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EFFECT OF MECHANICAL STIMULATION ON RABBITS' EYES: RELEASE OF ACTIVE SUBSTANCE IN ANTERIOR CHAMBER PERFUSATES

BY N. AMBACHE, L. KAVANAGH AND JUDITH WHITING
From the Medical Research Council, Department of Physiology, Royal College of Surgeons of England, London, W.C.2

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The rabbit is the animal most frequently used in ophthalmological research. In some respects its eyes may be less suitable than those of other animals, owing to what may be termed a greater lability: they are especially prone, during experimental manipulations, to vasodilatation, increased capillary permeability with a rise in intraocular pressure, and prolonged miosis. Duke-Elder & Duke-Elder (1931) have drawn attention to these changes, induced in their experiments by stroking the iris, but also known to occur after other forms of mechanical stimulation; they attributed these phenomena to the initiation of axon reflexes within the eye, especially in the iris. Indeed, it is possible to draw an analogy between these peripherally induced effects and a similar combination of events evoked 'antidromically' by the trigeminal nerve in this species. Mechanical (surprisingly, the most effective form of) stimulation of the fifth nerve outside the eyeball, for example by intracranial section (Magendie, 1824; Bernard, 1858; and many other authors since) or by rubbing (Maurice, 1954), elicits a prolonged constriction of the pupil, which is not prevented by atropine in rabbits known to be atropinesterase-free (Ambache, 1956); as shown later by Perkins (1957), this miosis is also associated with vasodilatation and a rise in intraocular pressure.

Assuming these interrelated events to be due, respectively, to 'axon reflex' or to 'antidromic' stimulation of sensory fibres in (or of some other structure associated with) the trigeminal nerve, the unusual feature of the 'triple response' in this situation is the contraction of a non-vascular smooth muscle, the sphincter pupillae. The histological findings of Langworthy & Ortega (1943) on the albino-rabbit iris reveal a possible anatomical basis for this complex response, including its unusual feature. Their paper, abundantly illustrated, describes in some detail the manner in which the myelinated fibres in the iris, supplied in all probability by the trigeminal nerve, 'tend to branch profusely and extend centrally to the

margin of the pupil. They apparently end in all portions of the iris . . ., but particularly around the pupillary margin'. These fibres end in three ways: (*a*) in disk-like endings or 'complex sprays of endings terminating in the superficial layers of the stroma of the iris', (*b*) endings which follow the course of the blood vessels and are always in intimate relation with them, especially near the pupillary margin; these are also referred to as 'large and diffuse terminations', and (*c*) groups of large, complicated endings with terminal plaques, lying between the smooth muscle fibres of the sphincter pupillae muscle; these are somewhat larger than and quite distinct from, the profuse cholinergic motor innervation of this muscle, which is supplied by unmyelinated fibres from the oculomotor nerve.

In fact, the miotic response and vasodilatation on mechanical stimulation of the iris do not depend upon the action of the cholinergic nerve supply to the iris, since we have now shown that they still occur when the animals have been effectively atropinized, the rabbits for these experiments having been carefully selected beforehand to eliminate all those with serum atropinesterase, in which spurious results are obtained with atropine. Mepyramine also does not appear to suppress these responses.

Former work from this laboratory has shown that an atropine-resistant active substance of an unusual type is present in the rabbit iris and can be extracted from it merely by grinding the iris in distilled water. This substance, provisionally named irin, has been identified as ether-soluble unsaturated hydroxy fatty-acid(s) and shown to possess smooth-muscle-contracting and vasodilator properties (Ambache, 1957, 1959, 1961). A similar acid was found in rabbit brain (Ambache, Reynolds & Whiting, 1963). In the present experiments an active substance of this kind, detectable by histamine-insensitive muscle preparations, has been found in the aqueous humour of rabbits after paracentesis, which is one of the mechanical stimuli known to induce the vasodilatation and miosis. A method has been devised for perfusing the anterior chamber without loss of pressure, which has made it possible to study the release of small amounts of this active substance following various forms of stimulation. This suggests that the substance(s) may in some way be concerned with the reactions of the iris mentioned above.

These results have already been reported in brief (Ambache, Kavanagh & Whiting, 1964) at a demonstration given to the Physiological Society.

