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# BIOLOGICALLY ACTIVE SUBSTANCES IN BRAIN EXTRACTS

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The investigations reported in this paper show that four substances which act on smooth muscle preparations are present in chloroform–methanol extracts of brain. These can be distinguished from acetylcholine, noradrenaline, histamine, 5-hydroxytryptamine (5-HT) and Substance P, whose presence in brain is already well established. Recently aqueous extracts of brain have been shown to contain a spasmogenic unsaturated hydroxy-acid (Ambache & Reynolds, 1960, 1961); a lysophosphatidic acid, a ganglioside, and an unsaturated fatty acid which contract the isolated rabbit intestine have been found in chloroform–methanol extracts by Kirschner & Vogt (1961).

## METHODS

*Preparation of brain extract.* The method of extraction was a modification of the procedure described by Folch, Lees & Sloane-Stanley (1954). Cats or dogs were anaesthetized with veterinary 'Nembutal' (pentobarbitone sodium 60 mg/5 lb. (2·27 kg) body wt. Abbott Laboratories) and bled to death. The brain was dissected out, macerated and extracted with a 2:1 (v/v) chloroform–methanol mixture, 6 ml. mixture/g tissue. The extraction was carried out at about 10° C with continuous stirring for 6 hr. The extract was filtered through glass wool and the tissue residue was re-extracted with another 0·5 vol. of the chloroform–methanol mixture for 3 hr. Each extract was next separately shaken up in a separating funnel with one tenth its volume of distilled water and left overnight at 10° C. The upper aqueous methanol phases were separated off, clarified by centrifugation, pooled together and concentrated *in vacuo* at a temperature not exceeding 45° C. The concentrate was an aqueous solution whose pH varied from 6·4 to 7·2 in different experiments. In a typical experiment 58 g brain from one dog gave 29 ml. extract of pH 6·85 and containing 38·5 mg solids/ml.

*Preparation of nasal mucosa extract.* The procedure of Toh & Mohiuddin (1958) was modified as follows. The mucosa was cut into fine pieces to which 2 ml. of distilled water was added for each gram of tissue. The mixture was alternately frozen and thawed four times and then centrifuged. The supernatant, which had a pH of 7·1, was adjusted to pH 3·6 with 0·2N-HCl. The acidified solution was then extracted twice with 1 vol. of ether, the separation of the phases being assisted by centrifugation. The ether extracts were pooled together and stored in the cold. In an experiment 24 g of nasal mucosa from one sheep head gave 62 ml. ether extract containing 1·3 mg solids/ml.

*Paper chromatography.* Ascending chromatography was carried out at 23–25° C. Extracts were applied along the origin as a band that did not exceed 1 cm in width. Development was stopped when the solvent front reached 24 cm. In this way chromatograms were readily reproducible. The location of active principles on the chromatogram was found by dividing the chromatogram into strips and dipping each strip for 3 min in the organ bath which

contained the isolated biological test preparation. Areas were cut from chromatograms and eluted with distilled water or 70 % (v/v) acetone, using 1 ml. solution/10 $cm^2$ of paper. The elution was carried out with constant shaking for 40 min and the paper strips were re-eluted with 0·6 ml. solvent/10 $cm^2$ for another 20 min. The eluates were pooled and concentrated *in vacuo* or by freeze-drying.

*Solvent systems.* 80 % methanol–acetic acid was a monophasic mixture of methanol, water and glacial acetic acid in the proportions 80–20–2 (v/v).

80 % propanol–acetic acid was a monophasic mixture of *n*-propanol, water and glacial acetic acid in the proportions 80–20–2 (v/v).

Butanol–acetic acid (100:2). 2 ml. of glacial acetic acid was dissolved in 100 ml. *n*-butanol, and water added to the mixture until it became just saturated.

Butanol–ammonia (100:2). 2 ml. of ammonia solution (sp.gr. 0·910) was added to 100 ml. *n*-butanol, and water added to the mixture until it became just saturated.

70 % acetone–acetic acid was a monophasic mixture of acetone, water and glacial acetic acid in the proportions 70–30–2 (v/v).

Ethylmethyl ketone–diethylamine–water (60–3–20 (v/v)). The upper phase of the mixture was used.

Chloroform–lutidine–acetic acid was a monophasic mixture in the proportions 4–4–1 (v/v).

*Paper electrophoresis.* Electrophoresis was carried out on Whatman filter paper No. 3 MM stretched horizontally in a closed chamber. The voltage gradient was 12 V/cm. The buffers used for electrophoresis were 0·02 M acetate–acetic acid buffer (pH 3·6), 0·02 M phosphate buffer (pH 6·7 and 7·8) and 0·02 M glycine-NaOH buffer (pH 9·4). Glucose was used as a marker to indicate movement due to electro-endosmosis.

