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VASOACTIVE SUBSTANCES IN THE NASAL MUCOSA

BY

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Besides histamine another smooth muscle stimulating substance has been found in the nasal mucosa of dog and sheep. It is an acid which is readily oxidized by neutral aqueous potassium permanganate and osmium tetroxide, and is thermostable at *p*H 10·8 but not at *p*H 2·8. The purification of this acid has been followed by using the atropinized tortoise intestine as a test object, which is insensitive to acetylcholine, 5-hydroxytryptamine and small amounts of histamine. Both the tortoise intestine and rat colon are contracted by this acidic substance. The amount of histamine found in sheep nasal mucosa ranged from 12 to 23 μg./g. tissue and in dog nasal mucosa from 6 to 13 μg./g. tissue.

The presence of large numbers of blood vessels in the nasal mucous membrane lends the mucosa readily to engorgement and oedema as in allergic vasomotor rhinitis. The factors which control vasomotor tone in the nasal mucosa have not been extensively investigated. Of the various vasoactive substances known to be present in animal tissues only histamine has so far been reported to occur in the nasal mucosa (Buhrmester and Wenner, 1936) and in nasal secretions of allergic rhinitis (Troescher, Ancona, and Kerr, 1945). The experience of one of us (C.C.T.) that the antihistaminic drugs are not completely effective in controlling the nasal congestion of allergic rhinitis has prompted us to investigate the possibility that other pharmacologically active substances besides histamine may be present in the nasal mucosa. This paper reports the finding of an unidentified vasoactive substance besides histamine in extracts of the nasal mucosa.

METHODS

Collection of Nasal Mucosa.—The heads of sheep obtained from the abattoir or of dogs which had been anaesthetized with pentobarbitone and bled to death were used. The heads were split sagittally and the mucosa was stripped from the nasal septum and turbinates. The mucosa was rapidly washed in running tap water, dried between filter paper, weighed and extracted.

Acid Extraction.—Nasal mucosa was minced with 3 ml. 0.1 N-HCl/g. tissue and the mixture briefly heated to boiling point. The extract was neutralized, centrifuged to remove insoluble residue and the supernatant solution used.

Phosphate Extraction.—Nasal mucosa was minced with 1 ml. M/15 phosphate buffer (*p*H 7.6)/g. tissue. The mixture was alternately frozen and thawed 3 times and centrifuged. The residue was re-extracted with an equal volume of phosphate buffer and the two extracts pooled.

Acetone Extraction.—Nasal mucosa was minced with 4 ml. acetone/g. tissue and the mixture left in the refrigerator overnight. The acetone extract was filtered off and the residue was re-extracted with an equal volume of 80% (v/v) acetone for another 3 hr. The acetone extracts were pooled and the acetone removed by evaporating *in vacuo*. The aqueous residue was used. For paper chromatography the aqueous solution whose *p*H was 5.6 to 6.3 was extracted 3 times with an equal volume of *n*-butanol previously saturated with water. The butanol extracts were pooled and evaporated to dryness *in vacuo*. The residue was taken up in ethanol and applied to filter paper for chromatographic development.

Paper Chromatography. — Chromatography was carried out by the ascending method at 25°. The solvent systems used were: Butanol-ammonia (100:1). 1 ml. ammonia solution (sp. gr. 0.910) was added to 100 ml. *N*-butanol. Water was then added to the solution until it became just saturated.

Butanol-acetic acid (100:1). 1 ml. glacial acetic acid was dissolved in 100 ml. *N*-butanol and water added to the solution until it became just saturated.

Areas were cut out from the chromatograms and eluted with M/100-sodium bicarbonate solution, using 1 or 1.5 ml. solution/10 cm.^{2} of paper. The elution was carried out with constant shaking for 1 hr.

Pre-treatment of Filter Paper.—Paper was treated with M/10-ammonium bicarbonate or M/10-am-

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monium acetate by passing the paper through the solution, removing the surplus fluid by pressing between blotting papers and hanging the filter paper up to dry.

Spray Reagents.—After spraying a chromatogram with neutral aqueous 1% $KMnO_4$ the chromatogram was allowed to dry in the air until the pink colour of the $KMnO_4$ had disappeared and the chromatogram had turned brown. This was important as traces of $KMnO_4$ contracted the tortoise intestine.

Treatment of a chromatogram with osmium tetroxide vapour was done in a closed vessel for 1.5 hr., after which the chromatogram was hung up in air for 1 hr. to remove traces of the reagent.