METHODS

Animals

Since delicate manoeuvres in the anterior chamber and pupillary measurements are more difficult in the obscurity created by the dark background of a heavily pigmented iris, adult rabbits (2–4·2 kg) were chosen with albino, blue or grey irides, as in the New Zealand

White (or Californian), Beverens, and Chinchilla breeds, respectively. They were anaesthetized with either (1) sodium pentobarbitone up to 40 mg/kg I.V., supplemented when necessary by further doses given I.V., I.P., or subcutaneously, the amounts required varying from animal to animal; a fresh 40 mg/ml. solution of this anaesthetic in 0·9 % NaCl solution was prepared daily, or (2) urethane 1·6–2 g/kg, administered I.V. as a warm 20 % solution in 0·8 % NaCl solution (Wright, 1952, p. 251), or (3) a combination of sodium pentobarbitone with urethane. In many of the experiments a depth of anaesthesia was reached such that reflex blinking could still be elicited from the eyelid margins but not from the cornea; anaesthesia was not further deepened for fear of arresting respiration. The use of a local anaesthetic, instilled into the conjunctival sac, was avoided throughout. In a few experiments in which the corneal reflex was not completely abolished, 1–2 ml. of 0·9 % NaCl solution at 2–4° C was dripped on to the cornea just before it was pierced.

After tracheal cannulation respiration was monitored by means of a microphone (a 20 mm deaf-aid earpiece). A 5 cm length of tubing connected one limb of the Y-shaped tracheal cannula to the central 6 mm metal ring on the face of the earpiece. Perforations were made in the metal case to allow a free movement of the respired air through the microphone. The sound of breathing was thus amplified to an audible level; this gave ample warning on the rare occasions when artificial respiration became necessary. When a record of the respiration was required the second limb of the tracheal cannula was connected to a transducer (see below).

Before cannulation of the anterior chamber the rabbits usually received heparin 250–1000 i.u./kg I.V. and in some of the perfusion experiments atropine sulphate 1–10 mg/kg.

Selection of atropinesterase-free rabbits. A proportion of our rabbits were taken at random, without knowledge of their atropinesterase content, for experiments in which there was no intention to use atropine. But in thirty of the forty-one experiments, whether atropine was used or not, the rabbits had been submitted to a preliminary test, which determined whether they were atropinesterase-free or not. For experiments in which effective and lasting atropinization of the animals was desired after a single dose of 1–10 mg/kg, selected atropinesterase-free rabbits were used. Certainty about the absence of atropinesterase was obtained either by examination of a sample of serum in a Warburg manometric apparatus, or by the rapid procedure described by Ambache, Kavanagh & Shapiro (1964); this is based on a mydriatic test which has been correlated with manometrically determined serum-atropinesterase levels.

Rabbits known to contain atropinesterase were also used either in experiments without atropine, or even when atropine was given initially merely in order to dilate the pupil at the time when the anterior chamber was being cannulated.

Pupils were measured in the horizontal diameter with a stainless-steel Castroviejo's combined caliper and rule graduated in mm.

Recording of intraocular pressure (I.O.P.)

Before recording I.O.P., the animals were prepared as follows: (1) A superficial vein lying under the deep fascia postero-laterally to m. extensor digitorum pedis communis was exposed 5 cm above the left ankle and some of its tributaries were tied. A fine Portex nylon intravenous cannula (0·63 mm outer diameter (O.D.)) was tied into this vein and fitted at its other end to a Gordh adaptor, which was strapped to the foot by a rubber band. All further injections were given by this route in order not to move the animal's head when needles were present in the anterior chamber. (2) To record arterial pressure the right femoral artery was cannulated with another nylon cannula, usually 1·34 mm O.D. and 0·98 mm bore, connected through a metal three-way tap (allowing local injection of 0·2 ml. = 1000 i.u. heparin before releasing the arterial bulldog clamp) to a capacitance transducer with a phosphor-bronze diaphragm.

The animal was then laid prone on a thermostatically heated operating table and a

thermocouple was placed in the rectum. The head was fixed in a holder moulded from Stellon denture material; it was secured on the plastic chin-plate (suitably incised in the mid line to allow for the tracheal cannula) by two leather straps, one passing behind the ears and the other over the snout. To secure the top of the head a strip of brass lined with foam rubber, and moulded to the shape of the head, was placed sagitally over the head and fixed by the two leather straps slotted into hooks on top of the strip at either end. The eyelashes were usually cut close to the lid margin.