*Tests for functional groups.* Extracts were spotted on strips of Whatman filter paper No. 1 and then treated with different reagents. Tests for loss of activity in the extracts were made by dipping the paper strips for 3 or 3·5 min in the organ bath containing the biological test preparation. The active principle in some extracts was strongly adsorbed on untreated filter paper, so that when the paper strip was tested on the biological preparation little or no effect was observed. This problem was solved by using acid-washed paper or paper which had been previously exposed to bromine vapour for 10 min and then hung in a stream of air to remove excess bromine.

*Reagents.* $KMnO_4$. A neutral aqueous solution was used, 0·2 g/100 ml. Paper strips that had been sprayed with the reagent were allowed to dry until the pink colour of the permanganate had disappeared and the paper strip had turned brown. This was important, as traces of permanganate contracted the tortoise intestine.

Bromine. The paper strip was placed in a closed vessel containing a few drops of bromine for 10 min. It was then hung in air to remove traces of the reagent.

Phenyl isocyanate. The reagent was directly applied to the paper strip with a Pasteur pipette. The treated paper was then exposed to air until the pungent smell of the reagent had disappeared.

Ninhydrin. A w/v solution in chloroform, 0·1 g/100 ml., was used. After the paper strip had been sprayed with the reagent it was hung in the air at room temperature for 1 hr before being tested on the biological preparation.

Pauly's reagent. This was made up as follows: sulphanilic acid, 1 g in 10 ml. concentrated HCl and 100 ml. water (1 vol.); $NaNO_2$ in water (5 g/100 ml., 1 vol.); anhydrous $Na_2CO_3$ in water (10 g/100 ml., 2 vol.). The solutions were mixed and used immediately.

Schiff's reagent. $SO_2$ was passed through a solution of fuchsin (*p*-rosaniline HCl, 0·1 g/100 ml.) until the colour was discharged.

Periodate oxidation was carried out according to the method of Buchanan, Dekker & Long (1950). The paper strip was sprayed with aqueous solution of $NaIO_4$, 1 g/100 ml., and oxidation was allowed to take place for 10 min at room temperature in the open air. A 10 % (v/v) aqueous solution of ethylene glycol was then sprayed on the paper and 10 min was allowed for the destruction of the excess periodate.

*Isolated tortoise jejunum.* This preparation has been described by Toh & Mohiuddin (1958). In the presence of atropine it is not stimulated by acetylcholine, 5-HT or by small amounts of histamine. Noradrenaline even in large amounts has only a very weak inhibitory effect on some preparations, but a weak stimulating effect on others.

*Isolated rabbit jejunum and guinea-pig ileum.* The isolated intestine was suspended in a 20 ml. bath containing Tyrode solution aerated with oxygen; 0·5 $\mu$g each of atropine and of lysergic acid diethylamide (LSD) were added to the bath. Although LSD did not abolish the stimulating effect of large amounts of 5-HT on the rabbit jejunum, it did reduce the size of the contractions produced by 5-HT. Crystalline chymotrypsin and alkaline phosphatase in doses of 5–10 mg contracted the rabbit intestine. Before solutions which contained these enzymes were tested the enzymes were first inactivated by keeping the solutions in a boiling water-bath for 30 sec and then cooling rapidly to room temperature. Control solutions of extracts were similarly treated. Both chymotrypsin and alkaline phosphatase were commercial preparations obtained from Mann (New York). The alkaline phosphatase contained 150 Bodansky units/mg.

Substance P was a sample given by Dr B. Pernow in 1954. It contained 5 units/mg (Pernow, 1953).

*Abbreviations.* Adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), guanosine-5′-triphosphate (GTP), inosine-5′-triphosphate (ITP), uridine-5′-triphosphate (UTP), cytidine-5′-triphosphate (CTP).

## RESULTS

### *Fractionation of brain extract by paper chromatography*

40 mg of a brain extract was applied along a 4 cm line on Whatman No. 3 MM paper for ascending chromatography in 80% propanol–acetic acid. The chromatogram was subdivided transversely into 2 cm strips which were tested on the atropinized tortoise gut or on the rabbit jejunum in the presence of atropine and LSD. Figure 1 shows the distribution of activity along the chromatogram into two main zones, as detected by contractions of the tortoise gut. One zone included the origin and strip 1 ($R_F$ 0–0·08), and the other included strip 12 ($R_F$ 0·92–1·00).

When the rabbit intestine was used as the test object both excitatory and inhibitory effects were observed (see Fig. 2). Material located in strips 4–6 ($R_F$ 0·25–0·50) of the chromatogram produced contractions accompanied by peristalsis and of mucus secretion. With some extracts this contraction was partly obscured by the presence of an inhibitory substance, so that inhibition of the rabbit gut was observed to precede the contraction. Another substance that contracted the rabbit gut was located in strip 12 ($R_F$ 0·92–1·00). The origin and strip 1 ($R_F$ 0–0·08) of the chromatogram contained material that inhibited the rabbit intestine, as is also shown in Fig. 2.