A 0.1% ninhydrin solution in chloroform was used. After the chromatogram had been sprayed with the reagent it was heated at 95° to 105° for 5 min.

The nitrous acid used was a solution of 1% $NaNO_2$ in 0.1 N-HCl.

Paper Electrophoresis.—Electrophoresis was carried out on Whatman filter paper No. 1 stretched horizontally in a closed chamber. The buffers used for electrophoresis were 0.033 M phosphate buffers (*p*H 8.5 and *p*H 5.3) and 0.05 M-glycine in HCl buffer (*p*H 3.5).

Isolated Tortoise Jejunum. — The land tortoise (*Cyclemys amboinensis*) was used. It was killed by pithing. The plastron was removed and the animal bled by removing the heart. A piece of jejunum was dissected out and suspended in a 20 ml. bath containing fluid of the following composition: NaCl 6.5 g., KCl 0.3 g., $CaCl_2$ 0.02 g., $NaHCO_3$ 0.10 g., glucose 0.50 g., water to 1,000 ml. The fluid was designed to prevent spontaneous contractions of the tortoise intestine so that it could be used for quantitative assays. Oxygen was used to aerate the medium. When first placed in the bath the gut was strongly contracted, but if the recording lever to which the preparation was suspended was weighted so as to keep the intestine under a tension of about 5 g. it soon relaxed, relaxation being aided by frequent changes of bath fluid. The weight of the lever was finally adjusted so as to keep the gut under a tension of 2 to 3 g. A loop of intestine which had been removed from the tortoise could be used the next day if the gut was covered with the solution described above and stored in the refrigerator at 10°. Substances tested on the tortoise intestine were kept in the bath for 45 to 60 sec. and were added to the bath at 4 to 5 min. intervals. Some pieces of intestine relaxed only slowly after contracting. In these instances the time interval between addition of substances to the bath was suitably increased.

Isolated Rat Colon.—The preparation used was similar to that described by Dalgliesh, Toh, and Work (1952).

Histamine Assay.—Histamine was assayed on the isolated guinea-pig ileum suspended in a 20 ml. bath containing Tyrode solution and 0.2 μg. atropine sulphate.

RESULTS

Histamine in Nasal Mucosa

The amounts of histamine found in sheep and dog mucosa are given in Table I. In the sheep the amount of histamine ranged from 12 to 23 μg./g. tissue while in the dog it varied from 6 to 13 μg./g. In four experiments on sheep tissue and four experiments on dog tissue the amounts of histamine extractable by acid and phosphate were compared. The values obtained by phosphate extraction were of the same order as those obtained by acid extraction, although they tended to be slightly lower.

TABLE I

HISTAMINE IN NASAL MUCOSA

Animal	μg. Histamine/g. Tissue	
	Acid Extraction	Phosphate Extraction
Sheep 1	12	11
,, 2	14	13
,, 3	16	12
,, 4	19	14
,, 5	19	—
,, 6	18	—
,, 7	23	—
Dog 1	7	6
,, 2	10	7·5
,, 3	13	10
,, 4	6	7

The Presence of an Unidentified Smooth Muscle-stimulating Substance in Extracts of Nasal Mucosa

Evidence that there was present in extracts of dog and sheep nasal mucosa a smooth muscle-stimulating substance which was not acetylcholine, histamine, or 5-hydroxytryptamine was obtained when the extracts were tested on the isolated rat colon and the isolated tortoise intestine.

Tortoise Jejunum.—The isolated tortoise jejunum is little stimulated by histamine and 5-hydroxytryptamine. Relatively large amounts of histamine (1 to 10 μg.) were needed to contract the tortoise gut in a 20 ml. bath. The effect of histamine was to produce a slow contraction of the gut, which could not be readily abolished by the antihistamine compounds mepyramine and promethazine without at the same time depressing the contractions obtained with acetylcholine. 5-Hydroxytryptamine in amounts of 0.2 to 20 μg. inhibited the contractions of the tortoise intestine to acetylcholine (Fig. 1). Although the amounts of 5-hydroxytryptamine tested varied 100 times, the inhibition due to 20 μg. 5-hydroxytryptamine was not much greater than that due to 0.2 μg. The tortoise intestine did not contract to 0.2 or