To record I.O.P. (without perfusion of the eye), a needle fitted with a stop was shot into each eye as described below. These were unmodified Solila dental needles as used in cartridge-type syringes, usually of S.W.G. 22 (0·7 mm O.D., 0·4 mm bore and 59 mm long). Each needle was connected by a Portex nylon intravenous cannula (0·6 mm bore and 23 cm long) to the three-way tap of one of the differential pressure transducers.

The inductive differential pressure transducers, Type 1025 supplied by New Electronic Products (now Honeywell Controls) Ltd., have two Perspex 3 ml. chambers in which pressures impinge upon an electro-plated iron diaphragm. When recording arterial, venous or intraocular pressures, one of these chambers was filled with the perfusing solution and the other was left open to the atmosphere and contained air. When recording respiratory air flow through the tracheal cannula both chambers were filled with air and one was left open to the atmosphere.

Each transducer was connected through its amplifier (Type 644B) to a pressure-monitoring meter on the panel of the instrument, and to a miniature galvanometer (Type BB 130A) in one of the six channels of the recorder, Type 1160, in which ultra-violet light is reflected from the galvanometers to record on Kodak Linagraph 'direct print' paper. A box of amber Perspex, fitted over the outlet of the recorder, allowed viewing of the records as they emerged, but was dark enough to protect them from overdevelopment by exposure to daylight until they were treated with Kodak MX 517-1 and Unifix solutions. In some of the records, contrast was increased by photocopying. On the sensitivity range most frequently used (0–40), a 1 cm deflexion on the paper represented 4 mm Hg; two adjustable reference lines were used to divide the paper into three. Calibration of the transducers was provided by a sphygmomanometer and a pressure-bottle containing occasionally 0·9 % NaCl solution but usually glucose-free de Jalon's solution to which heparin (500 i.u./l.) was added in a few experiments, to prevent clotting in the anterior chamber. A Palmer clock connected to the additional event marker in the recorder signalled minutes on the trace for slow paper speeds; at faster speeds the recorder's own time-marker registered 0·2 and 1 sec (or 2 and 10 sec) intervals on the paper.

After levelling the transducers with the fluid in the pressure bottle and with the rabbits' eyes, when both pressure-monitoring meters read zero, the needles were fired into the anterior chamber from either canthus, but preferably from the temporal side, away from the nictitating membrane. To avoid undue stimulation by grasping the conjunctiva or rectus tendons the needles were shot (from a distance of 0·5–1 mm) through the cornea by a gun of the type designed by Sears (1960) but constructed with minor modifications. To prevent the needles from reaching the iris or lens they were previously fitted with 4 mm sleeves of silicone rubber tubing which acted as stops 3–4 mm from the tip. To avoid a fall in I.O.P., manometer fluid was allowed to flow from each needle during its firing at a pressure of around 20 mm Hg, approximately that of the rabbit's anterior chamber (Sears, 1960; Kupfer, 1961). The needles were then fixed as described below, or sometimes left without further support.

The arterial blood pressure was recorded simultaneously from the capacitance transducer, through an amplifier circuit described by Donaldson (1958, Fig. 33.36 on p. 502); the output of this was fed to a BB 130 galvanometer (or to the more sensitive BB 40, shunted to a sensitivity of 100 μA full scale) in one of the remaining free channels of the recorder, in series with a monitoring 100 μA meter.

Samples of aqueous humour

Under sodium pentobarbitone anaesthesia a self-sealing valvular track, 2 mm long, was made in the cornea by a short-bevelled 26 or 30 S.W.G. hypodermic needle and aqueous humour was withdrawn from the anterior chamber into a syringe graduated to 0·01 ml. Further samples of the aqueous humour, now plasmoid, were obtained by paracentesis through the same track. The volume of each sample was read at once in the syringe before injection into a 5 ml. organ bath as soon as possible for assay against an irin standard.

Perfusion of anterior chamber

(*a*) *de Jalon's solution.* This was perfused without prior oxygenation. Its composition was that specified for rat colon (g/l.: NaCl, 9; KCl, 0·4; $CaCl_2$, 0·03; $NaHCO_3$, 0·15; glucose, 1).