In order to study these fractions in greater detail larger amounts of brain extract (720–2280 mg) were fractionated by paper chromatography. Three main fractions were obtained. (1) Fraction A; this was eluted with distilled water from the origin and strip 1 ($R_F$ 0–0·08). (2) Fraction B;

strips 4–6 ($R_F$ 0·25–0·50) were eluted with water in the early experiments, but with 70% (v/v) acetone in later experiments, when it was found that the latter solvent gave a better recovery of active material. (3) Fraction C; strips 11–12 ($R_F$ 0·83–1·00) were eluted with distilled water.

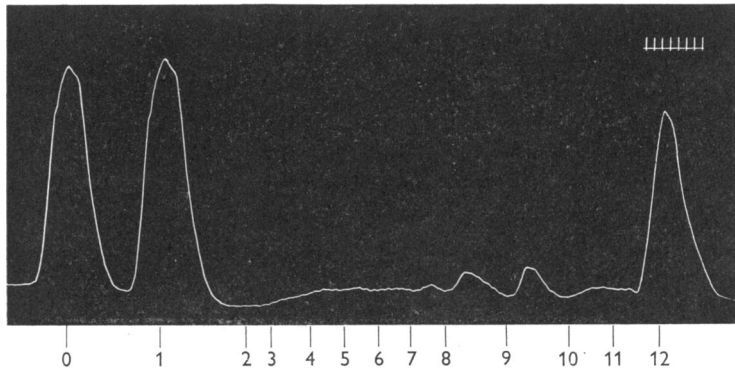


Fig. 1. Ascending chromatography of brain extract in 80% propanol–acetic acid; location of activity by the atropinized tortoise jejunum. The chromatogram was 24 cm long and was divided into twelve successive transverse strips of 2 cm, each of which was left in the bath for 3 min. Strip numbers are given below; strip 0 contained the origin and 12 the solvent front. Time 1 min.

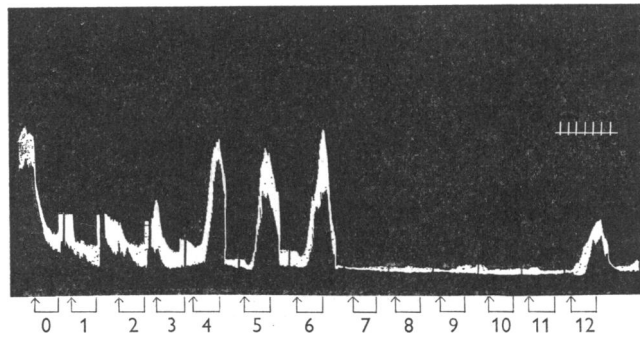


Fig. 2. Same chromatogram and strip numbering as in Fig. 1; location of activity by the rabbit jejunum preparation in atropine and lysergic acid diethylamide (LSD). Contacts 3 min. Time 1 min.

*Fraction A: subdivision into fractions A 1 and A 2 by paper chromatography.*

20 mg of Fraction A was applied along a 4 cm line on Whatman No. 3 MM paper and rechromatographed in 80% methanol–acetic acid. As is shown in Fig. 3, two components were now found, both of which contracted the atropinized tortoise gut. One of these was located at the origin and in strip 1 ($R_F$ 0–0·08) and is referred to as Substance A 1. The second com-

ponent, Substance A 2, appears to be the major active material and was located in strip 6 ($R_F$ 0·42–0·50). In other experiments in which larger amounts (10 mg/cm) of Fraction A were applied along the origin, Substance A 2 had the slightly lower $R_F$ value of 0·33–0·42 (strip 5 of chromatogram) but the $R_F$ value of Substance A 1 was not altered. Both Substances A 1 and A 2 could be eluted with water from these chromatograms for further investigation.

Substance A 1 strongly inhibited the rabbit intestine, while Substance A 2 had only a slight inhibitory effect. As ATP and AMP also contract the tortoise gut and inhibit the rabbit jejunum, their $R_F$ values on chromatograms developed in 80% methanol–acetic acid were determined. ATP had an $R_F$ value of 0–0·08 and AMP 0·17–0·33.

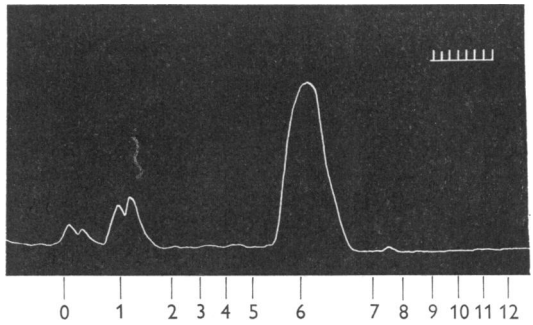


Fig. 3. Atropinized tortoise jejunum; 24 cm chromatogram of 20 mg of Fraction A, developed in 80% methanol–acetic acid. Strip numbering and contacts as in Fig. 1. Time 1 min.

