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# PHYSIOLOGICALLY ACTIVE CHOLINE ESTERS IN CERTAIN MARINE GASTROPODS AND OTHER INVERTEBRATES

By M. J. KEYL,* I. A. MICHAELSON* and V. P. WHITTAKER

*From the Department of Physiology, University of Cincinnati College of Medicine, Cincinnati, Ohio, and the A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

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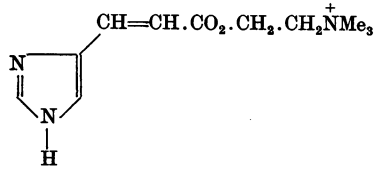
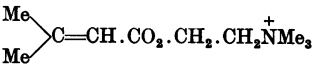
It is known that acetylcholine is not the only physiologically active ester of choline to occur in nature. Propionylcholine, present in ox spleen, was the first such ester to be chemically identified (Banister, Whittaker & Wijesundera, 1953). Another such ester is urocanylcholine (murexine) (I) discovered by Erspamer and co-workers (Erspamer & Dordoni, 1947; Erspamer & Benati, 1953) in relatively high concentration in the hypobranchial glands of three species of Mediterranean whelks of the family Muricidae.

These glands have attracted the interest of zoologists for many years, for they secrete a chromogen which in the presence of light and air is oxidized to an intense purple dye, well known to the ancient world as Tyrian purple. This chromogen is a sulphur-containing conjugate of 6-bromoindoxyl; one species also contains the unbrominated derivative (Bouchilloux & Roche, 1955). It was Dubois (1909) who first showed that extracts of these glands, besides containing the purple chromogen, were also extremely toxic to both warm- and cold-blooded animals, causing muscular paralysis. Vincent & Jullien (1938) drew attention to the high acetylcholine equivalence of these glands and the possible relationship of this to their toxicity, a connexion which was clarified by the discovery of urocanylcholine, which in addition to possessing certain properties in common with acetylcholine, is also an effective neuromuscular blocking agent (Erspamer, 1953).




(I)




(II)

* Present address, see p. 454.

In the experiments to be described in this paper the sensitive chromatographic methods developed for the identification of choline esters in biological material (Whittaker & Wijesundera, 1952; Gardiner & Whittaker, 1954) have been applied to a survey of a number of marine gastropods and other species. These methods permit analyses to be made on 1 g or less of tissue in contrast to the much larger quantities used by Erspamer & Benati (1953). The investigations were begun with four species of Muricidae readily available on the American North Atlantic coast. Two other species, *Busycon canaliculatum* and *Buccinum undatum*, were taken as representative of non-dye-secreting families of the same division of predatory gastropods (Rachiglossa) as the Muricidae. *Venus mercenaria* was included as a representative lamellibranch mollusc, *Littorina litorea* as a vegetarian, non-predatory gastropod. The neuromuscular blocking action of urocanylcholine (Erspamer, 1953; Keyl & Whittaker, 1955) suggested that this substance might be a transmitter at inhibitory nerve endings in crustacean muscle. The ventral cord and peripheral axons of the lobster were therefore included in the survey.

Our results show that the hypobranchial glands of all Muricidae so far examined, when tested on the frog rectus abdominis muscle, have a high acetylcholine equivalence; that this is a feature not of the Muricidae alone but also of at least one species of a different family of Rachiglossa; and that even within the Muricidae there are species variations in the nature of the active substance responsible for the high acetylcholine equivalence. In three of these species of Muricidae, as in the three other species investigated by Erspamer, the high acetylcholine equivalence has proved to be due to urocanylcholine, in the fourth, to the hitherto unknown compound $\beta$, $\beta$-dimethylacrylylcholine (II).

Our findings emphasize that physiologically active esters of choline have a wider significance in nature than is suggested by the classical concept of acetylcholine as a transmitter at cholinergic nerve-endings, a conclusion already implicit in the wide but sporadic distribution of acetylcholine itself in both the plant and animal kingdoms, often in relatively high concentration.

Parts of this work have formed the subject of preliminary communications (Whittaker & Michaelson, 1954; Whittaker, 1955, 1957).

## MATERIALS AND METHODS

### *Species investigated*

The species investigated are listed in Table 1, which also indicates their taxonomic relationship. *Urosalpinx cinereus* (oyster drill), *Busycon canaliculatum* (channelled whelk), *Venus mercenaria* (quahog) and *Homarus americanus* (lobster) were obtained from the Cape Cod region of the U.S.A., *Thais lapillus* (dog whelk), from Cape Cod or Plymouth, England, *Buccinum undatum* (common whelk) and *Littorina litorea* (periwinkle) from Plymouth and *Murex fulvescens* and *Thais floridana floridana* (Southern oyster drill) from the North Carolina and Florida coasts.

TABLE 1. Acetylcholine equivalence of tissues of species investigated

| Family* | Species* | Organ | No. specimens pooled | Acetylcholine equivalence† (u./g fresh tissue) |
|---|---|---|---|---|
| Phylum: Mollusca; Class: Gastropoda; Order: Prosobranchiata; Division: Rachiglossa | | | | |
| Muricidae | *Urosalpinx cinereus* (Say) | Whole organism | 20 | 118 |
| | *Murex fulvescens* (Sowerby) | Viscera less HG | 1 | 11 |
| | | | 1 | 5 |
| | | HG | 1 | 280 |
| | | | 1 | 480 |
| | *Thais lapillus* (L.) | Whole organism | 10 | 76 |
| | | | 10 | 113 |
| | | Organism less HG | 10 | 13 |
| | | HG | 10 | 1800 |
| | *Thais floridana floridana* (Conrad)‡ | Viscera less HG | 2 | 1 |
| | | | 1 | 6 |
| | | HG | 2 | 3880 |
| | | | 1 | 4000 |
| Buccinidae | *Buccinum undatum* (L.) | Viscera less HG | 4 | 6 |
| | | HG | 4 | 270 |
| Turbinellidae | *Busycon canaliculatum* (Say) | HG | 2 | 0 |
| Division: Taenioglossa | | | | |
| Littorinidae | *Littorina litorea* (L.) | Whole organism | 7 | 7 |
| Class: Pelecypoda; Order: Teleodesmacea | | | | |
| Veneridae | *Venus mercenaria* (L.) | Whole organism | 1 | 5§ |
| Phylum: Arthropoda; Class: Crustacea; Order: Decapoda | | | | |
| Homaridae | *Homarus americanus* (Milne-Edwards) | Ventral nerve cord | 4 | 90 |
| | | Limb nerves | 4 | 46 |

HG = Hypobranchial gland.

* Classification and nomenclature (where given) according to Pratt (1935).

† Determined on TCA extracts.

‡ For discussion of nomenclature see Butler (1953); also known as *Thais haemastoma* var. *floridana* (R. T. Abbott, personal communication).

§ Treatment with alkali (pH 12 for 10 min at 100°) increased activity 2·5 fold.

*Reference materials*

*Choline esters.* Acetylcholine perchlorate was prepared from commercial acetylcholine chloride by the method of Bell & Carr (1947) and twice recrystallized from ethanol. Nicotinoylcholine perchlorate was prepared by heating solid nicotinoyl chloride (obtained from the acid and thionyl chloride) with solid dry choline perchlorate under boiling toluene until the evolution of HCl was complete. The product was twice recrystallized from ethanol. Synthetic urocanylcholine bromide was kindly supplied by Dr J. A. Aeschlimann, Hoffman-La Roche Inc., Nutley, N.J., β-3-indolylpropionylcholine iodide, by Dr A. Cohen, Roche Products Ltd., Welwyn Garden City, Herts, and β-4(5)-imidazolylpropionylcholine dipicrate (Pasini, Vercellone & Erspamer, 1956) by Dr C. Pasini, Farmatalia S.A., Milan. Other choline esters were prepared as the bromides by the method of Fourneau & Page (1914) from the corresponding 2-bromoethyl esters which were usually obtained by refluxing 2-bromoethanol and the appropriate acid in benzene in an apparatus incorporating a water trapping device; a small amount of *p*-toluene sulphonic acid was added as a catalyst.