(*b*) '*Eye solution*' of the following composition: (g/l.) NaCl, 7·3; KCl, 0·4; $CaCl_2$, 0·2; $MgCl_2.6H_2O$, 0·158; $NaHCO_3$, 2·2; glucose, 1·24. The approximation in composition of this 'eye solution' to that of rabbit aqueous humour or plasma is shown in Table 1. Continuous bubbling in the reservoir with a 95 % O_2–5 % CO_2 mixture adjusted the pH of this solution to 7·6 before its entry into the eye.

TABLE 1. Composition of de Jalon's solution and of the 'eye solution' (m-moles/l.) compared with that of rabbit aqueous humour and plasma (Davson, 1963; m-moles/kg water)

	de Jalon's solution	'Eye solution'	Aqueous humour	Plasma
Na	155·8	151·5	143·5	151·5
K	5·35	5·35	5·25	5·5
Ca	0·27	1·8	1·7	2·6
Mg	—	0·78	0·78	1·0
Cl	159·9	135·83	109·5	108
HCO_3	1·79	26·18	33·6	27·4
Glucose	5·55	6·9	6·9	8·3

The 'eye solution' and the de Jalon's solution were prepared in water of high purity obtained by passing the distillate from a Manesty still through a glass column containing the same deionizing resin mixture as is supplied in the Griffin–Raleigh water deionizer. The conductivity of the water was measured daily; it was usually *ca.* 0·15 (and always < 1) μmho/cm^3 on emergence from the resin, and stabilized at 3 μmho/cm^3 during storage in an airtight, siliconed glass vessel; storage was not extended beyond 2–3 days, and whenever possible the water was prepared on the same or on the preceding day.

Procedure. The perfusing solution (without drugs) flowed from a raised 1 l. reservoir bottle connected by silicone-rubber tubing to a warming device, consisting of a 10 cm stainless-steel lumbar puncture needle buried subcutaneously between the scapulae and emerging in the mid line behind the ears. From this point the connexion to the inflow needle in the anterior chamber was by some 10 cm of fine but thick-walled silicone-rubber tubing (0·45 mm bore and 2·3 mm O.D.). The inflow needle was a 22-gauge Solila needle, modified thus: a 1 cm stainless-steel side arm was brazed on at a point 7 mm away from the blunt end, midway between it and the ball carried by this type of needle. The side arm was connected by polyethylene tubing to one of the two transducers for continuous recording of the I.O.P.

The perfusion circuit of the anterior chamber was completed by an outflow 22-gauge Solila needle without side arm, connected by some 45 cm of the fine silicone tubing to a 22-gauge needle (Fig. 1, *A*) inserted through a silicone-rubber bung into the top of a glass cannula (*B*) which was part of the drop-chamber shown in Fig. 1. A further 20 cm of the fine silicone tubing (*H*) carried the outflow from the drop-chamber to the platinum–iridium side arm (*I*) at the lower end of the miniature organ bath.

The drops forming on the tip of the needle (*A*) in the drop-chamber interrupted a light beam falling on to a cadmium sulphide photoconductive cell (*E*) from a capless lamp with 'flying leads' (*C*) and a 17 mm concave reflector (*D*). The cell was covered by a mask (*F*) with a small aperture carefully aligned with the tip of the needle. The impulses in the light-dependent circuit of this photoconductive cell were suitably amplified (French, 1962) by a transistor, and closed a relay actuating a compact Ledex/NSF solenoid twelve-way rotary circuit selector, which was wired to select every tenth or twelfth drop-impulse for electro-

