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PROPERTIES OF IRIN, A PHYSIOLOGICAL CONSTITUENT OF THE RABBIT'S IRIS

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The experiments described in the present paper show that extracts of the freshly excised rabbit's iris contain a hitherto unknown smooth-muscle stimulating substance to which the name irin has been given. Irin has been distinguished from various active substances which are found in mammalian tissues, namely choline esters, histamine, 5-hydroxytryptamine, bradykinin and substance P. Several methods for purifying this substance are described and its acidic nature has been demonstrated. A preliminary account of these experiments appeared in the *Proceedings of the Physiological Society* (Ambache, 1955, 1956).

METHODS

Rabbit iris extracts

Rabbits of mixed stock, including several albinos, were used. The eyes were excised under ethyl chloride anaesthesia; some of the animals were first bled from the carotid arteries. Excision of the eyes resulted in constriction of the pupils. The eyeballs were opened equatorially and the irides were pulled out with fine forceps together with their outer frill of ciliary tissue. They were dried thoroughly by rubbing firmly between successive Whatman No. 1 filter-papers, thus removing much pigment and most of the ciliary body. The dried tissue was weighed on a torsion balance fitted with a Perspex cup. In some experiments the use of filter-paper was avoided; the irides were weighed wet and then ground.

As it was probable that the iris contained traces of blood, for the preparation of several extracts the eyes were removed after the head had been perfused from both common carotid arteries with 0·5–1 l. of warm glucose-free Locke's solution until the venous return was clear. It was usual to transect the spine and cervical cord at the root of the neck halfway through this perfusion in order to allow a better outflow from the vertebral arteries.

Extraction in water. The dried tissue was ground in a mortar for several minutes with one of the following fluids (1 ml./50 or 100 mg tissue): (*a*) distilled water, (*b*) the de Jalon's fluid used for the rat colon preparation, (*c*) Tyrode's solution as modified by Ambache & Lessin (1955), (*d*) distilled water containing 0·015–0·06% $NaHCO_3$ or the buffer system of the above Tyrode's solution. The ground tissue emulsion (sometimes centrifuged and tested at this stage, before heating, and found to be pharmacologically active) was decanted into a centrifuge tube which was placed in a boiling water-bath for 2–3 min in order to coagulate proteins; it was then cooled at once under a cold tap. The extract was centrifuged at 3500 r.p.m. for 10 min and the slightly turbid supernatant was measured and decanted into a weighing bottle for storage at −15° C until use. Clearer extracts were

obtained by centrifuging for longer periods at 1° C and 13,000 r.p.m. Dilutions of the extracts for biological assay were made in the de Jalon's solution used for the rat colon.

Extraction in acetone. The dried irides were dropped into 1 ml. of acetone A.R. per 50 mg tissue. After ¼ hr the irides were snipped with scissors, and then stood at room temperature for another ¾ hr. After centrifugation the supernatant acetone was decanted and evaporated to dryness, either in a stream of air from a fan at room temperature or in a desiccator connected to a high vacuum ballast pump (Edwards type 2SC20). Unless otherwise stated, the dry residue was taken up before use in 1 ml./100 mg tissue of 0·9 % NaCl or of the de Jalon's fluid used for the rat colon.

Dosage of irin. In the biological assays the dose of iris extract is given as the 'tissue-equivalent' in ≡mg, i.e. as the weight in mg of original tissue to which the dose of extract corresponds.

Biological assays

Rat colon. Male rats were anaesthetized with ethyl chloride and bled out. The proximal 3–4 cm of colon were suspended in a 5 ml. organ bath at 23±2° C between platinum hooks as illustrated by Ambache & Lessin (1955). The oxygenated bath fluid was that used for the colon by Gaddum, Peart & Vogt (1949). Atropine and lysergic acid diethylamide (LSD) were added to the reservoir and were present in the bath fluid in concentrations of at least 10^{-7} throughout the irin assays. More than 1 hr was allowed for full relaxation before beginning assays.

Rat uterus. The technique followed was that of Amin, Crawford & Gaddum (1954). The oxygenated bath fluid was at 30° C and contained 10^{-6} atropine sulphate.

Rabbit and guinea-pig ileum preparations were set up in the above modified Tyrode's solution bubbled with 95 % O_2 and 5 % CO_2, at 35° C. In some of the experiments on the guinea-pig ileum the terminal end was used (omitting the Peyer's patch in the last 1–2 cm before the caecum) and the temperature was lowered to 30° C.

Ox sphincter pupillae muscle. Fresh ox eyes were opened by an equatorial incision. The iris was pulled out together with the ciliary body, which was dissected away with curved scissors from the outer margin of the iris. The unbroken ellipse of iris tissue was suspended lengthwise, either between two ligatures placed at each narrow end, or between one ligature and a hook slipped through the ellipse, and not the tissue itself, at its lower end. The preparation was maintained at 30–31·5° C in the same de Jalon's solution as was used for the rat colon. At 37° C and in the modified Tyrode's solution (95 % O_2 + 5 % CO_2) this muscle was found to be prone to spasm as already stated by Leyko (1935) and by Rüegg & Hess (1953). Records were taken at a lever magnification of ×8.

Cat iris. Pupillary measurements were made with callipers across the horizontal diameter in cats anaesthetized with 39 mg/kg pentobarbital sodium, intraperitoneally. Atropine SO_4 (>1 mg/kg) was administered intravenously. Irin was injected into the anterior chamber through 4–5 mm self-sealing oblique valvular tracks in the cornea by the method described previously (Ambache, 1951).

Incubation with chymotrypsin

Crystalline chymotrypsin, as supplied by Armour Laboratories and containing not more than 50 % $MgSO_4$, was dissolved in the de Jalon's fluid used for the rat uterus, to give a solution of 4 mg/ml. To 0·4 ml. of this solution in a quartz-stoppered tube I_D was added 0·4 ml. of a watery extract E, equivalent to 20 mg of iris tissue. This mixture was placed in a rotating test-tube holder in a water-bath at 37° C, where it was left for 1 hr together with a control tube I_C containing 0·4 ml. E + 0·4 ml. de Jalon's solution. At the end of the hour I_D and I_C were diluted to 2 ml. with the de Jalon's fluid used for the rat colon so as to contain ≡10 mg of iris/ml. For the assay an amount of chymotrypsin equivalent to that present in I_D was added to the organ-bath when I_C was tested.

For control experiments substance P and bradykinin were incubated similarly with the same chymotrypsin solution. The sample of substance P, kindly supplied by Dr B. Pernow, Stockholm, and stored at −15° C for 1 year, had an original activity of 50 Euler units/mg.

