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SMOOTH MUSCLE STIMULANTS IN EXTRACTS OF OPTIC NERVES, OPTIC TRACTS AND LATERAL GENICULATE BODIES OF SHEEP

BY

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It is usually considered that synaptic transmission of nerve impulses within the central nervous system is mediated by chemical substances, although direct evidence has not yet been presented to support this belief. The regional distributions of the peripheral transmitter substances, acetylcholine and noradrenaline, have been studied, together with estimates of their respective rates of synthesis and destruction, but no central transmitter function can be ascribed to them. Many other compounds, including 5-hydroxytryptamine and Substance P, have a variable distribution in central nervous tissue but none of these has met the requirements of a central nervous transmitter and their presence in brain remains a mystery. It is suspected that the presence of noradrenaline and 5-hydroxytryptamine in brain is important because of the changes in behaviour and function produced by drugs which are known to alter the normal concentrations of these amines.

All the known substances which have been postulated as central transmitters have effects upon smooth muscle, and more recently other smooth muscle stimulants of a lipid nature have been detected in brain tissue (Kirschner & Vogt, 1961; Ambache, Reynolds & Whiting, 1963; Toh, 1963). Whether or not these stimulants have a role in synaptic transmission is unknown.

Despite the ease with which tissue of the optic-geniculate complex of the mammalian brain may be obtained, pharmacological interest in this region has been slight. In this investigation, the optic nerves and lateral geniculate bodies of sheep have been examined for the presence of water-soluble smooth muscle stimulants, and attempts have been made to separate the individual stimulants by paper chromatography and to identify them pharmacologically. The effects of these stimulants upon transmission at the optic-geniculate synapse is being investigated.

METHODS

Optic nerves, optic tracts and lateral geniculate bodies of sheep were obtained either within 1 min or between 10 to 15 min after slaughter at the abattoir, and immersed in liquid air. The frozen tissue was brittle and easily ground to a fine powder under liquid air in a mortar. The powder was extracted with ethanol three times, and the extracts mixed, filtered and evaporated under reduced pressure. Attempts to remove lipid material by partitioning the residue between solvents and water led to the formation of stable emulsions. If, however, the residues were thoroughly dried *in vacuo* over phosphorus pentoxide,

partial removal of fats could be achieved in a Soxhlet apparatus with low-boiling-point petroleum ether or diethyl ether. The resultant residue was still opalescent when suspended in Tyrode or Krebs solutions.

Clear solutions could be obtained using a mixture of methanol and chloroform (1 : 2, v/v), 6 ml. of solution being added per g of nervous tissue, and extraction was allowed to continue for 6 hr at 10° C aided by continuous stirring (Toh, 1963). After filtering through glass wool, 3 ml. of solvent per g of tissue was added and extraction was continued for a further 3 hr. Distilled water was added in the proportion of one-tenth of the volume of extract and the upper aqueous-methanol phase was removed and evaporated to dryness under reduced pressure. The residues were hygroscopic but readily soluble in physiological solutions without opalescence.

In some experiments the nervous tissue was freeze-dried and the tissue then extracted in a Soxhlet apparatus with petroleum ether followed by ethanol. The ethanol extract was dried and further cleared of fat with diethyl ether, but suspensions rather than solutions resulted when attempts were made to dissolve the residues in physiological salt solutions.

Guinea-pig isolated ileum was prepared in the usual way and suspended in a 10-ml. organ-bath containing Tyrode solution, at a tension of 2 to 3 g and with a writing lever magnification of approximately tenfold. The bath temperature was 32° C and the solution was bubbled with 5% carbon dioxide in oxygen.

Rat isolated stomach preparations were prepared as described by Vane (1957) and were mounted in a 10-ml. organ-bath containing Krebs solution equilibrated with 5% carbon dioxide in oxygen at 37° C. The tension applied was 2 to 3 g and the lever magnification 15- to 18-fold.

All chromatography was done on Whatman No. 3 mm or 4 mm paper, using ascending solvent systems except in the case of catechol amines where Whatman No. 1 mm paper was used. Papers were washed in methanol and dried before use. The experiments were allowed to continue until solvent fronts had advanced 24 cm from the starting line. Extracts and marker substances were applied by micropipette to the starting line in the form of spots, or bands not exceeding 1 cm width, according to the quantities applied.

The following solvent systems were used: (1) butanol-acetic acid-water (60 : 15 : 25); (2) butanol-water (*n*-butanol saturated with distilled water); (3) isopropanol-ammonia-water (10 : 1 : 1; ammonia solution specific gravity=0.91); (4) methanol-acetic acid-water (80 : 20 : 2); (5) butanol-pyridine-water (1 : 1 : 1); and (6) *n*-propanol-ammonia-water (100 : 2 : water to saturate; ammonia specific gravity=0.91).