*Paper electrophoresis.* 10 mg of Substance A 1 and 20 mg of Substance A 2 were subjected to electrophoresis. At pH 3·6 Substance A 1 and ATP moved 5 cm and AMP 1·5 cm towards the anode in 2 hr; these values were corrected for endosmosis. Substance A 1 is thus strongly acidic, like ATP. At pH 3·6 and 6·7 Substance A 2 did not show any net movement, but at pH 9·4 it moved 5 cm towards the anode in 2 hr. Substance A 2 is therefore a weaker acid than Substance A 1 and AMP.

*Inactivations.* 6·8 mg portions of Substance A 1 and 5 mg portions of Substance A 2 were spotted on Whatman No. 1 paper. After treatment with various reagents the paper strips were tested on the atropinized tortoise gut. Substance A 2 is strongly adsorbed on ordinary filter paper and for these experiments it was necessary to use filter paper which had been either acid-washed or exposed to bromine vapour, as described in Methods.

The activity of Substance A 1 was not affected by Pauly's reagent or by bromine vapour but was destroyed by phenyl isocyanate (Fig. 4). These

reagents have a similar effect on ATP. From its chromatographic, electrophoretic, chemical, and pharmacological properties Substance A 1 thus appears to be ATP itself, or a nucleotide with similar properties. The sensitivity of the tortoise gut to the different nucleotides was found to be ATP, GTP, UTP, ITP, CTP in descending order. Creatine phosphate has no effect on the tortoise or rabbit gut.

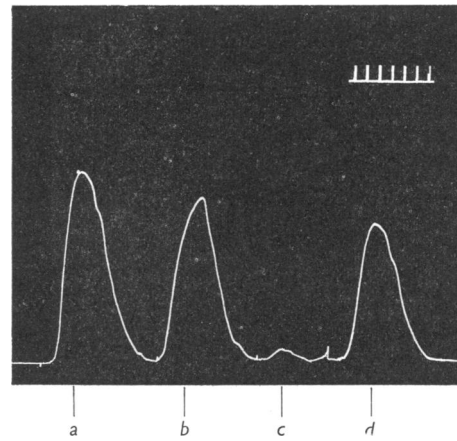


Fig. 4. Atropinized tortoise jejunum. Effect on the activity of 3·6 mg Substance A 1, spotted on filter paper, of (*b*) Pauly's reagent, (*c*) phenyl isocyanate and (*d*) bromine; (*a*) is the control. For details see text. Time 1 min.

Substance A 2 was destroyed in Pauly's reagent, phenyl isocyanate and by $NaIO_4$, but not by bromine vapour nor by $KMnO_4$. In fact, after exposure to bromine vapour the effect of Substance A 2 on the tortoise gut was potentiated (Fig. 5), but there was no potentiation by $KMnO_4$. The loss of activity after treatment with phenyl isocyanate and with periodate suggests the presence of a *cis*-polyhydroxy grouping such as is present in a carbohydrate. Substance A 2 probably contains a glycoside residue.

Substance A 2 is thermolabile. On incubating 10 mg of it for 1 hr at 35° C, the activity on the tortoise gut was reduced by about 40 % at pH 3·9, by about 80 % at pH 6·4, and was completely destroyed at pH 8·5. Substance A 2 is not deoxyuridine, inosine, xanthosine, guanosine, inosine-5′-monophosphate, guanosine-5′-monophosphate, dihydrodiphosphopyridine nucleotide, flavine mononucleotide, flavine adenine dinucleotide, or cytidine diphosphocholine, as these compounds have no effect on the tortoise gut. Adenosine and adenine contract the tortoise gut only when very large doses are used. Lastly, the electrophoretic properties of Substance A 2 preclude the possibility that it is adenosine or adenine.

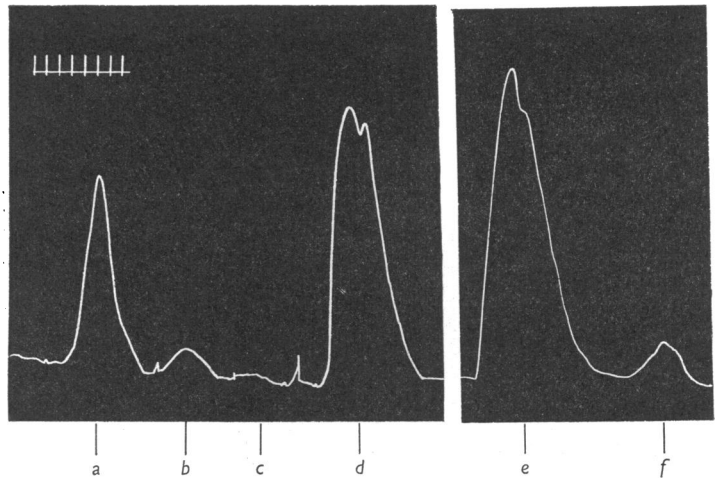


Fig. 5. Atropinized tortoise gut. Left-hand panel: effect on the activity of 5 mg Substance A 2, spotted on filter paper, of (*b*) Pauly's reagent, (*c*) phenyl isocyanate and (*d*) bromine; (*a*) is the control. Right-hand panel: destruction of 10·8 mg Substance A 2 shown at (*f*), after treatment with $NaIO_4$; untreated control at (*e*). Time 1 min.