Purification of watery extracts

The watery extracts described above were further purified in two stages:

Stage 1: *acetone treatment.* 20 vol. of acetone were added. A cloudy precipitate containing less than 5% of the pharmacological activity of the extract was removed by centrifugation. The supernatant acetone was evaporated to dryness as above. The residue could then be either used for biological assay, by re-dissolving in de Jalon's fluid, or further purified as follows.

Stage 2: *Chloroform re-extraction.* To the residue obtained at the end of Stage 1 a few ml. of washed chloroform were added. This was swirled round a few times and then decanted away from chloroform-insoluble impurities visible on the sides of the beaker. The chloroform was evaporated to dryness with a fan. Before use this second residue was redissolved in de Jalon's fluid.

Commercial chloroform contains 1% ethyl alcohol, added to prevent noxious oxidation products. This has been removed by shaking the chloroform 7–8 times with an equal volume of distilled water on the day of the experiment, as it has been found that washed chloroform which has been allowed to stand for several days becomes very acid, presumably through the formation of phosgene and HCl; these by-products appear to destroy irin.

Paper electrophoresis

Whatman No. 1 paper was cut in lengths of 42–46 cm by 3–4 or 7·5 cm wide. A small circular spot (*ca.* 1 cm) of material was placed on a transverse 'origin' at the middle of each paper, by applying 0·01–0·2 ml. of solution in tiny drops. The papers were then placed in the electrophoresis tank symmetrically astride a glass rod, with their origin over the rod and with their ends dipping in the solvent in two troughs, each containing a carbon electrode; the solvent level was about 18 cm below the glass rod. The papers were carefully wetted with solvent almost to the origin. The tank was sealed and the solvent level in the two troughs was equalized by a siphon. After ½–1 hr equilibration the current, from an Evans Electroselenium stabilized power pack, was switched on for 3–4 hr.

The solvent was 1l. NaCl 0·1% containing 1·56 g $NaH_2PO_4.2H_2O$ + a few ml. of N-NaOH to the desired pH, which was varied between 5·2 and 9·1 in different runs. The NaCl content was trebled in Expt. 1 of Table 1. The current varied between 2·5 and 6 mA at voltages of 150–270 V.

Controls. The following controls were carried out each time:

(*a*) 80–100 μg of glucose, as a 'neutral' marker. The endosmotic effect is such that glucose always shifts slightly towards the negative pole (0·6–2·25 cm). It is therefore necessary to apply this correction factor to all other migration values. The final position of the glucose was revealed as a brown spot by the method of Partridge (1949). The centre of this spot was taken as the corrected zero from which all migration measurements were made.

(*b*) 80 μg each of two amino acids, one acidic and the other basic. In Expt. 3 of Table 1 cysteic acid and arginine were used, but in all the others aspartic acid (or its NH_4 salt) and lysine HCl. The final position of the amino acids was revealed by spraying with 0·1% ninhydrin.

Extract details. The extracts used for electrophoresis were either acetone extracts or watery extracts purified by acetone or by acetone and chloroform. In Expt. 5 of Table 1 the distilled water extract, before being purified by acetone, was treated with ether as follows. The watery supernatant was shaken with 1 vol. of ether; the ether was then separated off and discarded. This stage introduces a loss of about 20% from distilled water extracts.

When the acetone was fanned down to one-tenth its volume (*ca.* 10 ml.), it was dispensed in equal amounts into 10 ampoules, which were pumped to dryness during the night. The sealed ampoules were stored at −15° C. The contents of one of these ampoules was taken up in 0·1 ml. of distilled water per ≡20 mg iris, and two 'spots' of 0·09 and 0·11 ml. respectively were placed on the paper about 2 cm. apart on the origin.

Location of irin. The position of the active substance on the dry papers was ascertained by assay on the rat colon in atropine with and without lysergic acid diethylamide (LSD). The paper was cut, at right angles to its long axis, into transverse slices 0·5 or 1 cm wide and 3–4 cm long. For

assay each successive slice was picked up with forceps and dropped into the organ bath where it was left for 2 min.

Electrophoretically purified irin was prepared in bulk by repeating the procedure outlined in section 2 for Expt. 5 of Table 1, but larger amounts of extract were applied to 15 cm wide papers as a continuous line at the origin. Markers were omitted; solvent pH was fixed at 8·8, voltage at 270 V, and duration at 4 hr. After electrophoresis a preliminary location of the irin was carried out by assay of a narrow (1·5 cm) marginal strip from this paper. Irin activity was confined to a 2 cm band at cm +3 to +5. The corresponding 2 cm band was then cut out from the remaining nine-tenths of the paper and the irin was eluted from it with the de Jalon's solution used for the rat colon and stored at −15° C.

RESULTS

I. *Distinction of irin from various pharmacologically active bases of natural occurrence*

The iris in enucleated rabbits' eyes is in a state of prolonged constriction. Watery extracts of such irides, whether pigmented or albino, contain a pharmacologically active substance which differs from most known tissue constituents. This substance, termed irin, is capable of contracting a number of isolated smooth-muscle preparations such as the diagonally striated portion of the rat's colon, the isolated ox sphincter pupillae, and guinea-pig and rabbit ilea. The contractions of the rat colon which are produced by irin are shown in Fig. 1; those of the ox sphincter pupillae in Figs. 3 and 8; and those of the guinea-pig ileum in Fig. 10. The rat colon and the ox sphincter pupillae were the most sensitive of these preparations. The routine preparation used in the present work has been the rat colon, which, in a 5 ml. bath, detects the irin-activity of as little as 0·25–1 mg of iris tissue.

Distinction from choline esters

The contractions produced by iris extracts on the various smooth-muscle preparations are resistant to atropine 2×10^{-8}–10^{-6}. Such concentrations of atropine render the rat colon insensitive to at least 0·2–1 μg of ACh; this is 40–200 times greater than the amount of ACh (about 5 ng) which is present in 1 mg of fresh rabbit iris (Ambache, Morgan & Payling Wright, 1948). Moreover, with the present method of watery extraction in the absence of eserine, most of the ACh in the iris is destroyed by the cholinesterase of that tissue when it is ground. In fact ACh could not be detected in two such extracts ($< 0{\cdot}05$ and $< 1{\cdot}66$ μg/g respectively) when assayed on the eserinized frog rectus abdominis muscle.

The atropine resistance of irin responses is shown in Fig. 1 (rat colon) and in Fig. 3 (ox sphincter pupillae). In the experiment of Fig. 1 the extract was 'blood-free' and had been made by grinding, in distilled water, the freshly excised irides of two albino rabbits whose heads had been previously perfused with 1 l. of warm glucose-free Locke's solution. The pH of the extract was between 6·1 and 6·7.