Reagents used to spray developed chromatograms were modified Dragendorff's reagent (Bregoff, Roberts & Delwiche, 1953), ninhydrin 0.3% in ethanol in aerosol (Sigma), Pauly's reagent (Toh, 1963) and 2% *p*-dimethylamine benzaldehyde in 3% hydrochloric acid (Ehrlich's reagent).

Chromatography for catechol amines was performed as described by Crawford & Outschoorn (1951).

All solvents were purified by redistillation before use. The concentrations of drugs are expressed as g/ml. and refer to the salts. The following salts were used: acetylcholine chloride (Roche), lysergic acid diethylamide (lysergide, Sandoz), mepyramine maleate (May & Baker), atropine sulphate (Burroughs Wellcome), histamine acid phosphate (B.D.H.), 5-hydroxytryptamine creatinine sulphate (Abbotts), 3,4-dihydroxyphenylethylamine (dopamine) hydrochloride (Sigma), (−)-noradrenaline bitartrate (Hoechst) and adrenaline tartrate (Burroughs Wellcome).

RESULTS

Examination of optic nerves, optic tracts and lateral geniculate bodies for known pharmacologically active substances which stimulate smooth muscle

Ethanol extracts of nervous tissue contained lipid material which could not be removed by ether or light petroleum ether and which formed emulsions with Tyrode solution. These extracts also contained substances which were capable of stimulating guinea-pig isolated ileum. The activity was assayed in terms of quantity of acetylcholine per g of tissue and the results are summarized in Table 1.

Optic tracts were collected on four occasions from several thousand sheep being slaughtered. On the first three occasions (batches 1, 2 and 3 in Table 1) the tissues were obtained in two lots, one of which was removed and frozen in liquid air within 1 min of slaughter, whereas the other portion was not removed and frozen until 10 to 15 min had

TABLE 1

THE AMOUNT OF ACETYLCHOLINE-LIKE ACTIVITY IN CRUDE EXTRACTS OF OPTIC NERVES, OPTIC TRACTS AND LATERAL GENICULATE BODIES OF SHEEP, DETERMINED BY ASSAY ON GUINEA-PIG ISOLATED ILEUM

Activity is expressed as μg of acetylcholine per g of tissue

Tissue	Extraction	Activity (μg/g) of tissue obtained: Within 1 min of death	Activity (μg/g) of tissue obtained: 10 to 15 min after death
Optic nerve	Methanol-chloroform	0·20	0·25
Optic tracts			
1st batch	Ethanol	0·20	0·01
2nd batch	Ethanol	0·30	0·03
3rd batch	Methanol-chloroform	0·18	0·02
4th batch	(*a*) Ethanol extract of freeze-dried tissue	—	0·15
	(*b*) Ethanol	—	0·18
	(*c*) Methanol-chloroform	—	0·25
Lateral geniculate bodies			
1st batch	Ethanol	0·10	—
2nd batch	Ethanol	0·10	—

elapsed. The two portions of each batch were processed in parallel and their acetylcholine-equivalents determined. It will be seen that tissues obtained within 1 min contained greater quantities of acetylcholine-like material. Some months later the fourth batch was obtained but the tissues were not removed and frozen until 10 to 15 min after death of the sheep. Three different extraction procedures were used on aliquots of the pooled optic tracts and, in this experiment, the acetylcholine-equivalents were similar to those obtained in the earlier batches for tissues collected immediately. We have no explanation for this discrepancy.

Both optic nerves and optic tracts appeared to contain an acetylcholine-like smooth muscle stimulant in concentrations of the order of 0.2 μg/g. Lateral geniculate bodies contained rather less acetylcholine-like substance, the concentration being of the order of 0.1 μg/g.

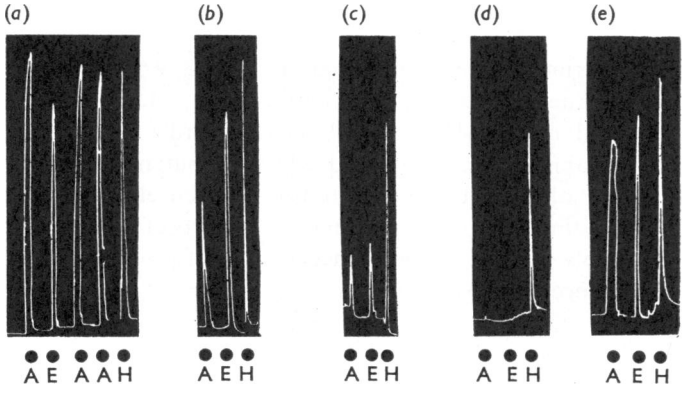


Fig. 1. The effect of atropine in small doses on the contractions of guinea-pig ileum to matched doses of acetylcholine (A, 10 ng), histamine (H, 1 μg) and crude ethanolic extract of optic tracts (E). (*a*) No atropine; (*b*) atropine, 1.5×10^{-10} g/ml.; (*c*) atropine, 2.5×10^{-10} g/ml.; (*d*) atropine, 5×10^{-10} g/ml.; and (*e*) no atropine.