### *Fraction B*

*Paper chromatography.* 15–20 mg of Fraction B was applied along a 4 cm line on Whatman No. 3MM paper and developed in 70% acetone–acetic acid. It was found that Fraction B contained a component which inhibited the rabbit gut as well as a component which contracted it (see Fig. 6). The inhibitory material was located in strips 6–9 ($R_F$ 0·50–0·75), and is probably adenosine, which had an $R_F$ value of 0·50–0·68 under comparable conditions. The activity of this inhibitory material was destroyed, as was that of adenosine, both by phenyl isocyanate and by periodate, which act respectively on the hydroxyl and on the 1:2 glycollic grouping present in the ribose part of the adenosine molecule. AMP also inhibits the rabbit gut, but it streaked on the chromatogram and had a lower $R_F$ value (0·25–0·50) than adenosine. It is possible that the adenosine present in the fraction was derived from AMP. In fact, it was noticed that the inhibitory activity in some preparations of Fraction B was considerably reduced after incubation with alkaline phosphatase. The substance which contracted the rabbit gut was located in strips 10–12, i.e. between $R_F$ 0·75 and 1·00, and will be referred to as Substance B.

With chloroform–lutidine–acetic acid as the developing solvent system Substance B remained at the origin. Darmstoff, a phosphatidic acid which contracts the atropinized rabbit jejunum, was found by Vogt (1958) to

separate into two biologically active components in this solvent system: one component remained at the origin, the other moved to an $R_F$ value of 0·85–0·9. With methylethyl ketone–diethylamine water as the developing solvent system Substance B could be differentiated from Darmstoff; Fraction B was found to have an $R_F$ value of 0–0·08 while Darmstoff has an $R_F$ value of 0·4–0·45 (Vogt, 1958).

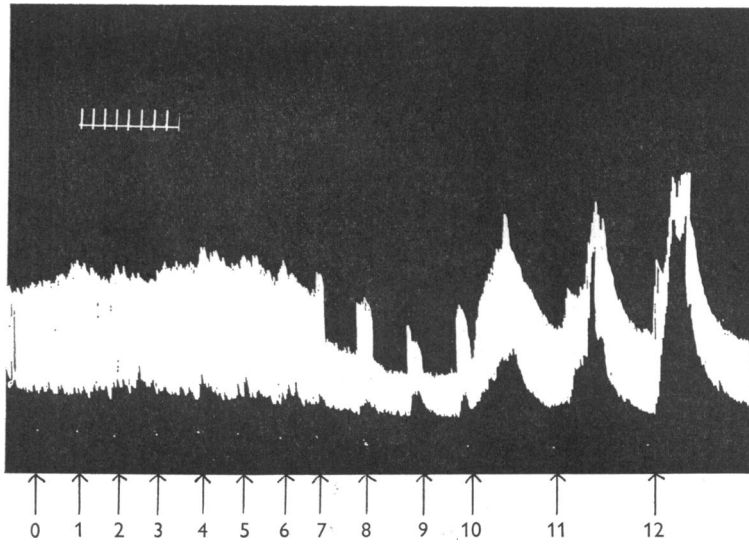


Fig. 6. Rabbit jejunum in atropine and LSD: 24 cm chromatogram of 15 mg of Fraction B, developed in 70% acetone–acetic acid. Strip numbering and 3 min contacts as in Fig. 1; administration at the arrows. For details see text. Time 1 min.

5 mg of Substance P was subjected to chromatography and the activity was located on the chromatograms by assays on the rabbit's intestine. With methylethyl ketone–diethylamine–water as the developing solvent system, Substance P was found to have an $R_F$ value of 0–0·08, like Substance B. In *n*-butanol–acetic acid both Substance P and Substance B had an $R_F$ value of 0–0·08. The two substances could, however, be clearly distinguished by electrophoresis and by their interaction with nupercaine (see below).

*Distinction from Substance P by paper electrophoresis.* 13–24 mg of Fraction B was subjected to electrophoresis. At pH 3·6 and 4·7, there was no net movement of Substance B. At pH 6·7, it moved 3 cm towards the anode in 2·5 hr (value corrected for electro-endosmosis). Substance B is thus an acidic material. On the other hand, when 4 mg Substance P was subjected to electrophoresis, the active material moved 4 cm in 2 hr towards the cathode at pH 3·6.