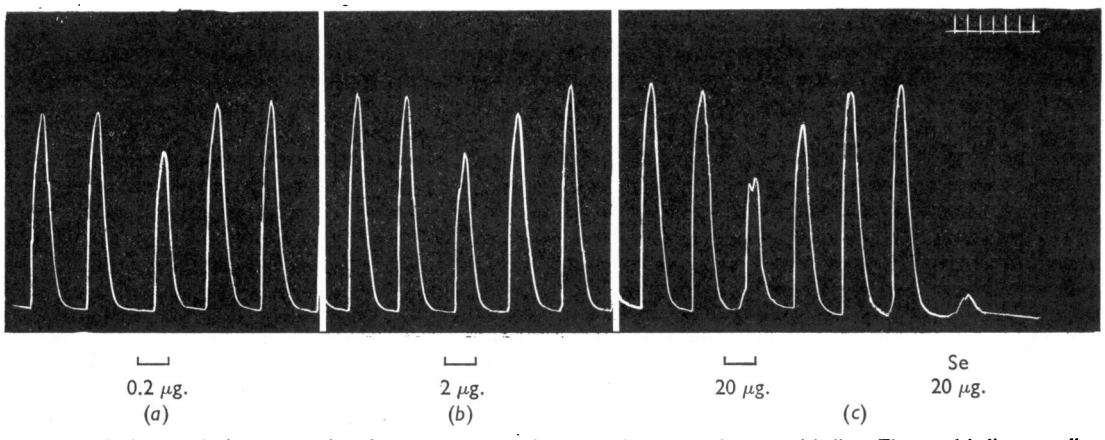

FIG. 1.—Inhibition by 5-hydroxytryptamine of the contractions of tortoise jejunum to 0·6 μg. acetylcholine. The acetylcholine was allowed to act on the gut for 45 sec. and repeated at 4 min. intervals. 5-Hydroxytryptamine was added to the bath 1 min. before the addition of acetylcholine and left in the bath during the period of action of acetylcholine as indicated. The amounts of 5-hydroxytryptamine tested were (*a*) 0·2 μg., (*b*) 2 μg., and (*c*) 20 μg. The gut did not contract to 0·2 μg. or 2 μg. but contracted slightly to 20 μg. as shown in (*c*) Se. Time: 1 min.

2.0 μg. of 5-hydroxytryptamine, but was slightly stimulated by 20 μg. Thus large amounts of 5-hydroxytryptamine had a weakly excitatory as well as an inhibitory effect on the gut. If the tortoise intestine is atropinized, it is not stimulated by acetylcholine, 5-hydroxytryptamine, or small amounts of histamine.

When acid, phosphate, or acetone extracts of nasal mucosa were tested on such an atropinized tortoise intestine it was found that the extracts caused the gut to contract strongly. In Fig. 2, X shows the contraction produced by a phosphate extract of 50 mg. sheep nasal mucosa. The effect of the extract was much greater than that produced by 0.75 μg. histamine, which was the amount present in this quantity of extract. In Fig. 2, Y indicates the contraction of the tortoise intestine to an acid extract of 50 mg. sheep nasal mucosa, which had previously been found to contain 1.0 μg. histamine. The stimulating effect of the extract, however, could not be accounted for by the histamine as the contraction due to 1.0 μg. histamine was again very much less than that due to the extract. The effect of the extracts on the tortoise intestine could not therefore be due to histamine, and since the gut was atropinized it could not be caused by acetylcholine. The effect could not have been caused by 5-hydroxytryptamine which, as already shown, had little or no stimulating effect on the tortoise intestine.

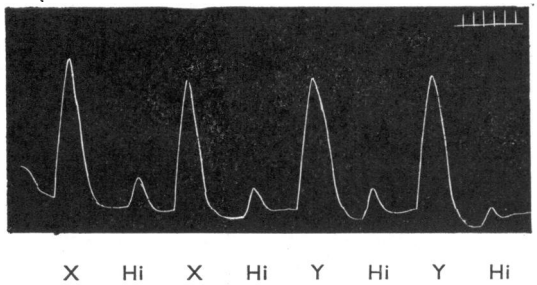

FIG. 2.—Contractions of isolated tortoise jejunum to (X) phosphate extract of 50 mg. sheep nasal mucosa, (Y) acid extract of 50 mg. sheep nasal mucosa and (Hi) histamine in the amounts indicated. The phosphate extract contained 15 μg. and the acid extract 20 μg. histamine/g. tissue. The bath held 20 ml. and contained 0·2 μg. atropine. Time: 1 min.