On the rat colon atropine not only failed to antagonize but actually potentiated the action of irin. As shown in Fig. 1, this potentiating effect consisted in a shortening of the latency (from 16 sec at *C* to 10 sec at *E*) and an increase in the height and duration of the contraction.

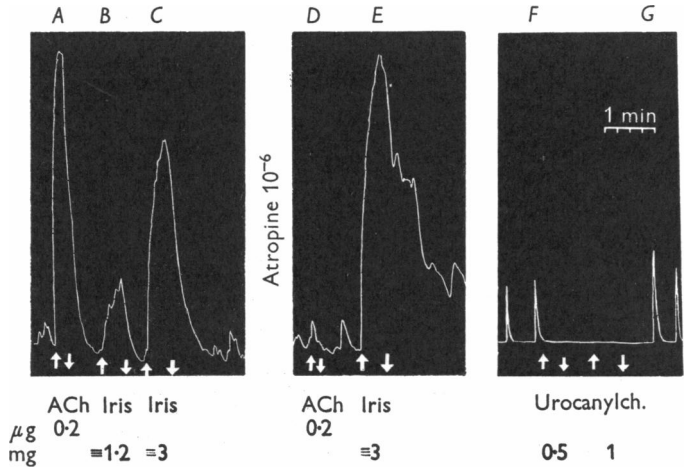

Fig. 1. Rat colon preparations suspended in 5 ml. baths. *A* to *E*, comparison of the effects of iris extract and of ACh before and after atropine given 20 min before *D*. *F* and *G*, inhibitory effects of urocanylcholine on the spontaneous activity of another preparation in the presence of atropine 10^{-6} and of LSD 10^{-7}. In all figures dots or ↑ indicate administration of drugs; vertical lines or ↓, bath washes; time marker 1 min except where specified.

Irin also differed from ACh by the fact that, in doses of ≡5–10 mg, it gave rise to prolonged contractions of the ox sphincter, which was insensitive to 10 μg of ACh. The irin-contractions of this preparation were also resistant (Fig. 3) to doses of atropine which were adequate to block the effect of the muscarine-like drugs 2268F (α-β-ethylal-γ-trimethyl-ammonium-propanediol) and 5-methyl-furmethide. The meiotic effect of intraocular injections of partially purified irin in atropinized rabbits has been reported previously (Ambache, 1956); the effect of purer material on cats' eyes is described below.

Urocanylcholine (*murexine*). When the optical density of watery iris extracts was measured, against a blank reference cell containing the solvent of the extracts, in a Unicam S.P. 500 spectrophotometer (0·6 ml. silica cells) over the range 200–350 mμ, it was found that their absorption spectra had a component peak at 250–260 mμ, which is close to the absorption maximum at 264–265 mμ of urocanylcholine (Erspamer & Benati, 1953). Although devoid of 'muscarinic' activity (Erspamer, 1948, 1953) urocanylcholine is reported to have a feeble motor action on some intestinal preparations; in rabbit gut and in some other preparations this effect is atropine-resistant (Erspamer, 1948). Its action on the rat colon has not been examined previously. In concentra-

tions of from 2×10^{-5} to 4×10^{-3}, urocanylcholine was found to inhibit this preparation (Fig. 1 *F*, *G*), whether atropine was present or not. This inhibitory action may be due to the imidazole portion of the murexine molecule.

Distinction from 5-hydroxytryptamine

The contractions produced by iris extracts are not due to 5-hydroxytryptamine (5-HT). Many of the extracts were made from irides freed of blood. But even the amounts of 5-HT which are known to be present in blood would be insufficient to account for the pharmacological action observed with extracts made from unperfused irides. The blood content of the rabbit's iris has been given by Palm (1951) as about 5%. It can be calculated from this value and from figures given by Humphrey & Jacques (1954) that in each mg of iris the blood content would contribute 0·25 ng of 5-HT, and about 0·1 ng of histamine.

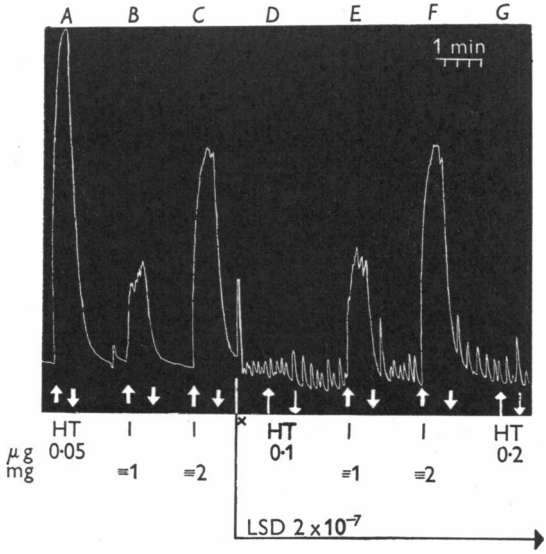

Fig. 2. Rat colon preparation suspended in 5 ml. bath containing atropine 10^{-7} throughout. Comparison of the effects of iris extract (I) and 5-hydroxytryptamine (HT) before (*A* to *C*) and after (*D* to *G*) adding LSD; the LSD was added at ×, 20 min before *D*, whilst the drum was stopped.

Irin differed from 5-HT in the following properties:

(1) It could be extracted from aqueous solutions by chloroform, whereas 5-HT remains in the aqueous phase (Dalgliesh, Toh & Work, 1953).

(2) The effect of irin on the rat colon was not abolished or reduced by the 5-HT-antagonist lysergic acid diethylamide as illustrated in Fig. 2. The extract used in this experiment was made from unperfused pigmented irides.

(3) The isolated ox sphincter pupillae was sensitive to irin but very insensitive to 5-HT (see Fig. 3 *B*, *D*).

(4) The atropinized rat uterus, although sensitive to 5-HT, did not respond to iris extracts in doses of up to ≡ 10 mg. However, such large doses of iris extracts potentiated the action of 5-HT when the two were administered together. This is illustrated in Fig. 4 (lower panel).

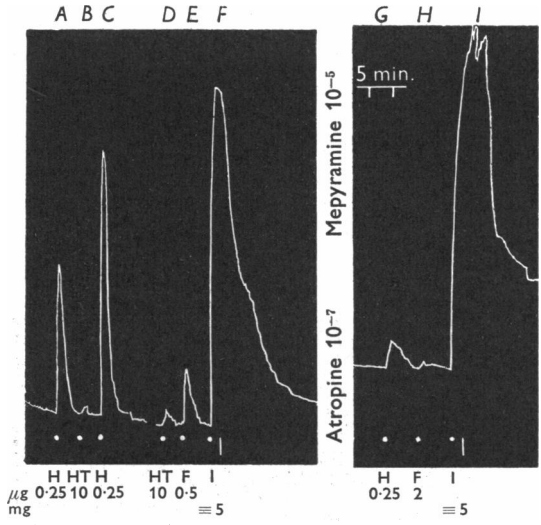

Fig. 3. Ox sphincter pupillae muscle suspended in 5 ml. bath. Comparison of the effects of histamine (H), 5-hydroxytryptamine (HT), 2268 F (F) and irin (I) before and after atropine plus mepyramine given 1 hr before *G* and kept in the bath to the end of the experiment. The mepyramine was first added in a concentration of 10^{-6} and increased to 10^{-5} 40 min later. Periods of contact 1 min except at *F* and *I* (2 min.).