*Inactivations.* 6 mg portions of Fraction B were spotted on Whatman No. 1 paper and treated with various reagents. Tests for activity were then made on the rabbit intestine (Fig. 7). Substance B was destroyed by bromine vapour and by neutral $KMnO_4$, but not by Schiff's reagent. Its activity is thus dependent on an unsaturated carbon bond. The active material was not destroyed by phenyl isocyanate nor by ninhydrin. Substance B was not destroyed on incubating at pH 3·7, 4·2, 6·4, 8·8 and 9·6 for 1 hr at 34° C.

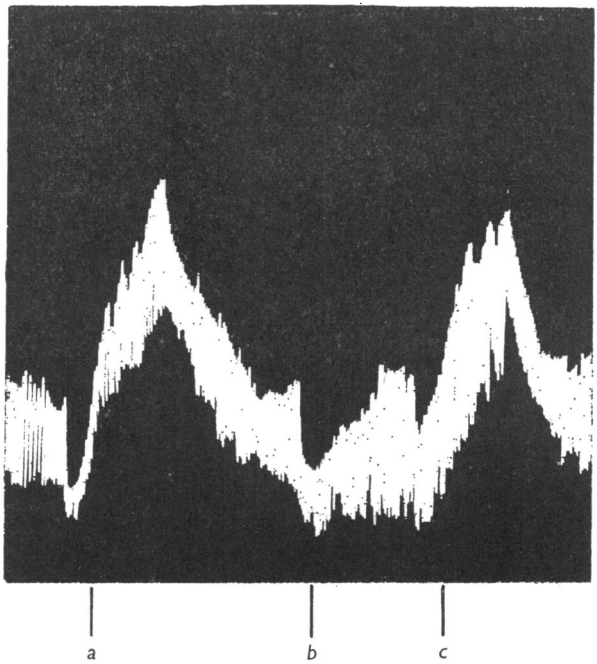


Fig. 7. Rabbit intestine in atropine and LSD. Effect on the activity o 6 mg Substance B, spotted on filter paper, of (*b*) $KMnO_4$ and (*c*) phenyl isocyanate; (*a*) is the control. For details see text.

6 mg portions of Fraction B were incubated at 34° C with 10 mg alkaline phosphatase (Mann) and with 5 mg crystalline chymotrypsin (Mann) respectively in 1 ml. solution at pH 8·6. At the end of 1 hr the activity of Substance B was considerably reduced by the phosphatase, and after 2 hr it was completely destroyed (Fig. 8*b*). Chymotrypsin completely destroyed the activity of Substance B after incubation for 1 hr (Fig. 8*c*).

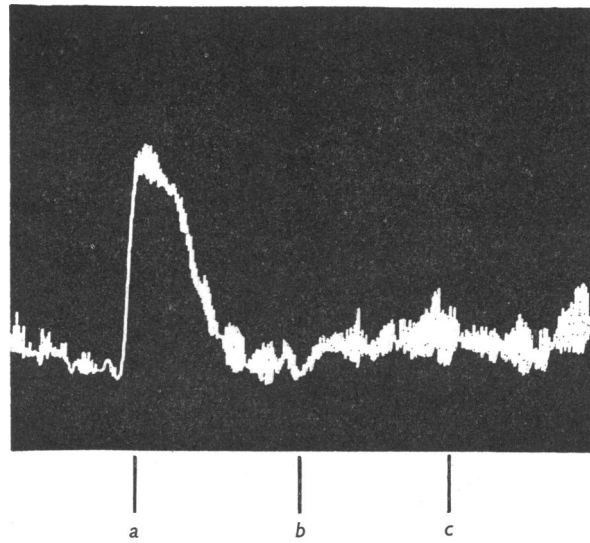


Fig. 8. Rabbit intestine in atropine and LSD. Destruction by alkaline phosphatase (*b*) and by chymotrypsin (*c*), of the activity of 2 mg of Substance B; control shown at (*a*). Incubation with the enzymes was carried out at 35° C and pH 8·6 for 2 hr.

### *Fraction C*

*Paper electrophoresis.* 2·7 mg Fraction C was used for electrophoresis. At pH 3·5 and 7·8 there was no net movement of the active material which contracts the tortoise gut. At pH 9·5 the active material migrated to a mean distance of 5 cm in 2 hr from the origin towards the anode; this value is corrected for electro-endosmosis. Fraction C is therefore a weak acid.