Tests were undertaken in order to show whether this stimulant activity was indeed due to acetylcholine. Equivalent submaximal responses were obtained to acetylcholine, histamine and ethanol extracts of optic tracts and lateral geniculate bodies. Small doses of atropine (1.5 to 5.0 ng) were added to the organ-bath (10.0 ml.) after each wash-out and the effects on the responses observed. Atropine inhibited the responses to doses of acetylcholine and the extracts of nervous tissue, but was not present in sufficient concentration to inhibit the response to histamine. In all tests, the crude ethanol extracts were inhibited to a slightly smaller degree than the standard acetylcholine (Fig. 1). Atropine (1.5×10^{-10} g/ml.) caused 52% inhibition of responses to acetylcholine and only 4% inhibition of the crude extract. When the concentration of atropine was increased to 2.5×10^{-10} g/ml. contractions due to acetylcholine and extract were inhibited by 80 and 60% respectively.

Chromatograms were prepared of extracts of optic tracts and developed in four solvent systems. The choline analogues were rendered visible by spraying the dried chromatograms with modified Dragendorff's reagent, which reacts with quaternary nitrogen atoms. Materials present in the crude extracts exerted no significant effect upon the mobilities of choline and its esters.

TABLE 2

R_F VALUES OF CHOLINE ESTERS AND SUBSTANCES PRESENT IN CRUDE ETHANOL EXTRACTS OF OPTIC TRACTS OF THE SHEEP

Spots are numbered in order from the starting line to the solvent front. * Spot 3 only appeared in extracts of tissues collected within 1 min of slaughter of the sheep

Material	Solvent system			
	Butanol-acetic acid-water	Butanol-water	Methanol-water	Butanol-pyridine-water
Choline	0·37	0·09	—	—
Acetylcholine	0·50	0·13	1·0	0·54
Propionylcholine	0·65	0·22	—	—
Butyrylcholine	0·80	0·38	—	—
Optic tract extract				
Spot 1	0·37	0·09	—	—
Spot 2	0·50	0·12	1·0	0·54
Spot 3*	0·78	0·52	—	—

Results of these experiments are summarized in Table 2, where it is apparent that substances resembling choline and acetylcholine were present. In addition, extracts of optic tracts obtained within 1 min of slaughter exhibited a third unidentified substance which may be a quaternary ammonium compound which in butanol-water as solvent moved at a faster rate than choline or the esters studied. When eluted from chromatograms, this substance failed to stimulate guinea-pig ileum. Extracts of tissue collected immediately after slaughter invariably showed greater concentration of acetylcholine-like material and less choline-like substance than were found in tissue collected 10 to 15 min *post mortem*.

The acetylcholine-like substance could be eluted from chromatograms and stimulated the guinea-pig ileum, and was antagonized by small doses of atropine (5×10^{-10} g/ml.) in a manner quantitatively similar to acetylcholine. The extract was 62% inhibited whereas acetylcholine was 59% inhibited.

Equipotent doses of acetylcholine and eluates of the substance resembling acetylcholine were adjusted to equal volumes by addition of Tyrode solution and the solution brought to

*p*H 12 by addition of 3% sodium hydroxide solution for 10 min at room temperature (22° C) and then restored to *p*H 7.4 with 1 N-hydrochloric acid. When tested on guinea-pig isolated ileum, both solutions were quantitatively similar in action and retained only one-quarter of their original potencies (Fig. 2). The inhibition of acetylcholine was 74%, and of the eluted extract 77%.

When incubated with guinea-pig serum at 34° C for 15 min, equipotent doses of acetylcholine and eluted " acetylcholine " showed similar reduction in activity when tested on the ileum. In this instance, contractions due to acetylcholine were reduced by 61% and those of the eluted material by 70%. Control experiments using acetylcholine and saline, serum and saline, and extract and saline showed no variation in activity after a similar incubation. These experiments were performed with acetylcholine equivalent to 50 ng/ml. and incubated with equal volumes of serum (Fig. 3).