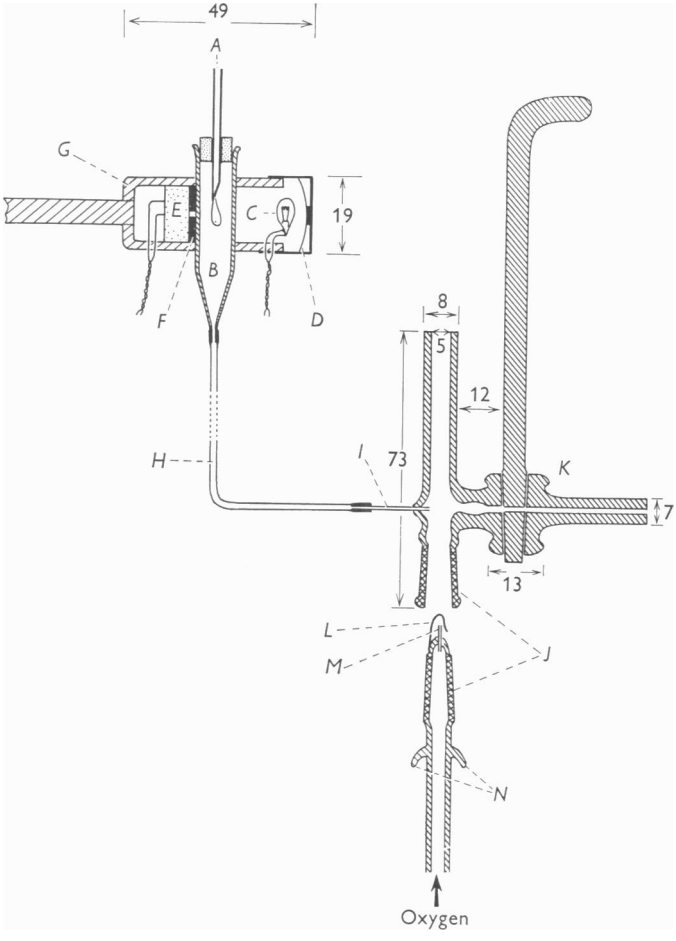

Fig. 1. Miniature organ bath before assembly and with drop-chamber connected. Dimensions in mm; parts of the drawing are not to scale.

Drop-chamber. *A*, 22-gauge Solila dental needle fixed in the glass cannula *B*; *C*, capless lamp supported by its leads; *D*, reflector; *E*, Mullard Type ORP 12 photoconductive cell with mask *F*; *G*, brass case; *H*, silicone tubing connecting drop-chamber to *I*, platinum-iridium side tube (0·8 mm O.D.) fused into organ bath.

Organ bath. *J*, B 7 ground glass cone and socket; *K*, 1 mm bore glass stopcock; *L*, platinum wire hook (29 S.W.G.); *M*, platinum bubbling tube (0·2 mm O.D.); *N*, glass hooks.

magnetic recording on the smoked drum in most of the experiments. The sound, coinciding with each drop, which was emitted by the Ledex switch gave audible warning of any cessation of flow. The total dead-space between the eye and the organ bath was 0·23–0·3 ml., representing 1–2 min flow.

The inflow needle was fired into the anterior chamber with manometer fluid flowing from it at a pressure of 20–25 cm H_2O, supplied by the perfusion reservoir, which was clamped off immediately afterwards. For the same reason, before firing the outflow needle, its 45 cm of tubing was temporarily attached to the second transducer, the three-way tap of which was opened to the calibrating pressure bottle at 20–30 mm Hg. After firing, this tap was closed at once, the outflow tube was connected to the drop-chamber, and flow was established by unclamping and raising the perfusion reservoir to a height such as gave an I.O.P. reading of approximately 20 mm Hg on the pressure-monitoring meter. For flow rates of 0·15–0·3 ml./min this height was found empirically to be 30–45 cm above the eye, which was level with the top of the organ bath.

The positions of the needles in the anterior chamber varied considerably. The ideal positions seemed to be near '3 o'clock' and '9 o'clock', respectively, and as far apart as possible in order to allow perfusion of as much of the chamber as possible. However, as the chamber is shallower at its outer edge, it was not practicable to pierce the cornea < 3 mm from the sclero-corneal margin. It was often necessary to change the inclination of and to advance or recede the needles by hand in order to place the tips away from the iris, and sometimes even to cross the needles in the anterior chamber so as to give a greater separation of the tips.

Once in position the needles were either (*a*) left without further support, as in the first seven experiments, or (*b*) fixed in miniature crocodile clips fastened to rigid but flexible arms of 13 S.W.G. solder. At their other end these arms were either screwed into the sagittal brass strip of the head-holder, or connected to a separate brass bar, 2–3 cm above and parallel to it, which was clamped to the supporting arm of the head-holder.

Temperatures of the cornea and of the perfusate were measured in one experiment with a Pye Scalamp thermocouple galvanometer.

Biological assays

Meriones *colon preparations*. Two species of jird were used in these experiments, *Meriones trystiami* and *M. libycus*. The original stock was supplied by Messrs May & Baker Ltd. and by St Bartholomew's Hospital Medical School, respectively. We are indebted to Mr C. Shorland for successful breeding of both colonies.