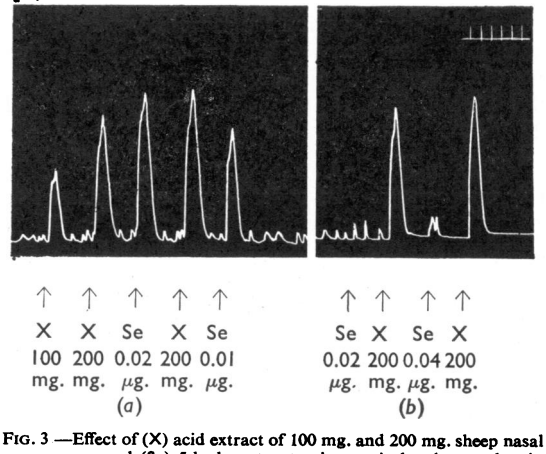

FIG. 3 —Effect of (X) acid extract of 100 mg. and 200 mg. sheep nasal mucosa and (Se) 5-hydroxytryptamine on isolated rat colon in 20 ml. bath containing 0·2 μg. atropine. In (*b*) the bath fluid contained 4 μg. BOL-148 as well as atropine. Time: 1 min.

Rat Colon.—Fig. 3 shows the effect of an acid extract of sheep nasal mucosa on the atropinized rat colon. In Fig. 3(*a*) the contraction produced by 200 mg. mucosa was matched by 0.02 μg. 5-hydroxytryptamine. In Fig. 3(*b*) the bath in which the gut was suspended contained D-2-bromo-lysergic acid diethylamide (BOL 148) as well as atropine. The effects of 0.02 μg. and 0.04 μg. 5-hydroxytryptamine were now abolished, but the contraction due to the extract remained. The effect could not therefore be due to 5-hydroxytryptamine or acetylcholine, nor could it be due to histamine, to which the colon is naturally insensitive.

Characteristics of the Unidentified Active Substance in Extracts of Nasal Mucosa

Dialysability.—The active substance could be dialysed from the crude extract into distilled water. However, even after 1.5 hr. of dialysis the greater part of the activity remained in the dialysing bag.

Effect of Trypsin and Chymotrypsin.—There was no loss of activity on incubating an extract of nasal mucosa at *p*H 8.0 with trypsin powder (crude preparation, British Drug Houses) or with crystalline chymotrypsin (Light). Both enzymes were used in a concentration of 10 mg./ml. of extract of nasal mucosa for 2 hr. at 39°. The active substance in the extract is unlikely to be a polypeptide as it was not destroyed by these enzymes.

Effect of Temperature.—An acetone extract of sheep nasal mucosa was adjusted to different *p*H and heated in a boiling water bath for 15 min. At the end of this period the samples were neutralized and their activity on the tortoise intestine tested. No loss of activity occurred at *p*H 10.8; there was a loss of 10% activity at *p*H 6.4 and 25% at *p*H 2.8.

Location of the Active Principle on Paper Chromatograms.—In order to separate the active principle on paper chromatograms 20 to 25 g. sheep nasal mucosa was extracted with acetone and the active principle taken up in *N*-butanol as described under "Methods." About ⅓ of the butanol extract was applied along a 32 cm. line on Whatman No. 1 filter paper for ascending chromatography. The position of the active principle on the chromatogram was located by dividing the chromatogram into zones and testing the eluates from these zones on the atropinized tortoise intestine.

On paper treated with 0.1 M-ammonium bicarbonate and using *N*-butanol-ammonia as developing solvent it was found that the active principle could be eluted from a zone lying between R_F 0.4 and 0.7. On paper treated with 0.1 M-ammonium acetate and using *N*-butanol-acetic acid as developing solvent it was found that the active principle lay in a zone between R_F 0.7 and 1.0.

If parallel strips from these chromatograms were sprayed with neutral aqueous potassium permanganate (1%) or exposed to osmium tetroxide vapour, no activity was present in the eluates of such treated chromatograms. On the other hand, spraying the chromatograms with ninhydrin, nitrous acid, or Pauly's reagent did not destroy the active principle. These results suggested that the active principle was a readily oxidizable substance but that it was not likely to be a base or a phenol.

Experiments were also made to purify larger quantities of extract on untreated Whatman No. 3MM paper using butanol-ammonia as developing solvent. The zone lying between R_F 0.4 and 0.7 was eluted with sodium bicarbonate solution and the eluate evaporated to dryness *in vacuo*. The residue obtained in this way was a mixture of sodium carbonate and purified extract. It was taken up in 0.9% NaCl solution for tests on the tortoise intestine. In one experiment (Fig. 4) the atropinized tortoise intestine was contracted by 0.5 to 1.0 mg. of solid.