Distinction from histamine

The contractions produced by iris extracts on the rat colon can naturally not be due to histamine since this preparation is insensitive to histamine and may even be slightly inhibited by it. The contractions produced in the histamine-sensitive ox sphincter pupillae and in atropinized ileum preparations of guinea-pigs and rabbits are also not due to histamine since they are resistant to mepyramine. This is shown on an ox sphincter in Fig. 3; and in Fig. 10 on a guinea-pig ileum, first with a crude iris extract and then with electrophoretically purified irin.

Distinction from bradykinin

The contractions produced by iris extracts cannot be due to the presence of the polypeptide bradykinin, as is shown by the following comparison:

Whereas the rat colon is sensitive to irin, and contracts, it was rather insensitive to bradykinin and was inhibited by it in large doses (50–400 μg). This difference is illustrated in Fig. 4 (top) on a preparation rendered insensitive to 5-HT by LSD.

The atropinized rat uterus, which is very sensitive to bradykinin, was relatively insensitive to irin as shown in Fig. 4 (bottom). Although iris extracts in doses of up to ≡10 mg did not by themselves contract the rat uterus, such large doses increased the response to bradykinin, if the two were given together (see Fig. 4, nos. 6–8).

Whereas bradykinin is insoluble in acetone and in methylethylketone (Prado, Beraldo & Rocha e Silva, 1950), irin was found to be soluble in 95 and 100% acetone, and to have a high R_F value in methylethylketone.

Unlike bradykinin irin was not destroyed by chymotrypsin.

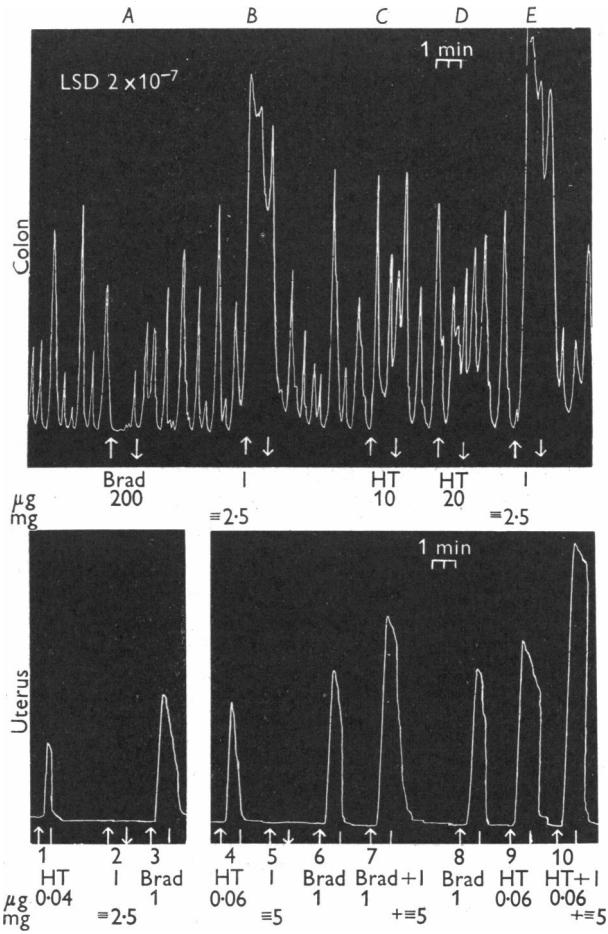

Fig. 4. Parallel assay on rat colon (upper panel) and rat uterus (lower panel) of bradykinin (Brad), iris extract (I) and 5-hydroxytryptamine (HT). Both preparations suspended in 5 ml. baths; bath fluid for rat colon contained atropine 10^{-7} and LSD 2×10^{-7}, and for rat uterus atropine 10^{-6} throughout. For details see text.

Distinction from substance P

Irin is not identical with the active polypeptide substance P, as is shown by the following differences:

As mentioned before, irin is soluble in acetone, whereas Amin *et al.* (1954, pp. 602 and 611) have shown that tissues extracted with 95% acetone retain 98–99·67% of their substance P content. On the other hand, when 20–24 vol. of acetone were added to watery extracts of rabbits' irides, irin was not precipitated. Moreover, irin was directly extractable from the tissues of the iris into 20 vol. of acetone. In one experiment irin from a dried residue was taken up in 100% acetone, freshly distilled after storing over $CaCl_2$; the amount which dissolved in this acetone was only slightly less than that which had dissolved into water from a parallel sample of the same residue. The solubility in acetone also distinguishes irin from the common phosphatides, since lecithin, cephalin and sphingomyelin are precipitated by acetone.

Whereas substance P is insoluble in ether and only slightly soluble in chloroform if alkaline (von Euler & Gaddum, 1931; Pernow, 1955), irin was extracted from water by acid chloroform and was found to be appreciably soluble in ether.

Substance P is known to behave as a base and, on paper electrophoresis at alkaline pH, to migrate towards the cathode (Vogt, 1953; Pernow, 1955). The converse was true of irin, as will be described in detail later on.

As shown by Eliasson, Lie & Pernow (1956) chromatograms of substance P in water-saturated *n*-butanol-acetic acid have to be run in an atmosphere of nitrogen and the R_F value is 0·38. Irin, on the other hand, could be chromatographed in air and its R_F in the above solvent was 0·91–0·98.

It has already been mentioned that irin is resistant to chymotrypsin-digestion, a treatment which rapidly destroys the pharmacological activity of substance P (Pernow, 1955). This difference is illustrated in Fig. 5.