*Inactivation.* 3·0 mg portions of Fraction C were spotted on Whatman No. 1 paper. After treatment with various reagents the paper strips were tested on the rabbit intestine and on the tortoise gut. The active substance was destroyed by bromine vapour and by $KMnO_4$. Substance C is thus probably an unsaturated acid. It was not destroyed by Schiff's reagent, Pauly's reagent, ninhydrin nor by phenyl isocyanate. With the tortoise gut as the biological test preparation it was found that phenyl isocyanate reduced the activity of Substance C, but did not completely destroy it; whereas on the rabbit gut there was no reduction in activity (see Fig. 9). It is unlikely that Substance C contains an –OH group. Incubation at pH 3·8, 5·0, 7·0 and 9·5 for 1 hr at 35° C did not destroy the active material. There was no destruction of Substance C when 2·4 mg of the material was incubated at 34° C with 10 mg of alkaline phosphatase (Mann) or with

10 mg of crystalline chymotrypsin (Mann) in 1 ml. solution at pH 8·8 for 2 hr.

*Paper chromatography.* 2·4–3·0 mg of Fraction C were applied along a 4 cm line on Whatman No. 3 MM paper for ascending chromatography. Using the tortoise intestine and the rabbit gut as the biological test preparations Substance C was located at $R_F$ 0·42–0·67 in methylethyl ketone–diethylamine–water. In *n*-butanol–ammonia the $R_F$ of Fraction C was 0·33–0·50.

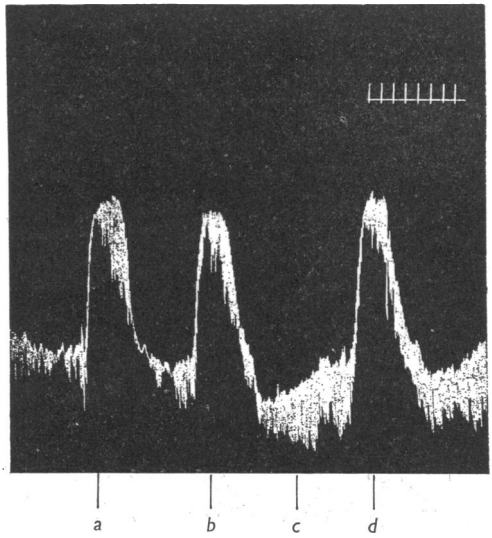


Fig. 9. Rabbit intestine in atropine and LSD. Effect on the activity of 1·2 mg Substance C, spotted on filter paper, of (*b*) Schiff's fuchsin reagent, (*c*) bromine vapour and (*d*) phenyl isocyanate; (*a*) is the control. Time 1 min.

An unsaturated acid capable of contracting the atropinized tortoise intestine has also been found in extracts of nasal mucosa (Toh & Mohiuddin, 1958). With 1·3–2·6 mg of such a nasal mucosa extract it was found that the active acid in the extract had an $R_F$ value of 0·50–0·58 in methylethyl ketone–diethylamine–water. There is thus also some similarity between the active substances in Fraction C and in nasal mucosa, in that both are unsaturated acids and have similar $R_F$ values on paper chromatograms. This is not absolute proof, however, that they are identical, as it is well known that fatty acids are not easily resolved on ordinary paper chromatograms.

Two other unsaturated acids that stimulate smooth muscle have been described. They are Darmstoff, a phosphatidic acid that contracts the atropinized rabbit jejunum and Irin, an unsaturated hydroxy-acid that

contracts the atropinized hamster colon and rat colon. On paper chromatograms run in methylethyl–ketone–diethylamine–water Darmstoff has an $R_F$ value of 0·4–0·45 (Vogt, 1958) and Irin an $R_F$ value of 0·8–0·97 (Ambache, 1959). It is unlikely that Substance C is Darmstoff or Irin.

*Effect of nupercaine (dibucaine) and hexamethonium*

When Substance B was tested on the rabbit intestine, it produced first a strong contraction of the circular muscle. This was then followed by contraction of the longitudinal muscle accompanied by peristalsis and expulsion of intestinal contents. Experiments were carried out to investigate whether local anaesthetics and ganglion-blocking agents could inhibit these effects. It was found that nupercaine, a local anaesthetic, in a concentration of 0·5–1·0 $\mu$g/ml., completely abolished the effect of Substance B.

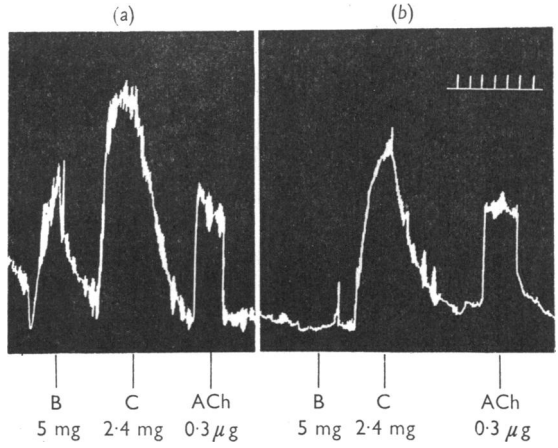


Fig. 10. Unatropinized rabbit intestine. Effects of Substance B (at B), of Substance C (at C), and of acetylcholine (ACh). Doses given below; contacts 2·5 min. In panel (*b*) the bath contained nupercaine 1 $\mu$g/ml. Time 1 min.