Other salts of choline esters were converted to the chlorides when necessary by passing them through a column of Deacidite FF (The Permutit Co. Ltd., London, England; −16+50 mesh) in a suitable solvent, usually 50% (v/v) aqueous acetone. Representative figures for the chromatographic behaviour and relative biological potency, as measured on the frog rectus abdominis muscle preparation for the various esters, are summarized in Table 2. Elution peaks obtained with the higher aliphatic esters were broad and varied somewhat from run to run. The high biological activity of imidazolylpropionylcholine is noteworthy.

*Other materials.* Synthetic homarine was kindly provided by Dr E. Gasteiger and urocanic acid by Dr H. Tabor. Harvard plasma fraction IV–6–3 (Surgenor & Ellis, 1954) was part of a sample kindly supplied some years ago by Dr D. M. Surgenor; under zero order conditions, 1 $\mu$g of the preparation hydrolysed 4 $\mu$moles acetylcholine/hr in 0·023 M bicarbonate buffer at pH 7·4 and 37° C. Other reagents and solvents were the best commercial grades.

TABLE 2. Chromatographic and biological characteristics of choline esters

| Choline ester | Retention volume (ml.) Elution peak (0·1 M-$NaH_2PO_4$) | Retention volume (ml.) Displacement peak (0·1 N-HCl) | $R_F$ in (*a*) | $R_F$ in (*b*) | $R_F$ in (*c*) | Relative molar potency on frog rectus (acetylcholine = 100) |
|---|---|---|---|---|---|---|
| Urocanyl | >160 | 2 | 0·06 | — | 0·54 | 10 |
| Imidazolylpropionyl | >240 | 4 | 0·15 | 0·37 | 0·29 | 300 |
| Indolylacetyl | >140 | 10 | 0·2 T | 0·63 T | 0·73 T | 6 |
| Indolylpropionyl | >140 | 30 | 0·26 | 0·60 | 0·73 | 14 |
| Nicotinoyl | >110 | 2 | 0·10 | 0·35 | 0·58 | 1 |
| Acetyl | 40 | — | 0·10 | 0·46 | 0·65 | 100 |
| Propionyl | 50 | — | 0·22 | 0·57 | 0·78 | 160 |
| *n*-Butyryl | 60 | — | 0·28 | 0·66 | — | 100 |
| *n*-Valeryl | 140 | — | 0·31 | 0·69 | 0·78 | 54 |
| 2-Methylbutyryl | 150 | — | 0·36 | 0·73 | 0·80 | 138 |
| *iso*-Valeryl | 125 | — | 0·38 | 0·78 | 0·82 | 38 |
| *n*-Caproyl | >160 | 2 | 0·36 | 0·68 | 0·79 | 20 |
| Benzoyl | 150 | — | 0·28 | 0·71 | 0·71 | 1 |
| Crotonyl | 90 | — | 0·20 | 0·50 | 0·69 | 22 |
| 2-*n*-Pentenoyl | 100 | — | 0·35 | 0·71 | 0·71 | 22 |
| $\beta,\beta$-Dimethylacrylyl | 200 | — | 0·35 | 0·70 | 0·71 | 20 |
| TF | 200 | — | 0·35 | 0·70 | 0·71 | 20 |

(*a*) *n*-Butanol saturated with water; (*b*) *n*-butanol–ethanol–acetic acid–water (8:2:1:3); (*c*) *n*-propanol–1·0 N acetic acid (3:1). T = tailing.

*Techniques*

*Extraction of tissues.* The shells were cracked with a hammer (molluscs) or cut away with scissors after removal of appendages (lobster), the tissues dissected out as rapidly as possible and physiologically active substances extracted therefrom with ice-cold 10% trichloroacetic acid (TCA) (MacIntosh & Perry, 1950) or acetone. For assay, chromatography or other tests, acetone extracts were evaporated to dryness *in vacuo* and the active material extracted into water or 0·05 M-$NaH_2PO_4$; TCA extracts were extracted with ether to remove TCA and partially evaporated under reduced pressure to remove ether. Results with TCA and acetone extraction were in good agreement and tests on the residue remaining after acetone extraction showed that this was 97–99% complete.

*Biological assays* were performed, unless otherwise stated, on the frog rectus abdominis muscle preparation of Chang & Gaddum (1933) as modified by Feldberg & Hebb (1947). The activity of extracts or chromatographic fractions was compared with that of standard solutions of acetylcholine perchlorate, one unit (u.) of activity being defined as that amount of active material giving a contraction equal to that produced by 1 n-mole ($10^{-9}$ mole) acetylcholine ($\equiv$0·18 $\mu$g acetylcholine chloride). The activity/g fresh tissue is referred to as the 'acetylcholine equivalence' of that

tissue. The use of this term must not be taken to imply that the activity is due to acetylcholine itself. When the active material is not acetylcholine the bioassay of such material in terms of acetylcholine as standard is subject to uncertainties which have been discussed previously (Gardiner & Whittaker, 1954). In some experiments the guinea-pig ileum (for details see Banister *et al.* 1953) and *Venus* heart (Wait, 1943) were used as test organs. Biological potency is expressed in percentage units in terms of the relative molar potency, i.e. the number of moles of acetylcholine giving a response equivalent to 100 moles of active substance.

*Ultra-violet spectrophotometry.* Homarine and urocanylcholine were estimated in chromatographic fractions from the optical density at their wave-lengths of maximum absorption ($\lambda_{max.}$) of a 1 cm thickness of suitably diluted solution and the molar extinction coefficient ($\epsilon_{max.}$) at those wave-lengths. For homarine, $\lambda_{max.} = 270$ m$\mu$, $\epsilon_{max.} = 6{\cdot}3 \times 10^3$ (E. Gasteiger, personal communication); for urocanylcholine, $\lambda_{max.} = 285$ m$\mu$ at pH 4·5, $\epsilon_{max.} = 1{\cdot}67 \times 10^4$ (Pasini, Vercellone & Erspamer, 1952).

*Chromatography.* Column chromatography was carried out, unless otherwise stated, on 12 × 0·6 cm columns of weak acid ion exchange resin (Rohm and Haas Co., Amberlite XE–97) buffered to pH 4·35 with 0·1 M-$NaH_2PO_4$ as described by Gardiner & Whittaker (1954). The effluent was collected in 2 ml. fractions by means of an automatic fraction collector at a flow rate of 12–20 ml./hr. Elution was performed with up to 240 ml. 0·1 M-$NaH_2PO_4$ followed by displacement of more strongly adsorbed cations by 0·1 N-HCl. Chromatographic behaviour is expressed as the retention volume, i.e. the volume of eluant required to attain peak concentration of the substance being eluted. Displacement peaks were measured from the point at which the pH of the effluent started to fall.

Paper chromatography was carried out by the ascending method on Whatman no. 1 filter paper at 20–22° C as described by Whittaker & Wijesundera (1952). Substances were located on filter paper by examination in ultra-violet light, by exposure to iodine vapour, by spraying with Pauly (diazotized 1%, w/v, sulphanilic acid in butanol followed by sodium carbonate), Ehrlich (2%, w/v, *p*-dimethylaminobenzaldehyde in 5% HCl), ninhydrin (0·25%, w/v, in acetone) or Folin–Ciocalteu reagents, and by bioassay of the material eluted from successive strips of the chromatogram. Recoveries were usually 75–95%, but occasionally exceeded 100%. Variations are attributed to uncertainties in the assays and the possible removal of interfering substances during chromatography. Vapour phase chromatography was carried out as described by James & Martin (1952).

*Other methods.* Esters were estimated by the ferric-hydroxamate method (Hestrin, 1949) at 520 m$\mu$. Choline was estimated by the method of Appleton, La Du, Levy, Steele & Brodie (1953). Volatile fatty acids were steam distilled at pH 3 in the presence of sufficient $MgSO_4$ to retain HCl and were aerated with $CO_2$-free air before and during titration with 0·01 N-NaOH. Bromine uptake (iodine value) of unsaturated fatty acids was determined on 1 ml. samples of the fatty acid in dry chloroform as described by Trappe (1938). Chloroform solutions of the acids were prepared from neutralized and freeze-dried alkaline hydrolysates of the corresponding esters by dissolving the hydrolysate in 2–3 drops 30% phosphoric acid and passing through a small column of anhydrous $Na_2SO_4$ and kieselguhr; the acid was washed through the column with chloroform and the washings made up to 3 ml. Microhydrogenation was carried out manometrically in the Warburg apparatus using colloidal palladium as catalyst as described by Harrison (1939).