In the experiment of Fig. 5 the activity of an iris extract incubated with chymotrypsin for 1 hr at 37° C was compared at two dose levels ($\equiv$0·5 and 1 mg) with that of controls of the same extract incubated without chymotrypsin. The extract used for incubation contained $\equiv$25 mg of iris per ml. and to half of it 2 mg/ml. of chymotrypsin was added. The chymotrypsin did not diminish the activity of the extract, as can be seen from a comparison of *A* with *B* and *C* with *D*. In contrast, when a solution of substance *P* was incubated with a much lower concentration of the same chymotrypsin (0·36 mg/ml.) its activity was greatly reduced. This is shown by comparing the effect of 100, 50 and 30 μg of 'control' substance P, incubated without the enzyme (*E*, *G* and *I*), with that of 100 and 191 μg of digested substance P at *F* and *H*. The effect of 191 μg of substance P incubated with chymotrypsin was smaller than that of 30 μg of undigested substance P. Thus more than

80% of the substance P was inactivated after 1 hour's treatment with chymotrypsin; a sample taken after the first half hour of incubation showed that already over 50% of the substance P had been inactivated. It might be mentioned in this connexion that when a solution containing 500 μg/ml. of bradykinin was incubated for 1 hr with 2 mg/ml. of chymotrypsin between 96 and 98% of the bradykinin was inactivated.

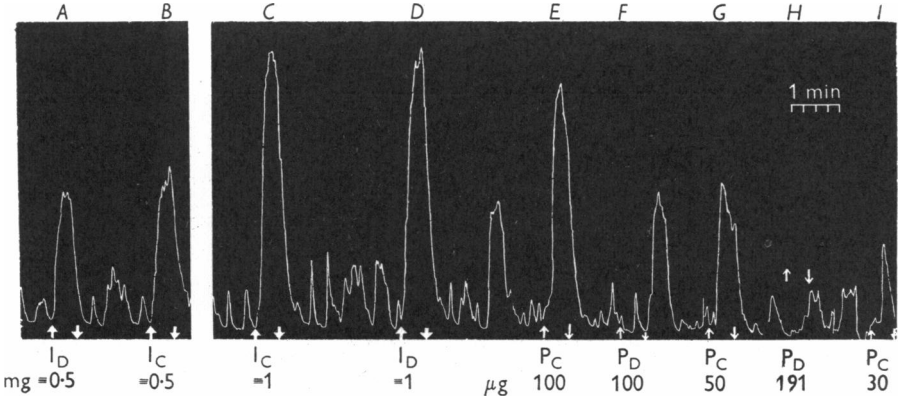

Fig. 5. Rat colon in 5 ml. bath containing atropine 10^{-7} and LSD 10^{-7} throughout. Effect of incubation with chymotrypsin on the activity of irin (*A* to *D*) and of substance P (*E* to *I*): I_D, irin incubated with chymotrypsin; I_C, irin incubated without chymotrypsin, but assayed in its presence (control); P_D, substance P incubated with chymotrypsin; P_C, substance P incubated without chymotrypsin, but assayed in its presence. For details see text.

II. *Purification of irin by acetone treatment and chloroform re-extraction*

The purification of irin by chloroform re-extraction is illustrated by the result in Fig. 6, which is representative of four experiments of this type. A distilled water extract of six pigmented 'blood-free' irides (250 mg) was treated with 20 vol. of acetone and centrifuged. The acetone-insoluble precipitate was found to contain less than 5% of the original pharmacological activity and was discarded. The clear acetone supernatant was evaporated to dryness in a beaker; the dry residue was then re-extracted with 10 ml. of washed chloroform. The chloroform was decanted and then shaken with one-tenth its vol. of distilled water. After separating from the water layer, the chloroform layer was dispensed in equal fifths into five weighing bottles and each was evaporated to dryness; each weighing bottle therefore contained the irin-equivalent of 50 mg of iris tissue. The content of one of these was taken up at once in 0·9% NaCl solution and assayed on the cat's iris and on a rat colon with and without LSD.

On the cat's iris the intraocular injection of $\equiv$0·5 mg of the purified material in 0·1 ml. of saline solution decreased the horizontal diameter of the pupil by 3 mm. The injection into the contralateral eye of $\equiv$2·5 mg in 0·05 ml. of

saline solution narrowed the pupil from 9·5 to less than 1 mm in 9 min; 7 min later it was still 1·5 mm. The assay on the rat colon is shown in Fig. 6. The activity of ≡0·5, 1 and 0·75 mg of this purified material is shown at *B*, *C* and *D* before, and at *G*, *I* and *J* after, treating the colon with LSD.

Another of these weighing bottles was used to determine the quantity of chloroform-soluble material present. For this purpose the bottle was weighed before and after removing the chloroform-soluble material in it with two lots of 1 ml. of washed chloroform. The difference between the two weighings was

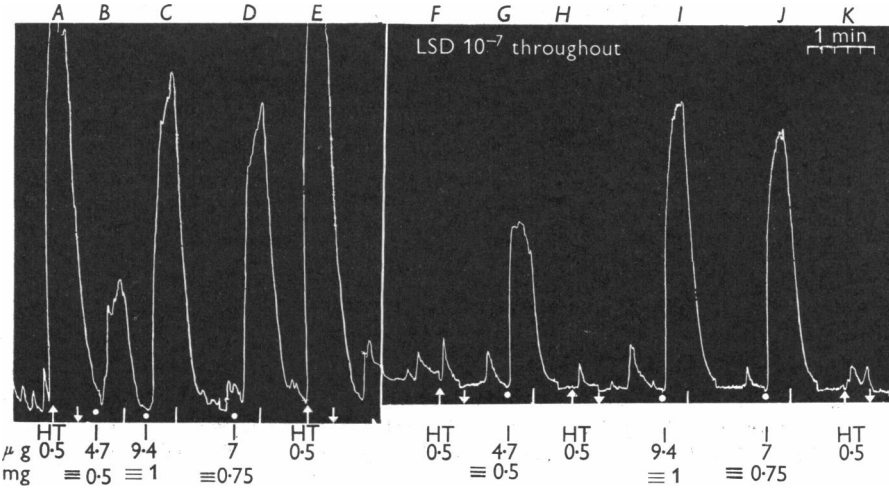

Fig. 6. Rat colon suspended in 5 ml. bath containing atropine 10^{-7} throughout. Effect of purified irin (I) and 5-hydroxytryptamine (HT) before (*A–E*) and after LSD 10^{-7} (*F–K*). The amounts of purified irin are given above in μg and, below, as the equivalents of fresh tissue in mg.

0·47 (±0·01) mg. Thus the 50 mg of iris tissue had yielded 0·47 mg of chloroform-soluble material. It could therefore be calculated that the responses obtained in Fig. 6 with ≡0·5–1 mg of tissue had in fact been elicited by 4·7–9·4 μg of the purified material; in a 5 ml. organ bath this represents a concentration of $1–2 \times 10^{-6}$ of the active substance at this stage of purification. The yield of 0·47 mg of active chloroform-soluble material from 50 mg of iris represents a purification of about 100 times. When this purification procedure was repeated in another experiment a similar result was obtained, 35·3 mg of iris yielding 0·38 mg of chloroform-soluble material. The amount of irin extractable from a rabbit's iris may therefore be taken as less than 1% of the weight of tissue.