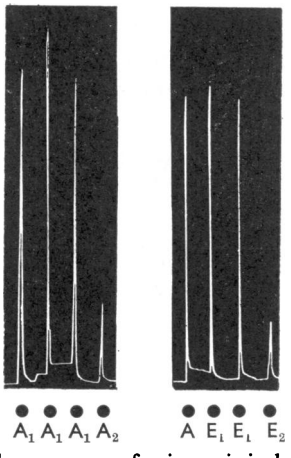


Fig. 2. The responses of guinea-pig isolated ileum to approximately equivalent doses of acetylcholine (A_1, 10^{-9} g/ml.; A_2, 10^{-9} g/ml. after exposure to alkali) and eluates of crude ethanolic extract of optic tracts (E_1, equivalent dose of eluate; E_2, same dose after exposure to alkali) before and after 10 min at *p*H 12 at room temperature (22° C).

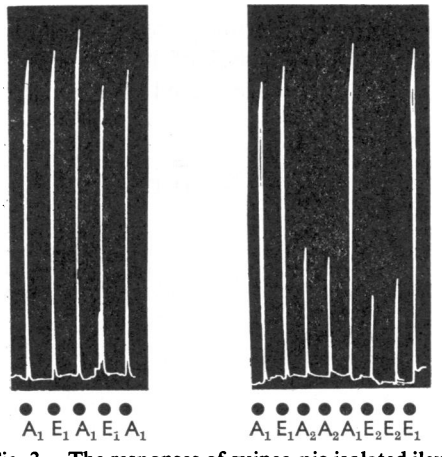


Fig. 3. The responses of guinea-pig isolated ileum to equivalent doses of acetylcholine (A_1, 10^{-9} g/ml.; A_2, 10^{-9} g/ml. after incubation with serum) and eluate of crude ethanolic extract of optic tracts (E_1, equivalent dose of eluate; E_2, same dose after incubation with serum) before and after treatment with guinea-pig serum for 15 min at 34° C.

The rat isolated stomach contracted after administration of crude ethanol or acetone extracts of optic tract tissue, but doses equipotent with a known dose of acetylcholine on guinea-pig ileum caused greater contractions than the known dose of acetylcholine, and took up to 4 min to reach a maximum in contrast to 2 min with acetylcholine. It was apparent that substances were present in the crude extract which were capable of stimulating the rat stomach but not the guinea-pig ileum. (See below.) Eluates of the area of chromatograms of crude extract where acetylcholine might be expected were matched with doses of acetylcholine on guinea-pig ileum and gave quantitatively similar responses on rat stomach.

In all tests, we have been unable to show that the acetylcholine-like substance differs from acetylcholine and conclude that it most probably is acetylcholine.

In an endeavour to determine whether or not histamine was present in crude tissue extracts, equipotent doses of acetylcholine, extract, and histamine were found for guinea-pig ileum. In the presence of small doses of diphenhydramine (20 ng) or mepyramine the response to histamine was much reduced but responses to acetylcholine and the crude extract were unaltered.

Chromatograms of crude extract and histamine were developed in four solvent systems and histamine was revealed by spraying with diazotized sulphanilic acid (Pauly's reagent). The crude extract showed spots with this reagent with similar mobilities to histamine in three solvent systems but in the fourth (*n*-propanol-ammonia-water) the R_F values were very different (Table 3). Both histamine and the substance in the chromatograms reacted to form red spots with Pauly's reagent, but ninhydrin coloured histamine a pink grey and the substance in the extracts reacted to form a purple complex. To react with Pauly's reagent histamine had to be present in amounts greater than 1 $\mu g/cm^2$. Elution of the tissue substance from chromatograms revealed no stimulant action on guinea-pig intestine which was sensitive to doses of histamine of the order of 25 ng. It was concluded that no histamine could be detected in these extracts of optic tracts or lateral geniculate bodies.

TABLE 3

R_F VALUES OF HISTAMINE AND A SUBSTANCE IN CRUDE EXTRACTS OF OPTIC TRACTS WHICH REACTS WITH PAULY'S REAGENT IN A NUMBER OF SOLVENT SYSTEMS

	Solvent system			
Material	Butanol-acetic acid-water	Methanol-acetic acid-water	*n*-Propanol-ammonia-water	Isopropanol-ammonia-water
Extract	0·15	0·65	0·19	0·05–0·10
Histamine	0·15	0·60	0·45–0·55	0·09

Chromatograms of crude extracts were sprayed with *p*-dimethylaminobenzaldehyde to detect the possible presence of 5-hydroxytryptamine. When developed in butanol-acetic acid-water, a substance reacted with the reagent in a position where 5-hydroxytryptamine was found, but in butanol-pyridine-water the unknown substance and 5-hydroxytryptamine were easily separated (Table 4).