The advantage of using these two desert rodents is apparent from Fig. 2, which contrasts the rhythmic activity and sensitivity to irin of two colon preparations, one from *M. libycus* (♂, 145 g), and the other from an albino rat (♂, 155 g). The two muscle preparations were almost identical in weight (320 and 375 mg, respectively) and in length and were set up simultaneously in parallel under identical conditions. It will be seen that at 30° C rhythmic activity is so great in the rat colon, especially after lysergic acid diethylamide (LSD), that it may mask the responses to small doses of irin. The jird preparations could always be relied on to be free from rhythmic activity even at 30° C and, as shown in Fig. 2, were more sensitive to irin and exhibited a steep slope in the dose–response curve.

The majority of the jird experiments were carried out on male *M. trystiami*. The preparation was as follows. The jirds were anaesthetized with ethyl chloride and bled out after cervical transection. The ascending colon was identified as in rats by its diagonally banded appearance, and excised between ligatures. The lumen was washed with a stream of de Jalon's solution from a syringe with a blunt needle until it was quite clear, and an open-ended length of colon, 4–5 cm for the experiments in the 5 ml. bath and 3–4 cm for the miniature organ bath, was suspended between two platinum hooks. The bath fluid was oxygenated de Jalon's colon-solution at 30° C with atropine 10^{-7} g/l. and LSD, usually

10^{-8} g/l.; D-2-bromo-*N*,*N*-diethyl-lysergamide bitartrate was used instead of LSD in a few experiments.

Miniature organ-bath experiments. A 1·4 ml. bath was made by Scientific Supplies, Ltd., London, in two detachable sections centred round a B 7 ground glass cone and socket joint (Fig. 1, *J*). To provide minute bubbles of gas for the oxygenation of the gut in this small space, a very fine platinum tube *M* (0·2 mm O.D.) was fused into the cone after its end was sealed, as also was a hook (*L*) of 29 S.W.G. platinum wire for attachment of the gut. The upper section of the bath, of internal diameter 5–5·5 mm, was built on the socket. At its

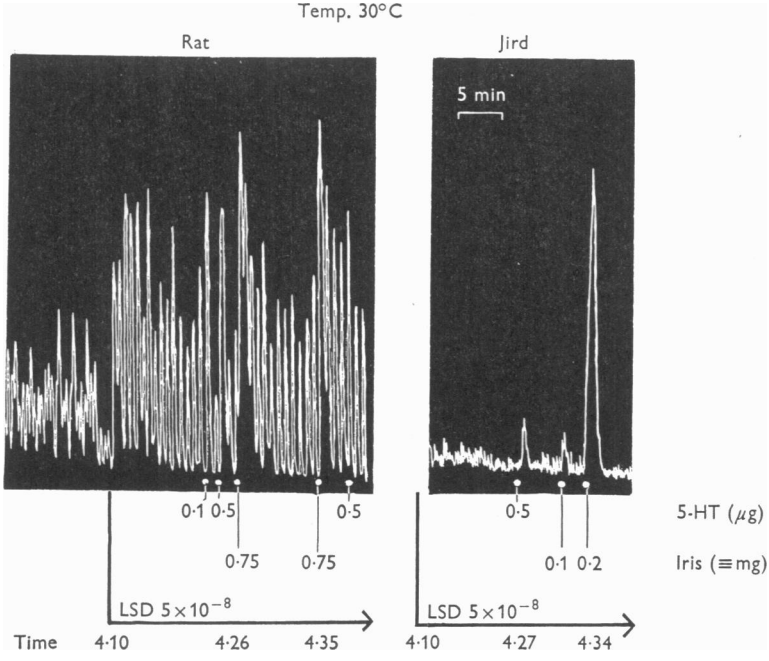

Fig. 2. Comparison in a parallel experiment under identical conditions of albino rat and jird (*Meriones libycus*) colon preparations. Atropine sulphate 10^{-7} g/l. present throughout, and lysergic acid diethylamide (LSD) 5×10^{-8} g/l. after 4.10 p.m.; 5 ml. baths; 30° C; lever loads 0·8 g. Contacts: iris extracts 1 min, 5-hydroxytryptamine (5-HT) 30 sec. Explanation in text.

lower end *ca.* 2 cm of platinum-iridium tubing (*I*; 0·8 mm O.D.) was fused into the side of the bath for the attachment of the outflow-connexion from the drop-chamber in the eye-perfusion experiments; and on the opposite side, a glass stopcock (*K*; bore 1 mm) was fused on for renewal of the bath fluid by overflow from a reservoir incorporating a drip. While the rabbit was being prepared for the eye-perfusion, the tap was left open and the bath fluid was automatically renewed at a slow rate.