## RESULTS

### *Acetylcholine equivalence of tissues*

Table 1 gives the biological activity, as assayed on the frog rectus, of tissues of the various species examined. The activity is expressed as acetylcholine equivalence as defined in the Methods section.

It will be seen that in the four species of Muricidae studied, very high acetylcholine equivalences are attained. In the three species in which the hypobranchial gland was extracted separately it was found that the activity is located almost entirely within the gland, the activity of other parts being in the range expected for cholinergically innervated tissues. The figures for the hypobranchial gland represent minimum values as, owing to the fragile nature of the glandular tissue, no attempt was made to free it from the surrounding portion of the mantle. The values for the hypobranchial gland of the two species of *Thais* are particularly noteworthy, being higher than those for the cerebral ganglion of the octopus (423 u./g) and insect ganglia (190–1100 u./g), calculated from data given by Prosser (1946). High activity is not, however, confined to the Muricidae, for the hypobranchial glands of *Buccinum undatum* were also highly active. By contrast, the hypobranchial gland of another predatory gastropod in the same division (Rachiglossa), *Busycon canaliculatum*, showed low activity, as did the vegetarian Taenioglossa, *Littorina litorea*, and the lamellibranch, *Venus mercenaria*. These species were not investigated further. As will be shown in the next section, the high acetylcholine equivalences of the hypobranchial glands of Muricidae and *Buccinum* are not due to acetylcholine itself, but to other physiologically active esters of choline.

*Identification of active substances*

Chromatography on columns of buffered ion exchange resin has proved an effective method of separating choline esters and other organic bases, particularly when the bases to be separated differ in the number of positive charges in the molecule. Thus, compounds ionizing as zwitter-ions show little affinity for the resin and are readily eluted, those ionizing as bivalent cations such as urocanylcholine show a high affinity and can only be eluted at low pH's (e.g. by 0·1 N-HCl), while univalent cations (e.g. acetylcholine) show an intermediate affinity for the resin and may be eluted under less acid conditions (e.g. by 0·1 N-$NaH_2PO_4$).

Fig. 1 illustrates the degree of separation obtained between the zwitter-ion homarine, acetylcholine and urocanylcholine. Homarine is included as a tissue base widely distributed in marine arthropods and molluscs. It will be seen that homarine emerges in the first few fractions on elution with phosphate. Acetylcholine emerges as a well-defined peak with a retention volume of about 40 ml. as the elution with phosphate continues, whereas urocanylcholine requires displacement with 0·1 N-HCl and emerges as a sharp peak when the pH of the effluent has fallen sufficiently, the peak concentration being attained in the first acid fraction.

Similar model experiments have been carried out with a variety of different choline esters. The results are summarized in Table 2. The table includes other chromatographic properties of these esters and their biological potency on

the frog rectus, since for the most part these properties have not previously been compared. It will be seen that the first five esters of this table, i.e. synthetic urocanylcholine and four other heterocyclic esters all of which can ionize as doubly charged cations in acid solution, resisted elution in 0·1 M-$NaH_2PO_4$ and required 0·1 M-HCl for displacement. The remaining esters in Table 2, all univalent cations of increasing chain length, can be eluted with phosphate, but in general with increasing difficulty as the chain length increases, as indicated by the increasing retention volumes.

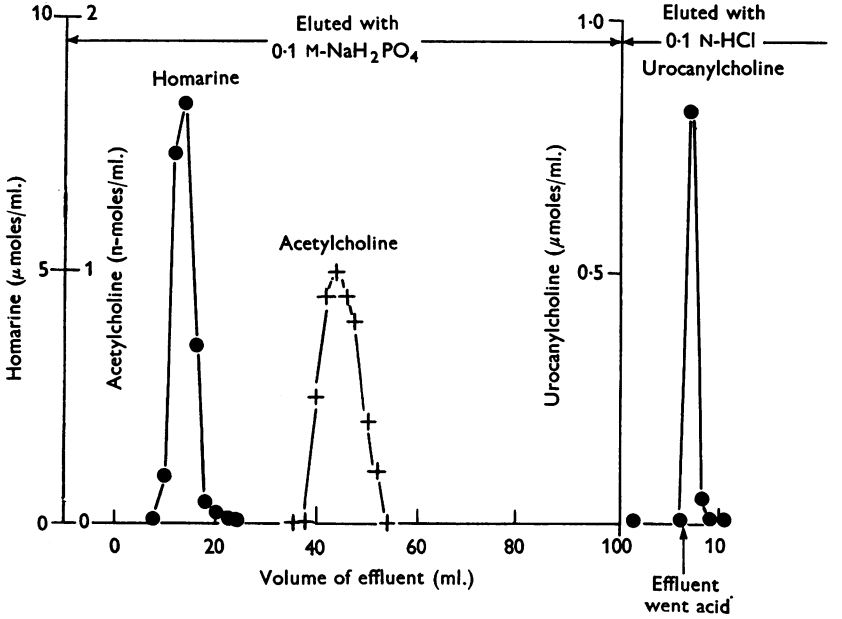



Fig. 1. Chromatographic separation of synthetic homarine (44 μmoles, 93% recovery), acetylcholine (0·015 μmoles, 70% recovery) and urocanylcholine (1·8 μmoles, 95% recovery). Acetylcholine was assayed in successive fractions by the frog rectus method, homarine and urocanylcholine spectrophotometrically.

*Results with Murex fulvescens, Urosalpinx cinereus and Thais lapillus*

The active ester in these species proved to be urocanylcholine accompanied by traces of acetylcholine. In addition chromatography revealed the presence of large amounts of homarine and other ultra-violet absorbing substances which, however, were biologically inactive in our test systems. The evidence for this is summarized, for *Murex fulvescens*, in Figs. 2 and 4.

*Homarine.* Fig. 2*a* shows the results of chromatographing a hypobranchial extract corresponding to 1·4 g. tissue. On eluting with buffer, two peaks (*A* and *B*) of intensely ultra-violet absorbing, biologically inactive material made their appearance. The main peak had an absorption maximum at 270 mμ (Fig. 2*b*), unchanged by variations in pH, and is attributed mainly to homarine.

There was also some fluorescent material in these fractions possibly related to the chromogenic substances (precursors of Tyrian purple) which are characteristic constituents of the hypobranchial glands of Muricidae. The 'homarine peak' was present in the chromatograms of crude tissue extracts of all species examined, but the second peak was seen only with extracts of *Murex fulvescens*.

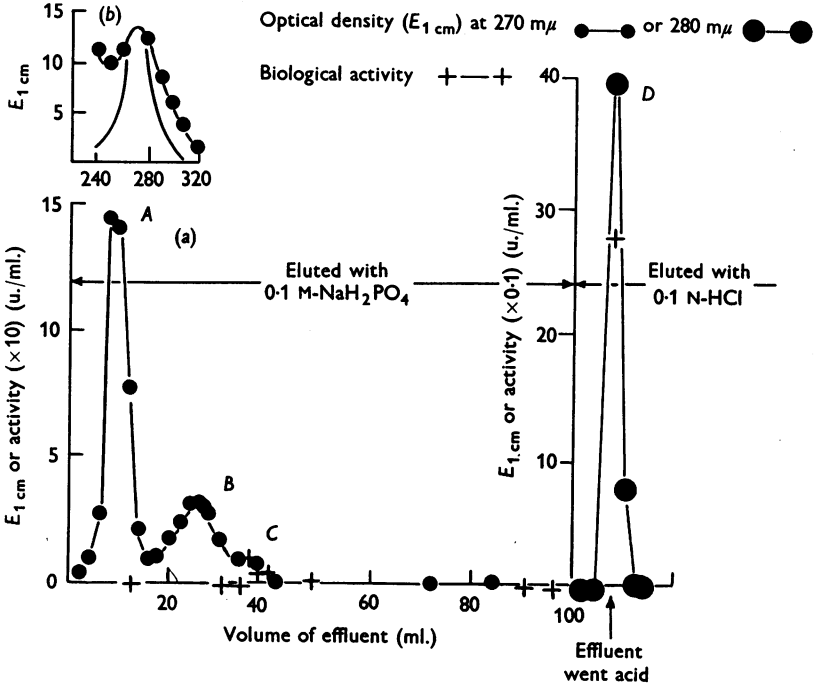



Fig. 2. (*a*) Chromatography of TCA extract (4 ml., corresponding to 1·4 g tissue and containing 666 u. of activity) of hypobranchial glands of *Murex fulvescens*. Recovery of biological activity (in acid fraction 4), 83%; recovery of ultra-violet absorbing material (buffer fractions 1–21 and acid fraction 4), 85%. (*b*) Absorption spectrum of peak ultra-violet absorbing buffer fraction (no. 5) (●—●), compared with homarine, (—).