The finding that after this purification procedure strong activity was present in the chloroform-re-extracted material, when administered in doses equivalent to 0·5 mg of tissue, suggested a high recovery of irin-activity by this method. The actual recovery was determined in separate experiments. In order to

obtain a good yield it was necessary to wash (or re-wash) the chloroform on the day of the experiment, as mentioned in Methods; otherwise recovery was low. When, however, this precaution was taken recovery of the activity was over 80%.

When dry carbon tetrachloride was used instead of chloroform for re-extraction the recovery was less; in one experiment it was 40%, in another over 65%. In one experiment in which an ampoule containing an active residue was re-extracted with crystallizable benzene, instead of with chloroform, the benzene dissolved more than 90% of the activity in the ampoule. On the other hand, when irin was partitioned between equal volumes of 0·9% NaCl solution and benzene, >90% of the activity remained in the watery phase.

III. *Evidence that irin carries a net negative charge*

A. Partition between chloroform and water; behaviour as a weak acid

The partition of irin between chloroform and water depends upon the pH of the water phase. The presence of $NaHCO_3$ in the water phase extracts irin from the chloroform; conversely, acid drives the irin from the water into the chloroform phase. Thus irin behaves like a weak acid since alkali would promote the dissociation of such an acid, and so increase its solubility in water; on the other hand, at low pH the acid would be present in its undissociated form, which would be more soluble in chloroform.

The fact that irin behaves as a weak acid explained an inadvertent finding. When 0·9% NaCl was used for partitioning with chloroform the result obtained was opposite to that of a partition in which de Jalon's fluid was used as the watery phase. De Jalon's solution contains enough bicarbonate (0·015%) to retain most of the irin in the watery phase, whereas 0·9% NaCl is sufficiently acid to allow escape of irin into the chloroform phase.

Fig. 7 illustrates the results obtained when irin was partitioned between chloroform and an acid, or an alkaline, watery phase. The acid watery phase consisted of bicarbonate-free de Jalon's solution acidified with HCl to pH 3·5; the alkaline watery phase, of de Jalon's solution, in which the bicarbonate was raised to 0·11%, to give a pH of 8·2. Incidentally, when these two modified de Jalon's solutions were mixed in equal proportions they neutralized each other to pH 7. The irin preparation used was a dried acetone extract of irides; it was dissolved in freshly washed chloroform so that 1 ml. contained the equivalent of 30 mg of tissue. To 1 ml. of this solution 1 ml. of acidified, and to another 1 ml., 1 ml. of alkaline, de Jalon's fluid were added in glass-stoppered tubes. After shaking the two tubes the contents of each were transferred to a 2 ml. syringe which was inverted for separation of the layers. When they had separated, the watery layer in each syringe was sucked off into a 1 ml. syringe with a fine needle and its volume was noted. The same was then done for the chloroform layers. The watery layers were immediately neutralized with an

equal volume of the appropriate modified acid or alkaline de Jalon's solution and the traces of chloroform present in them were removed by bubbling air through them for ½ hr. The chloroform layers were evaporated to dryness in a stream of air, and were re-dissolved in twice their volume of unmodified de Jalon's fluid. In this way all four final solutions contained the same equivalent of tissue per ml. (≡ 15 mg).

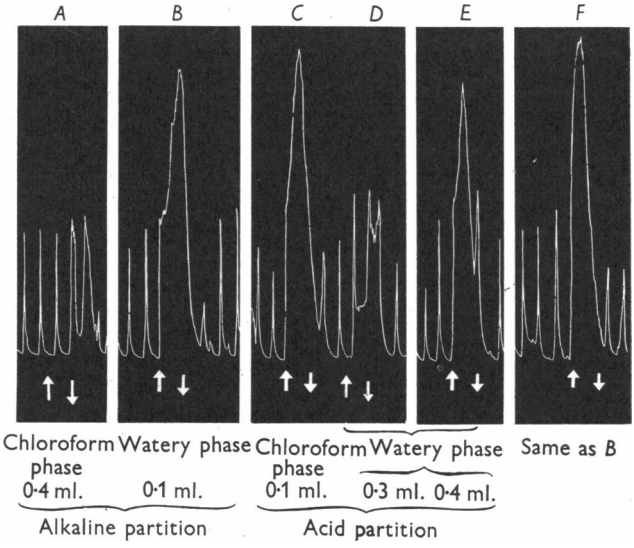

Fig. 7. Rat colon suspended in 5 ml. bath containing atropine 10^{-6} and LSD 10^{-7}. Partition of irin between water and chloroform under acid and alkaline conditions. Alkaline partition: *A*, *B*, and *F*; acid partition: *C*, *D* and *E*. For details see text.

Fig. 7 shows that in the alkaline partition more than 80% of the pharmacological activity was present in the watery phase, since the response to 0·1 ml. of this phase (at *B* and *F*) was considerably greater than to 0·4 ml. of the chloroform phase (at *A*); the latter was virtually devoid of activity. In the acid partition the response to 0·1 ml. of the chloroform phase (at *C*) was greater than that to 0·3 ml. of watery phase (at *D*) and still slightly greater than the response to 0·4 ml. of watery phase (at *E*). Moreover, the response to 0·1 ml. of this 'acid' chloroform phase was almost identical with that to 0·1 ml. of the 'alkaline' watery phase. In the acid partition, therefore, slightly more than 80% of the activity was found in the chloroform layer. This suggests that the pK of irin lies somewhere between pH 3·5 and 8·2, probably below the mean of these two values.

B. Paper electrophoresis

The pharmacologically active substance in iris extracts has a tendency, which is increased at alkaline pH, to migrate towards the anode on electro-

phoresis. When crude watery extracts were used they gave rise to diffuseness of the final active area on the positive side of the paper, but with purified extracts the pharmacologically active region on the paper was sharply delimited. There was a definite peak of activity confined to a zone of 0·5–1 cm, with a narrow band of reduced activity on either side of the peak. The results obtained with extracts purified in different ways are given in detail in Table 1; the migration values are corrected for the endosmotic shift shown by the glucose marker (see Methods). In none of the experiments was any pharmacological activity detected on the negative side of this marker, the position of which has been taken as the corrected zero. At acid (pH 5·2) as well as at neutral or slightly alkaline (7·6) pH (Expts. 1 and 2 of Table 1) the positive migration of irin was so small as to suggest a lack of movement from its origin. At more alkaline pH and with longer durations of current (Expts. 3–5) there was a definite anodal migration, and the peak of pharmacological activity was well away on the positive side not only of the glucose spot but also of the 'origin'. The extent of the anodal migration was less than that of the aspartic acid marker.