TABLE 4

R_F VALUES FOR ACETYLCHOLINE, 5-HYDROXYTRYPTAMINE AND A SUBSTANCE IN CRUDE EXTRACTS OF OPTIC TRACTS REVEALED BY *p*-DIMETHYLAMINOBENZALDEHYDE IN TWO SOLVENT SYSTEMS

	Solvent system	
Material	Butanol-acetic acid-water	Butanol-pyridine-water
Extract	0·51	0·54
5-Hydroxytryptamine	0·51	0·70
Acetylcholine	0·50	0·54

Doses of crude extract, acetylcholine and 5-hydroxytryptamine were matched on the isolated rat stomach and the effects of small doses of atropine and lysergide were examined. The effects of extracts and acetylcholine could be partially antagonized by doses of atropine which had no effect on responses to 5-hydroxytryptamine, and in the presence of lysergide the responses of the tissue to matched doses of extract and acetylcholine were not affected whereas the response to 5-hydroxytryptamine was greatly inhibited.

The areas of chromatograms of crude extract of optic tracts developed in butanol-acetic acid-water which would be expected to contain 5-hydroxytryptamine were eluted and tested on the rat stomach. The stimulant activity was not antagonized by doses of lysergide which caused greater than 50% inhibition of responses to equipotent doses of 5-hydroxytryptamine, but the stimulant activity was antagonized by atropine in a manner quantitatively similar to equipotent doses of acetylcholine. In butanol-acetic acid-water, 5-hydroxytryptamine and acetylcholine have similar R_F values (Table 4) and it was concluded that there was no detectable 5-hydroxytryptamine in the extract, all the activity being accounted for by the acetylcholine-like substance.

Chromatographic examination of the tissue extract for catechol amines failed to reveal their presence. The areas in which they would occur were determined by adding small quantities of adrenaline, noradrenaline and dopamine to a sample of tissue extract, and chromatographing as described by Crawford & Outschoorn (1951). Elution of the corresponding areas of chromatograms of tissue extract alone caused no inhibition of acetylcholine-induced contractions of guinea-pig isolated ileum.

An examination of optic nerves, optic tracts and lateral geniculate bodies for other substances which stimulate smooth muscle

The previous section has described the examination of ethanolic extracts of the brain tissues for substances known to effect smooth muscle. A disadvantage of ethanol extracts was that, despite attempts to remove lipid material with ether or light petroleum ether, clear solutions of the residue in Tyrode solution could not be obtained. When extracts were prepared using methanol-chloroform (2 : 1, v/v) to which was added one-tenth the volume of distilled water, the aqueous-methanol phase yielded an extract which, upon removal of the solvent, dissolved completely in physiological salt solutions.

Methanol-chloroform extracts were prepared and the water-soluble fraction was chromatographed in butanol-acetic acid-water until the solvent front had advanced 24 cm beyond the starting line. The chromatograms were cut transversely into 2-cm bands and each band was placed in an organ-bath containing a rat isolated stomach preparation, and allowed to remain for 3 min, after which the band was removed and the bath flushed out with fresh Krebs solution. Two significant areas of activity were detected—one occurring in the 6th, 7th and 8th bands from the starting line which was the substance which behaved similarly to acetylcholine as described above, and another which was concentrated in the 1st and 2nd bands from the starting line and which stained red when sprayed with Pauly's reagent, and yellow with Ehrlich's reagent. The latter area of activity was studied to attempt identification of the stimulant substance.

After 80 mg of the water-soluble fraction derived from a methanol-chloroform extract of optic tracts had been chromatographed in butanol-acetic acid-water, the first two bands (R_F=0.00 to 0.18) were eluted and the eluate concentrated *in vacuo* and rechromatographed in several solvent systems. The active area was located by spraying separate strips of the chromatogram with Pauly's and Ehrlich's reagents and confirmed by testing unsprayed strips on the rat stomach preparation. In each instance the stimulant activity was paralleled by the colour reactions.

When eluted from chromatograms the unknown substance had no action on guinea-pig isolated ileum, but caused strong contractions of the rat isolated stomach. When doses of

this substance equipotent with acetylcholine were tested on the isolated stomach, it was found that atropine (10^{-9} g/ml.) administered simultaneously caused approximately 50% inhibition of responses to acetylcholine but did not affect the responses to the eluted stimulant substance (Fig. 4). Mepyramine was without effect on contractions produced by this unknown substance, although the tissue was not very sensitive to histamine, doses in excess of 1 mg (in a 10-ml. organ-bath) being required to produce contractions.