Confirmation that the main ultra-violet absorbing component in these fractions was indeed homarine was obtained in experiments with *Thais lapillus*. Two-dimensional paper chromatography of acetone extracts of *Thais lapillus* (Fig. 3) in *n*-butanol–acetic acid–water (78:5:17) (Bouchilloux & Roche, 1955) followed by 20% (w/v) aqueous KCl, revealed three major and four minor ultra-violet absorbing substances and one prominent fluorescent substance. Of the three major components, one was identified as homarine, one as urocanylcholine (also identified by column chromatography; see below) and one as chromogen. These compounds accounted for 43, 36 and 14% respectively of the absorption at 270 mμ. Thus homarine accounts for nearly 70% of the non-murexine absorption of these extracts at this wave-length. Since the

chromogen has an absorption maximum at 292 mμ, its presence would account for the broadening of the ultra-violet absorption peak seen in Fig. 2(*b*) and similar experiments.

*Acetylcholine.* Returning to Fig. 2*a*, towards the end of the second peak, a trace of biologically active material made its appearance (peak *C*). This was probably acetylcholine, since a similar peak was obtained in parallel experiments with *Thais lapillus* and *Urosalpinx cinereus* and in these the active material was identified as acetylcholine by differential assay. This acetylcholine

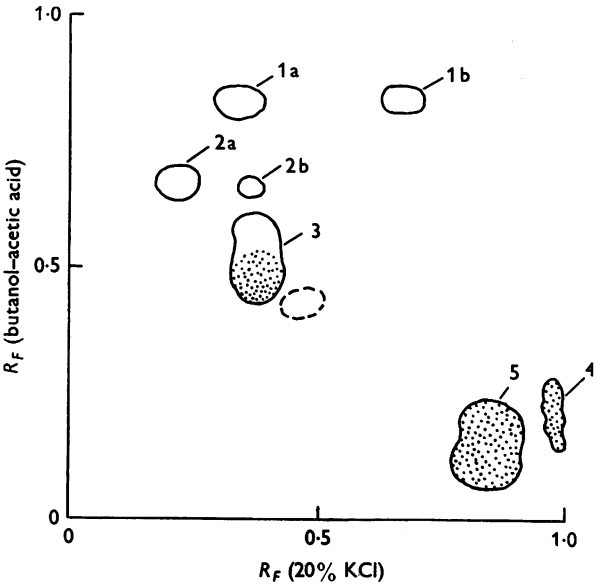



Fig. 3. Two dimensional chromatogram of acetone extract of *Thais lapillus* (whole organism less foot). Continuous rings indicate the position of strong (stippled) or weak (blank) ultra-violet quenching spots, the interrupted ring, a fluorescent spot. Identifications: 3, chromogen (conversion to Tyrian purple by N-HCl at 100° C); 4, homarine; 5, murexine (chromatographic and spectroscopic comparison with authentic specimens; 5 also gave the yellow Pauly reaction and had the biological activity characteristic of murexine).

would account for the whole of the low activity found in tissues other than the hypobranchial gland. After the appearance of peak *C*, further prolonged elution with phosphate removed no more active or ultra-violet absorbing material from the column.

*Urocanylcholine.* On changing over to 0·1 N-HCl, a biologically active and intensely ultra-violet absorbing material (peak *D*) made its appearance. This was identified as urocanylcholine. As seen in Fig. 4, the ultra-violet absorption spectrum coincides with that of authentic urocanylcholine and after treatment with alkali (100° at pH 12 for 10 min) activity was lost and the spectrum shifted to that of urocanic acid. The biological activity of the naturally

occurring urocanylcholine agreed to within about 10% with that of a solution of synthetic urocanylcholine of equal ultra-violet absorption, but was consistently slightly higher. This discrepancy may indicate the presence of another biologically more active choline ester (see Discussion).

The presence of urocanylcholine was similarly demonstrated in whole *Urosalpinx cinereus* and *Thais lapillus* and in the hypobranchial glands of the latter species. With *Thais lapillus*, further evidence for the identity of the natural product with urocanylcholine was obtained as follows. The same shifts

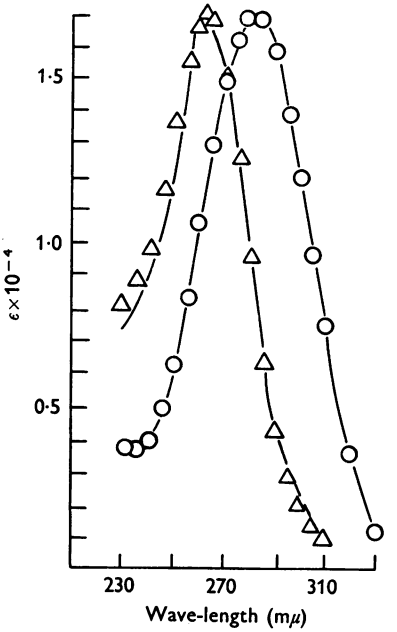



Fig. 4. Ultra-violet absorption spectrum of active material from *Murex fulvescens*, ○, and its alkaline hydrolysis product, △, at pH 4·5. The lines drawn through the points represent the spectrum of urocanylcholine and urocanic acid respectively at this pH.

in the absorption spectrum with pH were observed with the natural and synthetic esters (Fig. 5) and their hydrolysis products. The natural and synthetic esters gave the same yellow shade and intensity of Pauly reaction and both were hydrolysed by human plasma cholinesterase (Fig. 5) at pH 7·4.

Table 3 summarizes the results obtained for all three species and gives the concentrations of homarine, acetylcholine and urocanylcholine in their tissues. It will be seen that urocanylcholine accounts for almost all the acetylcholine-like activity of the organisms. Acetylcholine is present only in traces, mainly in the non-hypobranchial tissues. These traces may represent the contribution of cholinergic nerve fibres.

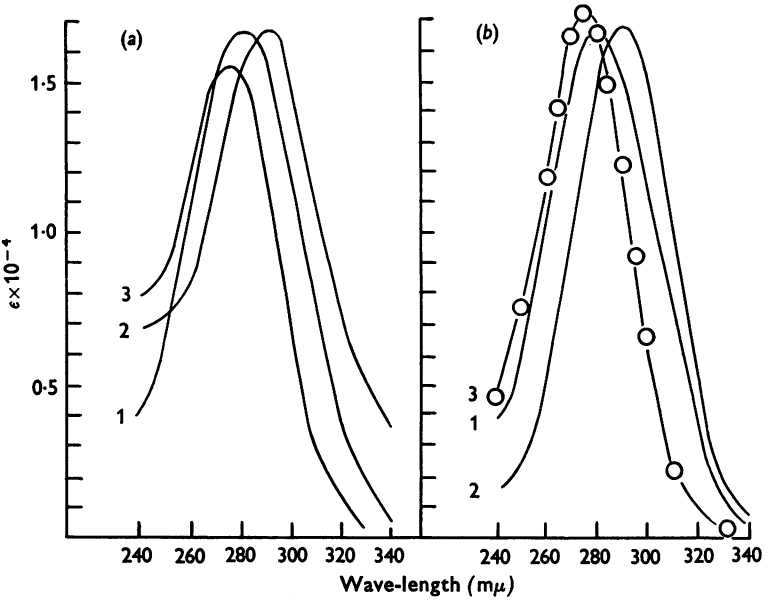



Fig. 5. (*a*) Ultra-violet absorption spectrum of active material from *Thais lapillus* (1) in 0·1 M acetate buffer, pH 4·5, (2) in 0·05 M phosphate buffer, pH 7·4, (3) the same after hydrolysis by purified human plasma cholinesterase at pH 7·4. For enzymic hydrolysis, 0·1 μmole ester/3 ml. buffer was kept during the night with 20 μg Harvard plasma fraction IV–6–3. There was no detectable hydrolysis under these conditions in the absence of enzyme. (*b*) Ultra-violet absorption spectrum of synthetic urocanylcholine (1) in 0·1 M acetate buffer, pH 4·5, (2) in 0·05 M phosphate buffer, pH 7·4, (3) the same after enzymic hydrolysis under similar conditions to (*a*). The circles show the absorption of an equimolar solution of urocanic acid at the same pH.

