sees only excitation of the *Venus* heart upon stimulation of the visceral ganglia (Welsh, 1953*b*). Such observations have led to the conclusion that the *Venus* heart is doubly innervated and when the mixed nerves to the heart are stimulated the cardio-inhibitory action is normally dominant and tends to mask the simultaneous action of cardio-excitatory neurons. It is quite possible that in some species cardio-excitation is dominant.

When Fredericq in 1947 reviewed the literature on cardio-regulatory nerves in invertebrates, he concluded that the cardio-inhibitor nerves of *Venus* probably released acetylcholine and that the cardio-accelerator nerves of *Aplysia* and of cephalopods probably released adrenaline. He further concluded that the cardio-moderator nerves of cephalopods might possibly act through the release of tyramine. From the present study, incomplete as it is, it appears probable that there are cholinergic inhibitor fibres to the heart of *Buccinum* and perhaps *Cyprina* as well. On the basis of this study it can only be said that possibly the cardio-excitor neurons of *Buccinum* and *Cyprina* act through the release of 5-hydroxytryptamine. The fact that this indole amine has been identified in nervous tissue of *Venus mercenaria* and the whelk, *Busycon canaliculatum* (Welsh, 1954*b*), lends support to this view. Twarog (1954) has also shown that 5-hydroxytryptamine is present in the anterior byssus retractor muscle (nerve endings?) of *Mytilus edulis*, where it perhaps acts in the mediation of inhibitory nerve impulses to this muscle.

The true nature and role of the cardio-excitor substance from the salivary glands of *Buccinum* remain to be determined. Since it acts like 5-hydroxytryptamine and since 5-hydroxytryptamine relaxes certain non-cardiac smooth muscle in molluscs (cf. Twarog, 1954), it is possible that the salivary secretion of *Buccinum* acts, in part, to relax its molluscan prey. The posterior salivary glands of *Octopus vulgaris* contain very large quantities of 5-hydroxytryptamine (Erspamer & Asero, 1952; Bacq, Fischer & Ghiretti, 1952), which is responsible for some of the toxic properties of the saliva of this species.

This paper is based on studies made at the Plymouth Laboratory while the author held a fellowship from the John Simon Guggenheim Memorial Foundation. I wish to express my deep appreciation to the Director and Staff of the Plymouth Laboratory for the excellent facilities placed at my disposal and for their kind hospitality and co-operation.

SUMMARY

Among the larger molluscs available in the Plymouth region, *Cyprina islandica* and *Buccinum undatum* have hearts which, when isolated, are suitable for the bioassay of acetylcholine and 5-hydroxytryptamine.

Mytolon, a vertebrate neuro-muscular blocking agent, is a highly effective antagonist of acetylcholine in the hearts of *Cyprina* and *Buccinum*. Lysergic acid diethylamide (LSD), a synthetic ergot alkaloid derivative, is an effective antagonist of 5-hydroxytryptamine in these hearts.

The nervous system of *Buccinum* contains a cardio-inhibitor material that resembles acetylcholine in its action on the *Buccinum* heart. It also contains a cardio-excitor material whose action on the heart resembles 5-hydroxytryptamine.

Hearts of *Pecten*, *Mytilus* and *Buccinum* also yield (from nerve endings?) similar cardio-excitor and inhibitor principles.

Salivary glands of *Buccinum* contain large amounts of a substance that has an action on the *Buccinum* heart resembling that of 5-hydroxytryptamine.

REFERENCES

ALEXANDROWICZ, J. S. & CARLISLE, D. B., 1953. Some experiments on the function of the pericardial organs in Crustacea. *J. Mar. biol. Ass. U.K.*, Vol. 32, pp. 175–92.

BACQ, Z.-M., 1935. L'acétylcholine dans les tissus des Invertébrés. *C.R. Soc. Biol., Paris*, T. 120, p. 246.

BACQ, Z.-M., FISCHER, P. & GHIRETTI, F., 1952. Action de la 5-hydroxytryptamine chez les céphalopodes. *Arch. int. Physiol.*, Vol. 60, pp. 165–71.

BUDINGTON, R. A., 1904. Nervous regulation of the heart of *Venus mercenaria*. *Biol. Bull., Woods Hole*, Vol. 6, pp. 311–12.

CARLSON, A. J., 1905*a*. Comparative physiology of the invertebrate heart. II. The function of the cardiac nerves in molluscs. *Amer. J. Physiol.*, Vol. 13, pp. 396–426.

—— 1905*b*. Comparative physiology of the invertebrate hearts. III. Physiology of the cardiac nerves in molluscs (continued). *Amer. J. Physiol.*, Vol. 14, pp. 16–53.

CORTEGGIANI, E., 1938. Contribution à l'étude de l'acétylcholine libre et dissimulée sous forme d'un complexe dans le cerveau. Thesis, University of Paris.

ERSPAMER, V., 1954. Il sistema cellulare enterochromaffine e l'enteramina (5-idrossitriptamina). *R.C. sci. Farmital.*, Vol. 1, pp. 5–193.

ERSPAMER, V. & ASERO, B., 1952. Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature, Lond.*, Vol. 169, p. 800.

FREDERICQ, H., 1947. Les nerfs cardio-régulateurs des invertébrés et la théorie des médiateurs chimiques. *Biol. Rev.*, Vol. 22, pp. 297–314.

GADDUM, J. H., 1953. Antagonism between lysergic acid diethylamide and 5-hydroxytryptamine. *J. Physiol.*, Vol. 121, 15*P*.

LUDUENA, F. P. & BROWN, T. G., Jr., 1952. Mytolon and related compounds as antagonists of acetylcholine on the heart of *Venus mercenaria*. *J. Pharmacol.*, Vol. 105, pp. 232–9.

PROSSER, C. L., 1940. Acetylcholine and nervous inhibition in the heart of *Venus mercenaria*. *Biol. Bull., Woods Hole*, Vol. 78, pp. 92–102.

TWAROG, B. M., 1954. Responses of a molluscan smooth muscle to acetylcholine and 5-hydroxytryptamine. *J. cell. comp. Physiol.*, Vol. 44, pp. 141–64.

VINCENT, D. & JULLIEN, A., 1938. La teneur en acétylcholine du cœur des mollusques. *C.R. Soc. Biol., Paris*, T. 127, p. 334.

WELSH, J. H., 1953*a*. The occurrence of an excitor amine in the nervous system of *Buccinum* and its action on the heart. *Anat. Rec.*, Vol. 117, p. 637.

—— 1953*b*. Excitation of the heart of *Venus mercenaria*. *Arch. exp. Path. Pharmak.*, Bd. 219, pp. 23–9.

—— 1954*a*. Marine invertebrate preparations useful in the bioassay of acetylcholine and 5-hydroxytryptamine. *Nature, Lond.*, Vol. 173, p. 955.

—— 1954*b*. Hydroxytryptamine: a neurohormone in the invertebrates. *Fed. Proc.*, Vol. 13, pp. 162–3.

WELSH, J. H. & SLOCOMBE, A. G., 1952. The mechanism of action of acetylcholine on the *Venus* heart. *Biol. Bull., Woods Hole*, Vol. 102, pp. 48–57.

WELSH, J. H. & TAUB, R., 1948. The action of choline and related compounds on the heart of *Venus mercenaria*. *Biol. Bull., Woods Hole*, Vol. 95, pp. 346–53.

—— —— 1953. The action of acetylcholine antagonists on the heart of *Venus mercenaria*. *Brit. J. Pharmacol.*, Vol. 8, pp. 327–33.