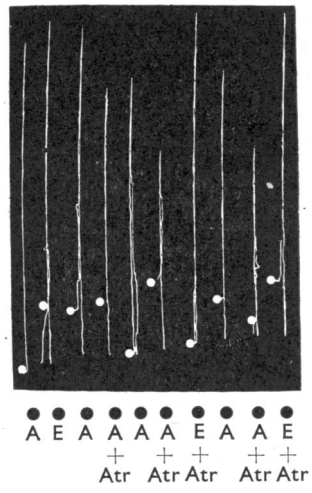


Fig. 4. The effect of atropine (Atr, 10^{-9} g/ml.) in small doses on the contractions of rat isolated stomach to matched doses of acetylcholine (A, 2×10^{-9} g/ml.) and unknown stimulant material (E) eluted from a chromatogram developed in butanol-acetic acid-water. The white spots indicate the level from which the responses commenced after stretching the preparation between doses. The drum was switched off when doses were added and the frontal writing lever was removed from the kymograph when the preparation was stretched beyond the baseline. The interval between doses was approximately 5 min.

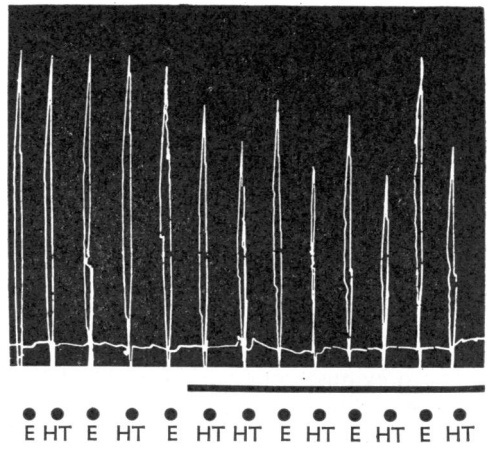
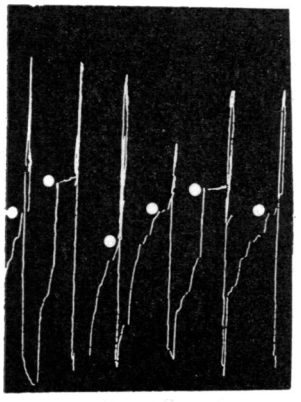


Fig. 5. The effect of small doses of lysergide (10^{-10} g/ml., indicated by the black line) on contractions of rat isolated stomach in response to matched doses of 5-hydroxytryptamine (HT, 2×10^{-10} g/ml.) and unknown stimulant material (E) eluted from a chromatogram developed in butanol-acetic acid-water. The drum was turned off during contraction and stretching, and doses were added at approximately 5-min intervals.

Eluates were matched approximately with 5-hydroxytryptamine on this preparation, and then were administered in the presence of lysergide. After 20 min, the responses to 5-hydroxytryptamine were reduced by approximately 50%, but those due to the eluted substance were much less affected (Fig. 5). When exposed to alkali at *p*H 12 for 10 min at room temperature with subsequent restoration to *p*H 7.4, the responses to eluted material were unaltered, but under the same conditions a previously equipotent dose of acetylcholine showed approximately 50% reduction in activity (Fig. 6).

The colour reactions of this substance resemble those of " Substance A-2 " extracted by methanol-chloroform from whole cat brains (Toh, 1963). Substance A-2 was thermolabile



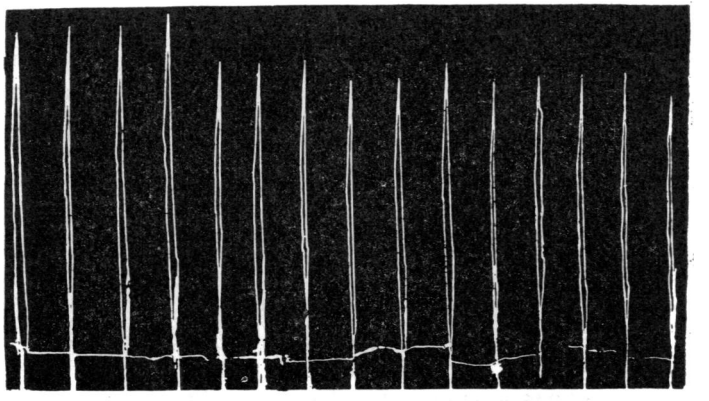


Fig. 6. The responses of rat isolated stomach to approximately equivalent doses of acetylcholine (A_1, 2×10^{-9} g/ml.; A_2, after exposure to alkali) and the unknown stimulant (E_1; E_2, after exposure to alkali) eluted from a chromatogram developed in butanol-acetic acid-water before and after 10 min at *p*H 12 at room temperature (23° C). The white spots indicate the level from which the responses commenced after stretching the preparation between doses, which were added at approximately 5-min intervals.