TABLE 3. Urocanylcholine, acetylcholine and homarine contents of certain Muricidae

| Species | Organ | Urocanylcholine* (mg/g) | Acetylcholine† (μg/g) | Homarine‡ (mg/g) |
|---|---|---|---|---|
| *Murex fulvescens* | Hypobranchial gland | 1·4 (100) | Trace? | 2·0 |
| *Thais lapillus* | Whole organism | 0·2 (95) | 0·7 | 2·6 |
| | Hypobranchial gland | 5·3 (100) | Trace? | — |
| | Remainder of organism | 0 (0) | 2·3§ | — |
| *Urosalpinx cinereus* | Whole organism | 0·3 (89) | 2·3‖ | 1·7 |

* As chloride, hydrochloride. Calculated from activity of original tissue extract, proportion of activity recovered after chromatography in urocanylcholine fractions and molar urocanylcholine: acetylcholine activity ratio (=10). Figures in brackets are the percentage of total activity in tissue due to urocanylcholine.

† As chloride. Calculated from activity of original tissue extract and proportion of activity recovered after chromatography in acetylcholine fractions. Identity of ester established by differential assay on frog rectus and guinea-pig ileum (§), or *Venus* heart (‖).

‡ Calculated from summed optical densities at 270 mμ of homarine fractions after chromatography.

*Thais floridana extracts*

In this species the active substance was found to be a choline ester distinct from acetylcholine, urocanylcholine and other known esters. Fig. 6 shows the result of chromatographing a hypobranchial extract of *Thais floridana*. In this experiment the optical densities at both 230 and 270 mμ, as well as the biological activity of successive fractions, were determined. Homarine once again emerged from the column in the early fractions along with biologically inactive material absorbing at 230 mμ. However, in contrast to results obtained with the other species of Muricidae, biological activity now made its

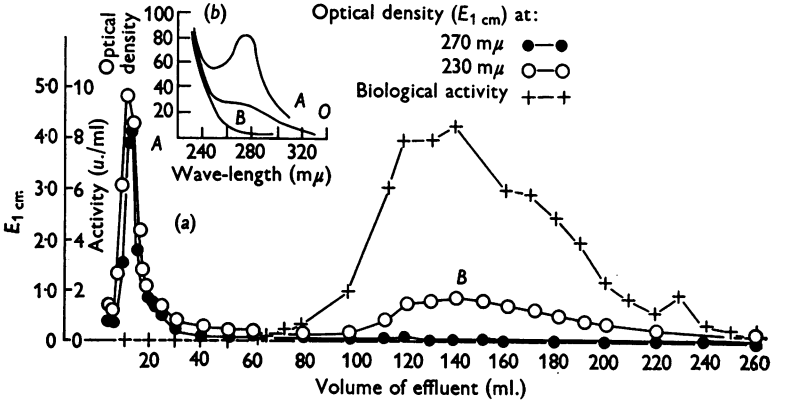



Fig. 6. (*a*) Chromatography of acetone extract (1·7 ml., corresponding to 1·8 g tissue and containing 1416 u. of activity) of hypobranchial glands from *Thais floridana*. Eluant: 0·1 M-$NaH_2PO_4$. Recovery of activity (fractions 30–130), 97%; material absorbing at 270 mμ (fractions 2–20), 75%. (*b*) Optical densities of extract before chromatography (curve *O*), fraction 6 (first peak) (curve *A*), and fraction 70 (second peak) (curve *B*). For plotting, the optical densities of curve *A* have been multiplied by 20, those of curve *B*, by 100.

appearance during elution with acid phosphate as a broad but well-defined peak of material which was associated with absorption at 230 mμ but not at 270 mμ or longer wave-lengths. A comparison of the spectra of the two peak fractions (Fig. 6*b*, curves *A* and *B*) with that of the original extract (curve *O*) showed that homarine, revealed as a constitutent of the original extract by the shoulder at 270 mμ, had been separated from a second, physiologically active component with an absorption maximum below 230 mμ. Elution with acid failed to reveal urocanylcholine or any other biologically active substance.

The new biologically active substance (designated as TF) was readily distinguishable from acetyl- and propionylcholine both by its pharmacological and chromatographic properties. Thus it failed to cause contractions of the guinea-pig ileum in doses, expressed in rectus activity units, at which acetyl- and propionylcholine were effective and its action on the frog rectus was

potentiated only twofold by eserine compared with a fivefold potentiation observed in the same experiment with acetyl- and propionylcholine. Table 2 shows that the substance also behaved differently from these esters on paper chromatography. TF was identified as described in a later section.

*Buccinum extracts*

The active substance in this species has also been found to be a new choline ester. Fig. 7 shows the results of chromatographing an extract from the hypobranchial glands of *B. undatum*. It will be noted that the homarine peak once again made its appearance in the early fractions; biological activity now made its appearance in the position corresponding to acetylcholine. This activity was alkali-labile and choline was identified chromatographically in

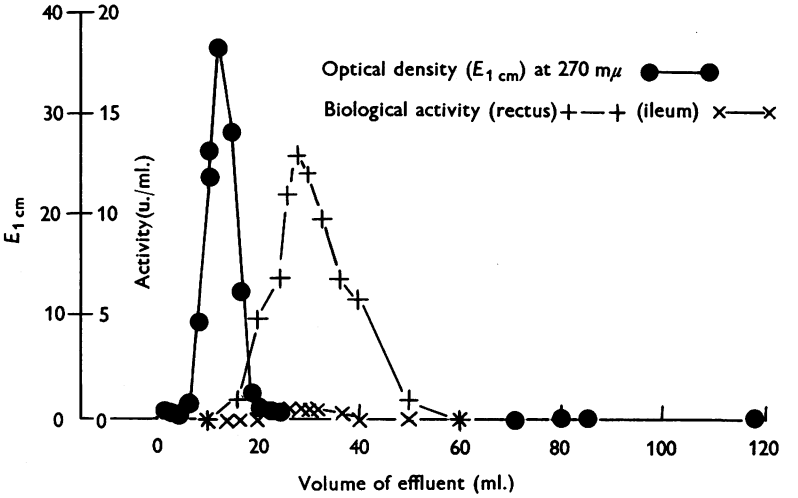



Fig. 7. Chromatography of TCA extract (3 ml., corresponding to 1·1 g tissue and containing 300 u. of activity) from hypobranchial glands of *Buccinum undatum*. Eluant: 0·1 M-$NaH_2PO_4$. Recovery of biological activity (rectus) (fractions 8–25), 72%.

the hydrolysate. However the activity was not acetylcholine, as was evidenced by its inability to cause contractions of the guinea-pig ileum and its much higher $R_F$ value (0·78 in *n*-butanol–ethanol–acetic acid–water (8:2:1:3)). The small amount of ileum activity shown in Fig. 7 was not alkali-labile and not atropine-sensitive. It may have been due to a trace of 5-hydroxytryptamine (5-HT) or similar substance, as 5-HT has a similar retention volume. Paper chromatographic characteristics of the active material in *Buccinum* extracts were similar to TF and in spite of the discrepancy between their retention volumes, the two substances may be related.

*Lobster nervous tissue*

In view of the neuromuscular blocking action of urocanylcholine (Erspamer, 1953; Keyl & Whittaker, 1955) this substance was considered to be a possible transmitter at inhibitory nerve endings in crustacean muscle. Extracts were made of lobster nervous tissue; the only choline ester detected in both the ventral cord and peripheral axons was acetylcholine. The acetylcholine equivalence of these tissues (Table 1) was similar to that reported by Schallek (1945) and the identity of the natural ester with acetylcholine was confirmed by column chromatography and differential assay on the frog rectus and guinea-pig ileum. It should be noted, however, that the assay method (frog rectus) is only one-tenth as sensitive for urocanylcholine as for acetylcholine and small amounts of the former might have remained undetected.