Paper Electrophoresis.—In order to study the electrophoretic behaviour of the active principle

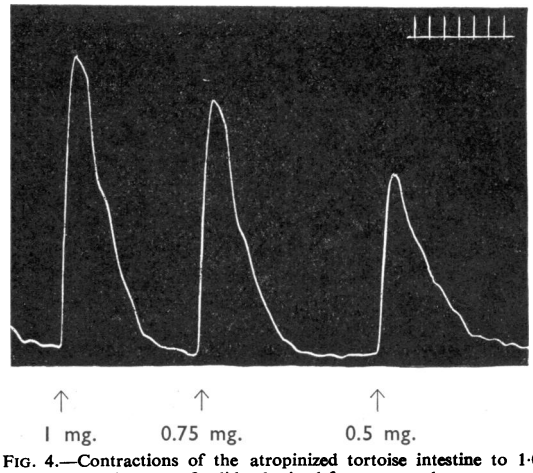

FIG. 4.—Contractions of the atropinized tortoise intestine to 1·0, 0·75 and 0·5 mg. of solids obtained from paper chromatograms of sheep nasal mucosa. The chromatograms were developed with butanol-ammonia and the zone lying between R_F 0·4 and 0·7 was eluted. Time: 1 min.

a certain degree of purification was necessary as it was found that crude extracts which contain lipid material do not show any movement on either acid or alkali buffered paper under a potential gradient of 12 V./cm. The active principle was therefore first isolated on paper chromatograms as described above, either using butanol-ammonia or butanol-acetic acid as developing solvent. In each case about 12 g. sheep nasal mucosa was used. The eluate from the chromatogram which contained the active substance was evaporated to dryness *in vacuo* over concentrated sulphuric acid. The residue was taken up in 0.2 ml. distilled water and about 60 μl. applied along a 4 cm. line drawn across a strip of Whatman No. 1 paper which had previously been soaked in buffer and equilibrated with the current turned on for 1 hr. Electrophoresis was then carried on for 4 hr. At the end of the experiment the paper strip was dried and divided into strips, 1.5 cm. long and 4 cm. wide. The position of the active principle after electrophoresis could therefore be located by testing the activity of each strip. This was done by immersing it for 2 min. in the bath in which the tortoise gut was suspended.

At *p*H 8.5 and under a potential gradient of 12 V./cm. the active substance was located on the anodic side of the origin. Using material purified on a butanol-ammonia chromatogram the active substance was found to have migrated 4.2 cm. from the origin towards the anode after 4 hr. On the other hand, with material obtained from a butanol-acetic acid chromatogram, the movement was only 1.0 cm. from the origin towards the anode. This smaller movement could be due to interference by lipid material which was not separated from the active substance on a butanol-acetic acid chromatogram. Endosmosis was in the direction of the cathode as shown by glucose, but no correction in the migration values has been made for this effect.

Using material purified on a butanol-ammonia chromatogram it was found that at *p*H 5.3 under a potential gradient of 12 V./cm. there was no movement of the active substance from the origin. At *p*H 3.5 the active substance was located 1.2 cm. from the origin towards the cathode after 4 hr. This movement, however, can be explained by endosmosis, because glucose was found to have migrated 2 cm. from the origin towards the cathode.

The tendency of the active principle to migrate towards the anode at alkaline *p*H and its lack of movement at acid *p*H suggested that it was probably an acid substance.

Discussion

The experiments described above show that there are two pharmacologically active substances present in the nasal mucosa. One of these is histamine and the other is an acid which is as yet unidentified. Neutral potassium permanganate and osmium tetroxide are used as reagents to detect unsaturated bonds, and as these compounds have been shown to destroy the activity of the acidic substance on the tortoise gut it would suggest that the acid is probably an unsaturated acid. The presence of unsaturated fatty acids in the lipids of olfactory mucosa has in fact been found from biochemical analyses by Heusghem and Gerebtzoff (1953). It has not yet been possible to identify the pharmacologically active acid of the nasal mucosa with Darmstoff (Vogt, 1957b), Irin (Ambache, 1957), the unsaturated G acid of human plasma (Gabr, 1956) or the unsaturated fatty acid of envenomed egg yolk (Vogt, 1957a), all of which stimulate smooth muscle.

The presence of another smooth muscle stimulating substance besides histamine in the nasal mucosa lends interest to the problem of allergic rhinitis. It may be that these substances are released in this condition to produce vascular engorgement and subsequent oedema.

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