Fig. 7. The effect of incubation at 37° C at *p*H 6.9 on the stimulant substance eluted from a chromatogram developed in butanol-acetic acid-water. The responses were recorded from a rat isolated stomach preparation and the drum was turned off during contraction and stretching. The interval between doses was approximately 5 min. E, Unincubated eluate; E_1, E_2, E_3 and E_4 are the same doses incubated for 15, 30, 45 and 60 min respectively.

and was 80% inactivated by incubation at 35° C for 1 hr at *p*H 6.4. Small samples of eluted material were brought to *p*H 6.9 and were incubated at 37° C for 15, 30, 45 and 60 min. When tested on the rat stomach no loss of activity was observed (Fig. 7) and it was concluded that the stimulant in optic tracts differed from Substance A-2.

In the various chromatographic systems employed, the unknown rat stomach stimulant had the following R_F values: butanol-acetic acid-water, 0.06 to 0.20; butanol-water, 0.08 to 0.12; isopropanol-ammonia-water, 0.05 to 0.10; and *n*-propanol-ammonia-water, 0.12 to 0.23. Comparison with Table 3 reveals that in two of these systems (butanol-acetic acid-water and isopropanol-ammonia-water) the unknown substance behaved like histamine. The stimulant also behaved like histamine or 5-hydroxytryptamine in its colour reactions both with Pauly's and Ehrlich's reagents and, indeed, these reactions are common to both indole and imidazole derivatives.

Comparison of this unknown stimulant with a number of indole derivatives on chromatograms developed in butanol-acetic acid-water revealed no similarity in R_F to the known compounds as indicated by Pauly's reagent. The unknown stimulant did not move as far as the other indoles from the starting line (Table 5).

The ultraviolet absorption spectrum of the active eluate was determined in a Perkin-Elmer 350 Spectrophotometer. Absorption maxima were recorded at 210 and 245 mμ in contrast to indoles which have a maximum at 270 mμ. Imidazoles also exhibit absorption maxima

TABLE 5

R_F VALUES AND COLOUR REACTIONS OF SOME INDOLE DERIVATIVES DEVELOPED IN BUTANOL-ACETIC ACID-WATER AND SPRAYED WITH PAULY'S REAGENT

* Solvent system was butanol-pyridine-water; † colour reagent was ninhydrin aerosol (Sigma)

Compound	R_F	Colour
5-Hydroxytryptamine	0·47	Red
3-Indolylacetic acid	0·95	Yellow
5-Hydroxyindolylacetic acid	0·75	Red
5-Hydroxytryptophane*	0·47	Brown
n-Acetyltryptophane	0·90	Yellow
Tryptamine	0·70	Pink-grey†
1-Hydroxy-2-dimethyl-5-hydroxyindole	0·64	Brown-red
Stomach stimulant substance	0·14	Red
Stomach stimulant substance	0·14	Pink†

at 210 and 245 mμ but with greater absorption at the former wavelength. The absorption at 245 mμ by histamine was very much less than by the eluate.

In the chromatographic systems, butanol-acetic acid-water, isopropanol-ammonia-water and methanol-acetic acid-water, the eluate and histamine displayed similar R_F values. In the system *n*-propanol-ammonia-water, however, the two could be clearly differentiated as the eluate had an R_F of 0.19 and the corresponding value for histamine was 0.45 to 0.55. Neither histamine nor histidine showed any stimulant action on the rat isolated stomach except in enormous doses, and it is therefore most unlikely that the activity of the eluate could be attributed to either of these imidazoles.

The unknown stimulant differed from γ-aminobutyric acid which had an R_F value of 0.35 to 0.40 in the butanol-acetic acid-water system and it was concluded that the stimulant activity on the rat stomach could not be attributed to this substance.

DISCUSSION

Extracts of optic nerves, optic tracts and lateral geniculate bodies of sheep prepared with ethanol, acetone or methanol-chloroform have yielded substances which are capable of stimulating guinea-pig isolated ileum or rat isolated stomach preparations. One such substance resembled acetylcholine in its behaviour in all the chromatographic systems examined and was readily distinguished from choline and its esters other than acetyl. This acetylcholine-like substance behaved quantitatively like acetylcholine on both smooth muscle preparations in the presence of small doses of atropine, and was destroyed at a comparable rate to acetylcholine when exposed to a *p*H of 12 for 10 min, or when incubated with guinea-pig serum at 37° C. In no test could the substance be distinguished from acetylcholine, and it is suggested that it is highly probable that it was acetylcholine.