*Identification of active ester from Thais floridana*

Im preliminary experiments, TF was found to be an ester of choline. The evidence is as follows:

(1) activity was lost in 10 min at pH 12 and 100° C;
(2) choline was identified in the hydrolysate by paper chromatography as a spot with the same $R_F$ value as authentic choline in four different solvents (in *n*-butanol saturated with water, 0·08; in *n*-butanol–ethanol–acetic acid–water (8:2:1:3), 0·4; in *n*-propanol–1·0N acetic acid (3:1), 0·5; in phenol saturated with water, 0·95);
(3) activity was destroyed by a highly purified human plasma cholinesterase preparation, and, very slowly, by bovine red cell cholinesterase (Fig. 8);
(4) a 1:1 molar correspondence was noted (Table 4) between the ester content of purified samples of TF as measured by the ferric hydroxamate method and the choline content of equivalent amounts of TF hydrolysate.

Hydrolysates of TF gave no Pauly or ninhydrin reaction, showing that the ester could not be an imidazole or amino acid derivative. Thus it is not histidinyl- or 4(5)-β-imidazolylpropionylcholine (dihydromurexine), compounds which might well occur in an organism closely related to species containing urocanylcholine. The chromatographic behaviour of the latter compound also differs from that of TF (Table 2).

On paper chromatography of our first preparations of TF, Ehrlich and Folin–Ciocalteu positive material was found to be associated with the biological activity. However, quantitative Folin–Ciocalteu estimations on TF eluted from the chromatograms showed that this material was present in amounts stoichiometrically equivalent to only one-sixth or less of the choline liberated on hydrolysis. Furthermore, the choline esters of indole acetic and indole propionic acids were found to have column chromatographic (Table 2) and

spectral properties distinct from those of TF. For these reasons it was concluded that the indolic material was an impurity (possibly derived from the pigment of the hypobranchial gland) and that TF could not be an indole derivative.

Larger quantities of TF were prepared from acetone extracts of *Thais floridana* hypobranchial glands by precipitation with excess saturated ammonium reineckate in the cold. The precipitate was taken up in 50% (v/v) aqueous acetone and reineckate ion replaced by chloride by treatment with IRA 400 (Rohm and Haas Co.) or Deacidite FF in the chloride form. The resulting colourless solution was freeze-dried and the residue stored at − 15° C. This material contained approximately 1 $\mu$mole ester/$\mu$mole choline and had a biological potency 20–25% of that of acetylcholine on the basis of its choline content (Table 4).

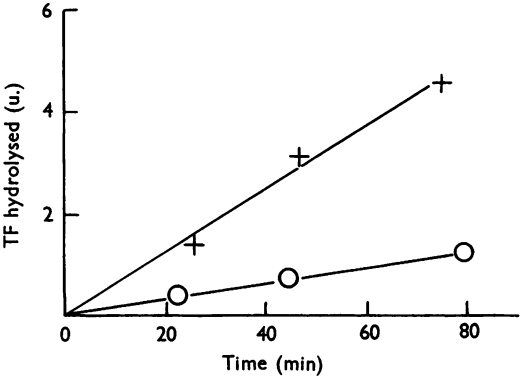



Fig. 8. Hydrolysis of TF by human plasma (+) and bovine red-cell (○) cholinesterase. The reaction mixture contained, in 2 ml.: 0·5 $\mu$l. 'Cholase' brand human plasma cholinesterase (Cutter Laboratories) or 100 $\mu$g bovine red cell cholinesterase (Winthrop Stearns Inc.), 5 u. TF and 10 $\mu$moles Na phosphate buffer, pH 7·4. A control tube containing ester but no enzyme showed no hydrolysis during the period of the experiment. Manometric determinations showed that these amounts of enzyme hydrolysed about 100 $\mu$moles of acetylcholine/hr under zero order conditions in 0·023 M bicarbonate buffer at pH 7·4 and 37° C, and in a control tube with 0·01 $\mu$mole acetylcholine in place of TF, hydrolysis was complete in less than 10 min.

Ultra-violet spectroscopy of the purified material showed that the absorption band below 230 m$\mu$ had a maximum at 222 m$\mu$, suggesting that TF is the ester of an $\alpha$, $\beta$-unsaturated fatty acid. Alkaline hydrolysis of TF followed by steam distillation of the acidified hydrolysate in the presence of $MgSO_4$ gave a volatile acid in 24–92% yield (Table 4). This acid (designated as TF-acid) had an absorption spectrum (circles, Fig. 9*b*) with a maximum at 220–222 m$\mu$ closely resembling acrylic acid and its derivatives (Ungnade & Ortega, 1951; Bueding, 1953), and other $\alpha$, $\beta$-unsaturated fatty acids. Titration data were also consistent with this conclusion. Confirmation of the presence of one double bond in the molecule was obtained from determinations of iodine value and hydrogen uptake, in which approximately 1 $\mu$mole bromine or hydrogen was taken up/$\mu$-equiv acid (Table 4). Difficulty was experienced in hydrogenating TF directly; this was found to be due to the presence in the preparation of a catalyst poison, possibly a sulphur compound, since the chromogen

in the hypobranchial glands of Muricidae is known to be a sulphur-containing 6-bromoindoxyl conjugate. The presence of this catalyst poison was evinced by the strongly inhibitory effect of our preparations of TF on the hydrogenation of a synthetic $\alpha, \beta$-unsaturated fatty acid ester of choline. Experiments with such esters also showed that on the 1–10 $\mu$mole scale, the $\alpha, \beta$-unsaturated fatty acids are partially destroyed under conditions sufficiently vigorous to bring about hydrolysis of their choline esters. This adequately accounts for our failure to recover TF-acid quantitatively from TF hydrolysates.

TABLE 4. Reactions of TF

| TF used | | | | | |
|---|---|---|---|---|---|
| Activity (u.) | Choline content ($\mu$moles) | Ester content ($\mu$moles) | $Br_2$ uptake*† ($\mu$moles) | Volatile fatty acid formed (+)* or used (−) ($\mu$-equiv) | $H_2$ uptake* ($\mu$moles) |
| 250 | 1·07 | 1·18 | 1·18 (100) | — | — |
| 250 | 1·1 | — | — | + 1·015 (92) | — |
| 1250 | 5·5 | — | — | + 2·81 (51) | — |
| 930 | 4·1 | — | — | + 3·56 (87) | — |
| 6000 | — | — | — | +18·42 (77) | — |
| — | — | 6·37 | — | + 1·79 (31) | — |
| — | — | 4·74 | — | + 1·14 (24) | — |
| — | — | — | — | − 2·93 | 2·36 (81) |
| — | — | — | — | − 9·2 | 8·4 (91) |

* As percentage of starting material in brackets. † By TF hydrolysate.

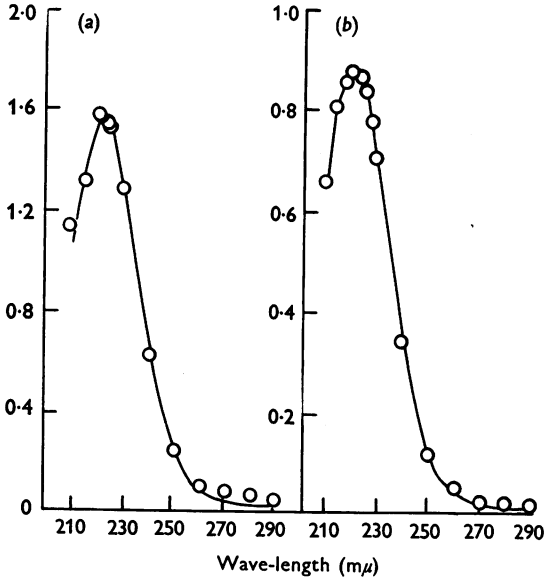



Fig. 9. Absorption spectra of (*a*) TF (○) and DMAC (———); (*b*) TF-acid (○) and DMA (———). The molar extinction coefficients for TF and TF-acid were calculated on the assumption that TF is a mono-ester and TF-acid is monobasic. Optical densities were measured in aqueous solution at 0·05–0·09 $\mu$moles/ml.