Several of these points are illustrated in Fig. 8, which shows the pharmacological assay of the electrophoresis paper from Expt. 5 of Table 1. In the experiment two 'spots' consisting of ≡18 and ≡22 mg of iris, respectively, were run in parallel, and at the end of the electrophoresis the dried paper was split longitudinally into two strips, each containing one of the spots. The strip containing the ≡18 mg of iris extract was assayed cm by cm lengthwise on the rat colon. The exact location along the axis of each transverse cm slice of paper is given in the figure after correction for the position of glucose, which in this experiment had shifted 1·8 cm towards the cathode. Pharmacological activity was detected only in slices 11, 12 and 13. The responses to these were clearly distinguishable from the normal spontaneous contractions of the preparation. All other slices were inactive. There was a clear peak of activity in slice 12, i.e. at +4·8 to 5·8.

The strip containing the ≡22 mg spot was stored till next day at −15° C and then split down the middle longitudinally into two strips, each thus carrying ≡11 mg of iris. One strip was used for a test on a preparation of ox sphincter pupillae (see Fig. 8, second panel) and the other on a cat's iris. For the test on the ox iris a transverse slice from the negative side and one from the positive side were added to the bath. The slice from the positive side corresponded in location to the two slices 11 and 12 in the assay on the rat colon. Whereas the slice from the negative side was inactive, that from the positive side elicited a prolonged spasm which was so powerful as to send the lever off the scale. The spasm persisted for over 15 min and the preparation had not relaxed fully after 30 min, although the bath fluid was changed after 2½ min and frequently thereafter.

For the test on the cat's iris one third was split off longitudinally from the margin of the second strip, thus carrying ≡3·6 mg iris. Again a transverse slice was cut out from the positive side (position: +3·8 to 5·8) and another from the negative side (position: −0·2 to −2·2). Each of the slices was then eluted with 0·1 ml. of de Jalon's fluid and 0·08 ml. of each eluate was injected

TABLE 1. Anodal migration of irin on paper electrophoresis

Expt. no.	Details of extracts *Q.*: ≡mg iris	Electrophoresis conditions: (*a*) pH of buffered solvent, (*b*) voltage, (*c*) duration	Corrected position of amino acids (cm) Aspartic	Lysine	Corrected location of irin (cm)
1	Blood-free, watery, purified by acetone and chloroform, *Q.*: ≡10 mg in 0·2 ml. saline	pH 7·6 270 V 2 hr	+5·4	−0·6	+0·3 to +1·3
2	Blood-free, watery, purified by acetone. *Q.*: ≡10 mg in 0·1 ml. saline	pH 6·7–7·0 270 V 3 hr	+8·8	−2·5	+0·5 to +1·0 (some up to +1·5)
3	Acetone extract (albino). *Q.*: ≡4·85 mg in 0·01 ml. dist. water	pH 9·1–9·4 170 V 3 hr	(cysteic) +3·1	(arginine) −4·6	+1·1 to +2·6, sharp peak at +1·1 to +2·1
4	18 day old acetone extract, *Q.*: ≡30 mg in 0·06 ml. dist. water	pH 8·4 270 V 4 hr	+9·0	−6·3	+3·9 to +5·9, peak +3·9 to +4·9
5 (see fig. 8)	Watery, purified by ether and acetone, *Q.*: ≡18 mg in 0·09 ml. dist. water	pH 8·8 270 V 4 hr	+7·1	−7·5	+3·8 to +5·8 (a little up to +6·8), sharp peak +4·8 to +5·8

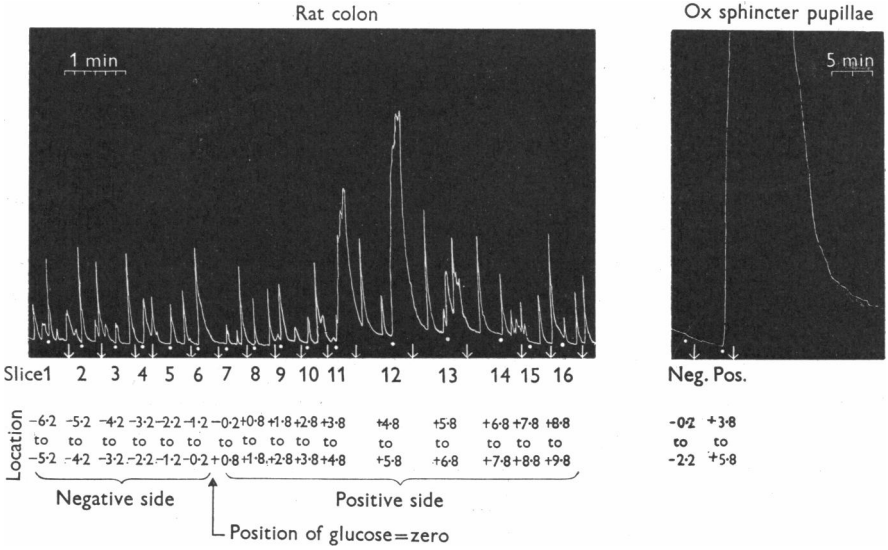

Fig. 8. Anodal migration of irin on paper electrophoresis (Expt. 5 of Table 1). Larger panel: location of the irin by assay of the paper on a rat colon in atropine 10^{-7} and LSD 2×10^{-7}; activity confined to slices 11–13. Smaller panel: isolated ox sphincter pupillae, showing effect of the electrophoretically purified irin from a corresponding positive slice of a parallel paper-strip at Pos., and absence of activity from a control negative slice at Neg.; time marker 5 min. The position of each slice in cm along the longitudinal axis of the paper is given below the slice numbers. For details see text.

into the anterior chambers of an atropinized cat through oblique self-sealing corneal tracks of about 5 mm length. The result is illustrated in Fig. 9, which also shows the corneal tracks. The eluate from the positive slice, which was injected into the left eye, produced a slit-like narrowing of the pupil, whereas that from the negative side, injected into the right eye, had no effect. The photograph in Fig. 9 was taken 10 min after the 'positive' injection; the pupil remained too narrow for measurement for over 30 min and was only 1·5 mm after 60 min and 3 mm after 100 min.