The acetylcholine equivalent of optic nerves was of the order of 0.25 μg/g of tissue, with a similar concentration in optic tracts removed from the animal within 1 min after slaughter. This figure compares favourably with 0.3 μg/g given by McIntosh (1941) for optic nerves in cats. In general, the acetylcholine equivalent of optic tracts was higher, the quicker they were removed from the animal and frozen. Lateral geniculate bodies had a lower acetylcholine equivalence of the order of 0.10 μg/g of tissue.

This figure for lateral geniculate bodies is lower than might be expected from the experiments of Hebb & Silver (1956), who showed that sheep lateral geniculate bodies have

choline acetylase activity more than 1,000-times greater than that in optic nerves of the rabbit or the dog. We can only suggest that the tissue does not necessarily synthesize acetylcholine at the maximum capacity found in *in vitro* experiments.

If the substance present in these extracts is acetylcholine, then its significance is not clear, for its concentration is low in comparison with other regions of the nervous system where cholinergic transmission is accepted, for example the anterior spinal roots (McIntosh, 1941). It seems to us that acetylcholine is unlikely to be the prime transmitter at the optic-geniculate synapse, although its presence in small concentrations may serve to modify the action of other transmitter substances.

Another quaternary ammonium substance was detected in extracts prepared from tissue frozen within 1 min of death of the animal, but never when 10 to 15 min were allowed to elapse following slaughter. This substance did not resemble any other esters of choline which were available to us and we cannot offer any suggestions as to its identity. The apparent lability may have significance in postulating a transmitter role for the material. When eluted from chromatograms this compound had no detectable action upon guinea-pig isolated intestine, but there is no reason to suppose that a possible central transmitter substance could act at any site other than the synapse to which it is appropriate, let alone on morphologically different tissue.

Although optic nerves of oxen do contain up to 9 μg/g of histamine (Werle & Weicken, 1949; Euler, 1956), we were unable to find evidence of any histamine in the optic nerves of sheep; this is in agreement with the finding for dog optic nerve (Adam, 1961). Similarly, we could find no traces of 5-hydroxytryptamine in our extracts, in agreement with Amin, Crawford & Gaddum (1954). Only traces of noradrenaline were found by Vogt (1954) and, in our less-sensitive estimates, we were unable to demonstrate the presence of noradrenaline at all.

Methanol-chloroform extracts of these tissues were capable of chromatographic separation into the acetylcholine-like substance and another substance which stained red with Pauly's reagent and yellow with *p*-dimethylaminobenzaldehyde. This substance resembled the material designated as Substance A-2 by Toh (1963) which was extracted from whole cat brains, but one important point of difference was found. Toh's Substance A-2 was thermolabile and was 80% inactivated following 1 hr of incubation at 35° C at *p*H 6.4. Under similar conditions, the substance present in our extracts suffered no change in activity and we suggest that Toh's substance must therefore arise from regions of the brain other than the tissues studied here.

The unknown substance in our methanol-chloroform extracts did not contract guinea-pig ileum but stimulated the rat stomach preparation, and was not antagonized by small doses of atropine, lysergide or mepyramine and was not destroyed by exposure to alkaline *p*H or incubation with guinea-pig serum. Although indoles and imidazoles react with Pauly's reagent to produce red compounds, we could not relate the unknown substance to any indoles available to us, or to histamine or histidine, because the unknown material in the extract had different chromatographic behaviour and different behaviour on isolated preparations. In view of the ultraviolet absorption maxima of the unknown, it is possible that an imidazole is present but we have no further evidence and the nature and significance of this substance is still obscure.

SUMMARY

1. The water-soluble fractions of ethanol or methanol-chloroform extracts of optic nerves, optic tracts and lateral geniculate bodies of sheep were examined for smooth muscle stimulant substances.

2. Stimulant activity was determined initially on guinea-pig isolated ileum and expressed as μg equivalents of acetylcholine per g of fresh tissue. A substance resembling acetylcholine was found which could not be distinguished from acetylcholine by pharmacological, chromatographic or chemical tests.

3. No histamine, 5-hydroxytryptamine or noradrenaline was detected in the extracts.

4. An unidentified water-soluble substance could be separated from the acetylcholine-like substance by paper chromatography, which was inactive on guinea-pig ileum but which contracted the isolated rat stomach.

5. The ultraviolet absorption maxima of this substance resembled those of imidazoles, but differed from those of histamine and histidine.

6. These findings are discussed in relation to possible roles of these substances as central nervous transmitters.

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