The volatile fatty acid obtained from the microhydrogenation of TF-acid ($TFH_2$-acid) was recovered from the reaction mixture by steam distillation, concentrated, and identified as *iso*-valeric acid by vapour phase chromatography (Fig. 10). A mixture of $TFH_2$-acid and *iso*-valeric acid came off the column as a single step showing no trace of separation such as can be seen even with two such closely similar acids as 2-methylbutyric and *iso*-valeric acids. Since $\beta,\beta$-dimethylacrylic (senecioic) acid (DMA) is the only $\alpha,\beta$-unsaturated fatty acid with the same carbon skeleton as *iso*-valeric acid, it was concluded that TF is $\beta,\beta$-dimethylacrylylcholine (DMAC) (II).

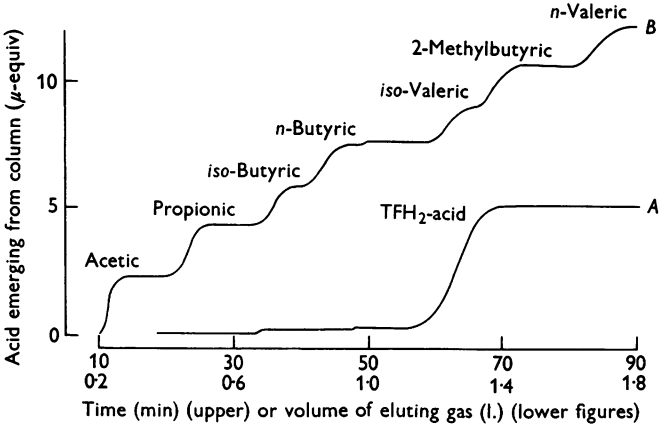



Fig. 10. Vapour phase chromatography of 6·73 $\mu$-equiv $TFH_2$-acid (curve *A*) and a mixture of approximately 2 $\mu$moles each of a number of volatile fatty acids (curve *B*). Length of column: 3·66 m; eluting gas, nitrogen. It will be seen that $TFH_2$-acid has the same retention volume as *iso*-valeric acid.

Fig. 9*b* shows the close spectroscopic resemblance between TF-acid and DMA. The identity of TF and synthetic DMAC was established as follows. Solutions of DMAC and TF having equivalent ester contents and biological activity had identical absorption in the range 210–250 m$\mu$ (Fig. 9). (The small peak at 270 m$\mu$ in the spectrum of TF is probably due to a trace of homarine.) Solutions of DMAC and TF remained biologically equivalent independently of dose level and preparation although their dose-response curves often differed from that of acetylcholine and their molar potency relative to acetylcholine varied somewhat from one preparation to another. They were equally potentiated by eserine (one-and-a-half- to twofold) but less than acetylcholine (four- to fivefold). DMAC had the same retention volume on columns of weak acid ion exchange resin as our purest specimens of TF, though it was observed that the retention volume of TF was reduced, sometimes considerably, by the presence of inactive material in the crude preparations. The $R_F$ values of the two substances were identical in three solvents (Table 2 and Fig. 11). It was

also noted that the ferric hydroxamates derived from DMAC and TF had identical absorption spectra with absorption maxima displaced 40 mμ towards the red as compared with ferric acethydroxamate. As the curves cross at 520 mμ no error results in the ester estimations at this wave-length, but when an absorptiometer with a green filter is used, TF and DMAC give readings which are about 20% higher than that of equimolar acetylcholine.

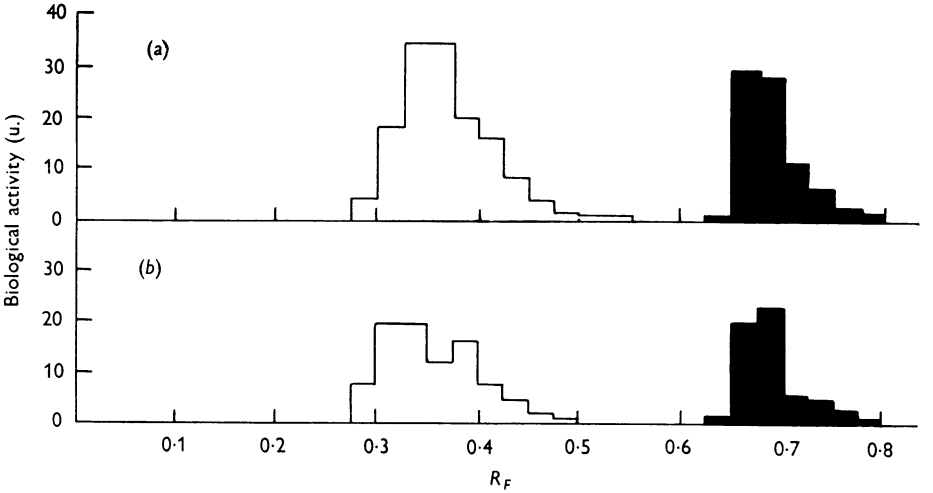



Fig. 11. Distribution of activity after paper chromatography of *ca.* 100 u. of (*a*) TF, (*b*) DMAC chlorides. White blocks, results with *n*-butanol saturated with water as mobile phase; black blocks, results with *n*-butanol–ethanol–acetic acid–water (8:2:1:3) plotted on same abscissae.

## DISCUSSION

The most interesting findings of the present survey are: that a high hypobranchial acetylcholine equivalence is not confined to the Muricidae; that even within the Muricidae, there are species variations in the nature of the active substance; and that one such active substance is the choline ester of dimethylacrylic acid.

DMA is known to occur in nature and has recently been implicated in the biosynthesis of terpenes, rubber and steroids. Its natural occurrence as a physiologically active choline ester has not previously been suspected although related synthetic esters are known to possess physiological activity. Thus β-diethylaminoethyl senecioate has been reported to have local anaesthetic action (Gilman, Heckert & McCracken, 1928) and the methyl ester of γ-crotonobetaine has properties similar to acetylcholine (Burgen & Hobbiger, 1949).

The presence of an active histidine α-deaminase in *Thais lapillus* (Whittaker, unpublished) suggests that the precursors of DMA and urocanic acid may be the corresponding α-amino acids, valine and histidine; the occurrence of different esters in two such closely related species as *Thais floridana* and *Thais lapillus*

may be due to differences in the dietary availability of amino acids. Preliminary attempts to obtain an active choline acylating enzyme system from *Thais lapillus* have been without success. Since pigeon liver was observed to activate urocanic acid in the presence of adenosine triphosphate, coenzyme A and hydroxylamine as a trapping agent, experiments with snail preparations fortified with pigeon liver extracts were carried out, but these were also unsuccessful. It is felt, however, that the failure to demonstrate urocanylcholine synthesis may be due to some technical difficulty analogous to those encountered by Taggart & Forster (1950) in the study of oxidative enzymes in marine organisms.

DMAC has not been identified in urocanylcholine-containing species. The finding, however, that snail urocanylcholine, isolated by column chromatography, was consistently 5–10% more active than synthetic urocanylcholine and had a greater absorption in the far ultra-violet, is consistent with the presence of small amounts of a physiologically more active ester with an absorption maximum below 230 m$\mu$ such as DMAC or imidazolylpropionylcholine.

The physiological significance of high concentrations of biologically active choline esters in the hypobranchial glands of some species is not understood. Erspamer (1953), like Dubois (1906), believes that the gland is a venom gland and has suggested that urocanylcholine is used to bring about relaxation of the closing muscles of *Mytilus* and other bivalves which are preyed upon by *Murex*. This is contrary to the accepted account of the feeding habits of the Rachiglossa which are supposed to gain access to the soft parts of bivalves by means of their long probosces, which are inserted through a hole bored through the shell of the prey by their powerful radulae. *Thais lapillus* has been observed to attack *Littorina* in the laboratory aquarium in this way, and there was no indication of paralysis in the latter, which made unsuccessful attempts to dislodge its enemy by vigorous swinging movements of its shell. The work of Bülbring, Burn & Shelley (1953) on the regulatory action of acetylcholine on ciliary movement in the gills of *Mytilus* suggests an alternative role for choline esters in hypobranchial glands. However, until more is known about the pharmacology and distribution of these esters, any theory of their action is necessarily tentative.