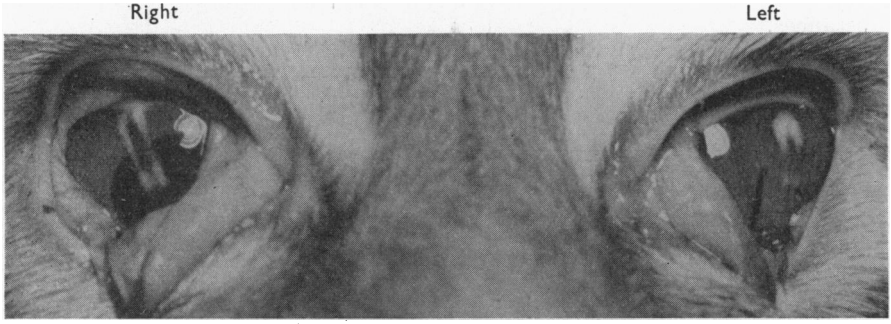

Fig. 9. Cat, 3 mg/kg atropine I.V. Pupils after injection of ≡2·9 mg of irin, purified by electrophoresis, into the left anterior chamber, and of an equal volume of eluate from an inactive region of the electrophoresis paper into the right anterior chamber. The injections were made into the left eye 10 min, and into the right eye 13 min, before the photograph was taken. For details see text.

In further experiments irin was purified electrophoretically for tests on the guinea-pig's ileum preparation. Watery extracts were first partially purified by ether and acetone treatment and then purified by electrophoresis in bulk on wide paper. The location of the active region was determined by a pilot assay, on the rat colon, of a marginal sliver of the paper. The bulk of the irin activity was then eluted with de Jalon's fluid from the active region, which was between +3 and +5 (uncorrected). The eluate produced strong contractions of the guinea-pig's ileum, which were not abolished by atropine. In fact in one experiment atropine (2×10^{-8} and 2×10^{-7}) greatly potentiated the response to the eluate, but in three others it was slightly depressed, as were also responses to histamine; the concentration of atropine used in these experiments varied between 2×10^{-8} and 2×10^{-6}. The irin contractions persisted after mepyramine was administered in concentrations of 2×10^{-8}, which were adequate to abolish equally strong or stronger histamine contractions. Fig. 10 shows the effect of 0·25 ml. (matched, before mepyramine, by 0·02 μg histamine) and of 0·5 ml. of eluate on the atropinized mepyramine-treated preparation. The effect of these doses of eluate corresponded to that of ≡2·5 and ≡5 mg of iris, as shown by a comparison with the effects of a crude extract (partially purified

by ether and acetone treatment), so that 1 ml. of eluate corresponded to ≡ 10 mg of iris. There was no sign of tachyphylaxis or desensitization to irin over several hours.

The finding that the guinea-pig's ileum responds to irin in the presence of atropine and mepyramine has to be taken into consideration when assaying tissue extracts either for histamine, on the atropinized preparation, or for acetylcholine (in the presence of mepyramine alone) or for substance P and bradykinin, in the presence of atropine and mepyramine. Should irin be present in such tissue extracts false values may be obtained in assays of these substances.

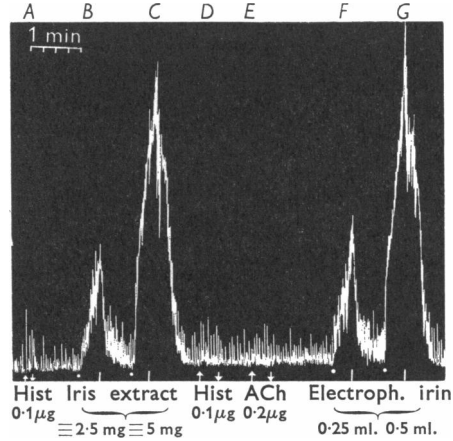

Fig. 10. Guinea-pig terminal ileum suspended in 5 ml. bath at 30° C in atropine 10^{-7} and mepyramine 2×10^{-8}. Responses to an iris extract purified by ether and acetone (*B* and *C*), and to electrophoretically purified irin (*F* and *G*). At *A* and *D*, histamine (Hist) and at *E*, ACh.

DISCUSSION

The present investigation arose out of a study of the prolonged, atropine-resistant, spasm of the sphincter iridis which occurs in rabbits on mechanical stimulation of the trigeminal nerve (Bernard, 1858; Maurice, 1953) or of the iris proper (Duke-Elder & Duke-Elder, 1931). The possibility was envisaged that this spasm was the effect of a smooth-muscle stimulating substance released from the trigeminal nerve when stimulated antidromically or, as in the experiments of Duke-Elder & Duke-Elder, when the iris itself was stroked, the effect then being the result either of an axon reflex or of local injury. The first step towards testing this possibility was to find out whether iris extracts contained such a substance, the release of which could account for the observed spasm. The present experiments show that this is so.

Rabbit iris contains a substance which strongly contracts various smooth-muscle preparations commonly used for the assay of active tissue constituents, and the contractions are resistant to atropine. On the sphincter pupillae this

substance produced a prolonged spasm, as was shown when iris extracts were injected into the anterior chamber of cats or rabbits, or tested on isolated preparations of the ox sphincter iridis. The experiments described in this paper are confined to the characterization of this substance. They show that the substance is different from other known smooth-muscle stimulating substances found in tissue extracts and that it is acidic in nature. To this substance the name irin has been given.

A number of acidic substances with smooth-muscle stimulating properties are known. Further experiments are in progress to distinguish irin from these substances and the problem whether release of irin is the cause for the spasm of the sphincter iridis on antidromic stimulation of the trigeminal nerve or of the iris proper will be dealt with elsewhere.

SUMMARY

1. Watery extracts of rabbit iris contain a pharmacologically active substance, irin, capable of contracting iridial and intestinal smooth muscles. The histamine-insensitive rat colon preparation has been used for the routine estimation of irin; it is capable of detecting the activity corresponding to (≡) 0·25–1 mg of iris.

2. Unlike choline esters, irin is not antagonized by atropine; on the colon it is in fact potentiated. Irin differs from acetylcholine in its ability to contract the ox sphincter pupillae, which is rather insensitive to acetylcholine (up to 10 μg).

3. Unlike 5-hydroxytryptamine (5-HT), irin is not antagonized by lysergic acid diethylamide. Irin differs further from 5-HT in its action on the ox sphincter, which is insensitive to 5-HT. On the other hand, the atropinized rat uterus, which is very sensitive to 5-HT, does not react to irin, tested in doses of up to ≡10 mg. On this preparation iris extracts augment the response to 5-HT and to bradykinin.

4. Irin differs from bradykinin and from substance P not only by the fact that the rat uterus is insensitive to it, but also in its solubility in acetone and in chloroform, in its resistance to chymotrypsin digestion and in R_F value.

5. Irin has been purified 100 × by treating watery extracts with 20 vol. of acetone and re-extracting the acetone-soluble, dried, fraction with chloroform.

6. Irin behaves as a weak acid on partition between water and chloroform; it is retained by alkali in the watery phase but driven by acid into the chloroform. It migrates towards the anode on paper electrophoresis.

7. Electrophoretically purified irin contracts the rat colon and the guinea-pig ileum, and causes prolonged spasms of the ox sphincter pupillae and of the cat iris.

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