Contractions of the rabbit gut to acetylcholine and to Substance C were only slightly depressed by the local anaesthetic (see Fig. 10). Substance B is thus distinguishable from Substance P which is not blocked by cocaine (Pernow, 1953).

The ganglion-blocking agent, hexamethonium, in a concentration of 45 $\mu$g/ml., did not block the effects of 8·4 mg Substance B and 3·3 mg Substance C, although it blocked the contracting effect of 30 $\mu$g nicotine. The effect of Substance B is thus not due to a direct action on ganglion-cells present in the enteric wall, but on some other nervous element.

*Tests on guinea-pig ileum*

Substance A 2 caused a slight inhibition of the guinea-pig ileum. Substance B did not contract this preparation in the presence of atropine, LSD and mepyramine; nicotine likewise failed to produce contractions in the presence of these inhibitors. On the other hand, Substance C produced contractions even in the presence of atropine, LSD and mepyramine.

## DISCUSSION

One of the active substances in Fraction A is probably ATP. The other active component in this fraction, Substance A 2, has not yet been identified. It resembles a nucleoside or a nucleotide in being inactivated by phenyl isocyanate and by periodate, and in stimulating the tortoise intestine and inhibiting the rabbit gut. Both ATP and Substance A 2 are probably present in their free form and not as components of brain nucleic acid because of the mild methods of extraction employed. In fact Schmitz, Potter, Huribert & White (1954) have reported the presence in rat brain extracts of ATP, CTP, GTP, UTP and their derivatives, which have not been produced by the hydrolytic break-down of nucleic acids. The presence of this group of pharmacologically active compounds in the free form have led to investigations as to their role in the central nervous system. Most of the work in elucidating their function have been devoted to their role in carbohydrate and lipid metabolism in brain tissue (Brady & Tower, 1960). It is also interesting to note that the nucleosides cytidine and uridine increase the survival time of perfused brain from 1 to 4 hr as shown by an increase in the electrical activity of the brain. This has been attributed to improved glucose utilization by the perfused brain (Geiger & Yamasaki, 1956). When ATP and adenosine are injected into the lateral ventricle of the cat, behavioural symptoms such as vomiting, defaecation and tachypnoea are produced (Feldberg & Sherwood, 1954). There is no doubt that the nucleotides do play an important metabolic role in the C.N.S., but no adequate evidence has yet been obtained to show that they also have a physiological role in regulating C.N.S. activity or in the transmission of the nerve impulse.

Substance B is destroyed both by phosphatase and by chymotrypsin. This suggests that it is a phosphopeptide. As it is also destroyed by bromine and by potassium permanganate, it is likely that its activity depends upon a C–C linkage. Such a linkage is common in lipids containing unsaturated acids, but not in peptides. If the destruction of activity by bromine and permanganate can be attributed to the presence of such an unsaturated lipid residue, then Substance B could be a lipophosphopeptide probably with the phosphorus group linking the lipid and

peptide residues. No firm conclusion can be made at this stage on the nature of Substance B. The fact that Substance B fails to contract the rabbit intestine in the presence of a local anaesthetic such as nupercaine suggests that it acts through stimulation of nerve tissue. If Substance B can stimulate intestinal nerve tissue, then it is also likely that it can stimulate nerve tissue elsewhere. What role it plays in the C.N.S. remains for future experiments to determine.

## SUMMARY

1. Brain extracts have been fractionated by chromatography and found to contain substances which contract the atropinized tortoise and rabbit intestines. They are not acetylcholine, histamine, 5-HT, noradrenaline or Substance P, which are already known to be present in brain.

2. One of these fractions contains ATP and an unidentified substance, both of which contract the tortoise gut. The unidentified substance, which is referred to as Substance A 2, is acidic and is destroyed by Pauly's reagent, phenyl isocyanate and $NaIO_4$. It probably contains a glycoside residue. Substance A 2 is thermolabile, especially at alkaline pH.

3. A second fraction contains material which contracts the rabbit intestine. This material, referred to as Substance B, is acidic. It is destroyed by bromine vapour and neutral $KMnO_4$. It is also destroyed by alkaline phosphatase and by chymotrypsin. It is suggested that Substance B is a lipophosphopeptide.

4. A third fraction contains an unidentified fatty acid which contracts both the tortoise and rabbit intestines. This acidic substance, referred to as Substance C, is destroyed by bromine vapour and neutral $KMnO_4$. It is thus unsaturated.

5. The effect of Substance B on the rabbit intestine is inhibited by nupercaine, but not by hexamethonium.

6. The effect of Substance C on the rabbit gut is not inhibited by nupercaine nor by hexamethonium.

7. Substance A 2 causes a slight relaxation of the guinea-pig ileum. Substance B fails to contract the guinea-pig gut in the presence of atropine, LSD and mepyramine. Substance C still contracts the gut in the presence of these inhibitors.

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