SUMMARY

1. The hypobranchial glands of a number of marine gastropods have been examined for the occurrence of physiologically active esters of choline.

2. Three out of the four species of Muricidae examined contained urocanylcholine, identified chromatographically and spectroscopically.

3. The fourth species, *Thais floridana*, contained a new ester, identified as $\beta$, $\beta$-dimethylacrylylcholine (senecioylcholine).

4. A high acetylcholine equivalence was found in the hypobranchial gland of *Buccinum undatum*, which although a member of the same division (Rachiglossa) as the Muricidae, is not a purple-secreting snail. This activity was not due to acetylcholine but the ester responsible for it has not been identified.

5 The only physiologically active choline ester identified in lobster nervous tissue was acetylcholine.

6. The acetylcholine equivalence of the other species and tissues examined was within the range for tissues with cholinergic innervation.

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## REFERENCES

Appleton, H. D., La Du, B. N., Levy, B. B., Steele, J. M. & Brodie, B. B. (1953). A chemical method for the determination of free choline in plasma. *J. biol. Chem.* **205**, 803–813.

Banister, J., Whittaker, V. P. & Wijesundera, S. (1953). The occurrence of homologues of acetylcholine in ox spleen. *J. Physiol.* **121**, 55–71.

Bell, F. K. & Carr, C. J. (1947). The preparation and properties of the perchlorates of some choline esters. *J. Amer. pharm. Ass.* **36**, 272–273.

Bouchilloux, S. & Roche, J. (1955). Contribution à l'étude biochimique de la pourpre des *Murex*. *Bull. Soc. Chim. biol., Paris*, **37**, 37–53.

Bueding, E. (1953). Formation of tiglic and *n*-valeric acids by bacteria-free *Ascaris lumbricoides*. *J. biol. Chem.* **202**, 505–512.

Bülbring, E., Burn, J. H. & Shelley, H. J. (1953). Acetylcholine and ciliary movement in the gill plates of *Mytilus edulis*. *Proc. Roy. Soc.* B, **141**, 445–466.

Burgen, A. S. V. & Hobbiger, F. (1949). The pharmacology of the methyl and benzyl esters of $\gamma$-crotonic betaine ($\gamma$-carboxyallyltrimethyl-ammonium chloride). *Brit. J. Pharmacol.* **4**, 229–233.

Butler, P. A. (1953). The Southern oyster drill. *Proc. nat. Shellfish Ass. Meet.* **44**, 67–75.

Chang, C. H. & Gaddum, H. J. (1933). Choline esters in tissue extracts. *J. Physiol.* **79**, 255–285.

Dubois, R. (1909). Recherches sur la pourpre et sur quelques autres pigments animaux. *Arch. Zool. exp. gén.* (5), **2**, 471–590.

Erspamer, V. (1953). Murexin. *Arch. exp. Path. Pharmak.* **218**, 142–144.

Erspamer, V. & Benati, O. (1953). Isolierung des Murexins aus Hypobranchialdrüsenextrakten von *Murex trunculus* und seine Identifizierung als $\beta$-[Imidazolyl-4(5)]-acryl-choline. *Biochem. Z.* **324**, 66–73.

Erspamer, V. & Dordoni, F. (1947). Richerche chimiche e farmacologiche sugli estratti di ghiandola ipobranchiale di *Murex trunculus*, *M. brandaris* e *Tritonalia erinacea*. III. Presenza negli estratti di un nuovo derivato della colina o di una colina omologa: la murexina. *Arch. int. Pharmacodyn.* **74**, 263–285.

Feldberg, W. & Hebb, C. (1947). The effect of magnesium ions and of creatine phosphate on the synthesis of acetylcholine. *J. Physiol.* **106**, 8–17.

FOURNEAU, E. & PAGE, H. J. (1914). Sur les éthers de la choline. *Bull. Soc. chim. Fr.* (4), **15**, 544–553.

GARDINER, J. E. & WHITTAKER, V. P. (1954). The identification of propionylcholine as a constituent of ox spleen. *Biochem. J.* **58**, 24–29.

GILMAN, H., HECKERT, L. C. & MCCRACKEN, R. (1928). Aromatic properties of some aliphatic compounds. Local anaesthetics derived from aliphatic carboxylic acids. *J. Amer. chem. Soc.* **50**, 437–439.

HARRISON, K. (1939). Metabolites of contracting muscle. Utilization of fumarate. *Biochem. J.* **33**, 1465–1469.

HESTRIN, S. (1949). The reaction of actylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. *J. biol. Chem.* **180**, 249–261.

JAMES, A. T. & MARTIN, A. J. P. (1952). Gas–liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. *Biochem. J.* **50**, 679–690.

KEYL, M. J. & WHITTAKER, V. P. (1955). Some physiological properties of urocanylcholine. *Amer. J. Physiol* **183**, 634.

MACINTOSH, F. C. & PERRY, W. M. L. (1950). Biological estimation of acetylcholine. *Meth. med. Res.* **3**, 78–92.

PASINI, C., VERCELLONE, A. & ERSPAMER, V. (1952). Synthese des Murexins (β[Imidazolyl-4(5)]-acryl-cholin). *Leibigs Ann.* **578**, 6–10.

PASINI, C., VERCELLONE, A. & ERSPAMER, V. (1956). Murexino-simili. Preparazione e proprietà di una serie di esteri colinici di acidi acrilici e propionici β-sostituiti con nuclei eterociclici diversi. *Gazz. Chim. Ital.* **86**, 266–274.

PRATT, H. S. (1935). *Manual of the Common Invertebrate Animals.* Philadelphia: Blakiston.

PROSSER, C. L. (1946). The physiology of nervous systems of invertebrate animals. *Physiol. Rev.* **26**, 337–382.

SCHALLEK, W. (1945). Action of potassium on bound acetylcholine in lobster nerve cord. *J. cell. comp. Physiol.* **26**, 15–24.

SURGENOR, D. M. & ELLIS, D. L. (1954). Preparation and properties of serum and plasma proteins. Plasma cholinesterase. *J. Amer. chem. Soc.* **76**, 6049–6051.

TAGGART, J. V. & FORSTER, R. P. (1950). Renal tubular transport: effect of 2, 4-dinitrophenol and related compounds on phenol red transport in the isolated tubules of the flounder. *Amer. J. Physiol.* **161**, 167–172.

TRAPPE, W. (1938). Modifikation der Jodzahlbestimmungsmethode nach H. P. Kaufmann für kleine und kleinste Fettmengen. *Biochem. Z.* **296**, 180–185.

UNGNADE, H. E. & ORTEGA, I. (1951). The ultraviolet absorption spectra of acrylic acids and esters. *J. Amer. chem. Soc.* **73**, 1564–1567.

VINCENT, D. & JULLIEN, A. (1938). Richesse de la glande à pourpre des *Murex* en esters de la choline. *C.R. Soc. Biol., Paris*, **127**, 1506–1509.

WAIT, R. B. (1943). The action of acetylcholine on the isolated heart of *Venus mercenaria*. *Biol. Bull., Woods Hole*, **85**, 79–85.

WHITTAKER, V. P. (1955). The identification of urocanylcholine in certain North Atlantic molluscs. *Abstr. III int. Congr. Biochem.* p. 131.

WHITTAKER, V. P. (1957). β,β-Dimethylacrylylcholine, a new naturally occurring, physiologically active ester of choline. *Biochem. J.* **66**, 35*P*.

WHITTAKER, V. P. & MICHAELSON, I. A. (1954). Studies on urocanylcholine. *Biol. Bull., Woods Hole*, **107**, 304.

WHITTAKER, V. P. & WIJESUNDERA, S. (1952). The separation of esters of choline by filter paper chromatography. *Biochem. J.* **51**, 348–351.

Present addresses (added in proof):

M. J. Keyl, Department of Physiology, University of Oklahoma School of Medicine, Oklahoma City, Oklahoma, U.S.A.;

I. A. Michaelson, Department of Pharmacology, The George Washington University School of Medicine, 1335 H Street, Washington, D.C., U.S.A.