Open-ended jird or rat colon preparations were made in the usual way; the rats were male albinos (< 100 g, preferably 50–75 g). A 5 mm loop of thread was then sewn through the lower end of the preparation, and the upper end was attached to a platinum-wire hook on the lever thread, which, for ease of manipulation, had already been passed down through the upper section of the bath. The colon was then attached to the hook (*L*) on the cone by the loop, and the upper section of the bath was lowered over the preparation for junction

with the cone. The lower half of the cone had been smeared previously with Vaseline to make a completely watertight joint, which was further secured by means of two small elastic bands looped over the tap and fixed to the glass hooks (*N*) below the cone.

The bath fluid was either (*a*) de Jalon's colon-solution bubbled with oxygen and maintained at 30° C for the *Meriones*, or at room temperature for the rat colons; or (*b*) the same 'eye solution' as was used for some of the anterior chamber perfusions; this was gassed with 95 % O_2 + 5 % CO_2 and was used only with rat colons at room temperature. Atropine and lysergic acid diethylamide, or 1-methyl lysergic acid butanolamide tartrate were present in these bath fluids in amounts indicated in the text. Contractions were recorded with Kavanagh's (1962) lever at a load of 0·5–0·6 g and a 12 × magnification.

Drugs and extracts. All drug dosages refer to the respective salts. The dosage of all iris extracts, crude or purified, is expressed in $\equiv x$ mg, i.e. in terms of the activity originally present in x mg of extracted iris tissue.

The sensitivity of the preparations was tested with the same irin standard in all the rat colon experiments, except that of Fig. 5; the extract was used without purification in the first three experiments, and after an ether-purification, with 60–66 % recovery, in all the others. In the *Meriones* experiments an older, unpurified extract was used as the standard; when compared on a jird preparation this was 2·5 × weaker than the later 'rat colon' irin standard.

RESULTS

Irritation reactions unaffected by atropine and mepyramine

By showing that the miosis and increase in I.O.P. which occur in rabbits' eyes after irritation of the iris are not prevented either by atropine or by mepyramine, our results extend the observations of previous workers and suggest that these phenomena occur independently of the release of acetylcholine or of histamine and are therefore likely to be mediated by some other active substance. Since about 45 % of rabbits destroy atropine, often with amazing rapidity, the present experiments were all performed on animals which were known to be atropinesterase-free, and in which effective atropinization of the iris with mydriasis and complete paralysis of the light reflex had been observed to last for several hours in a preliminary test.

Miosis after stroking the iris (during which there was frequently an audibly detectable change in the animals' respiration), or after collapse of the anterior chamber, was noted in all the experiments of this series and in the perfusions described below, whether the animals were effectively atropinized or not, and also after intravenous administration of mepyramine maleate 1–5 mg/kg. It also occurs after enucleation in rabbits' (but not in cats') eyes, as already reported (Ambache & Whiting, 1963); this too is not prevented by atropine.

Likewise, the increase in I.O.P. after stroking the iris occurred in fourteen atropinesterase-free rabbits injected with atropine ± mepyramine intravenously. The dose of atropine sulphate was 1 mg/kg in thirteen of these animals and 10 mg/kg in the other; mepyramine maleate was administered in nine of these experiments, the dose being 1 mg/kg, 2 mg/kg and 5 mg/kg in

three groups of three rabbits. With atropine alone, responses to stimulation occurred in eight of ten trials, and 'spontaneously' in one rabbit, i.e. without deliberate stimulation (but perhaps due to the mere presence of the I.O.P.-recording needle in the cornea), first in one eye and then in the other. With atropine and mepyramine together responses were obtained in nineteen out of twenty trials and once 'spontaneously'. The results are given in detail in Table 2; as in Sears' (1960) observations, the magnitude of the I.O.P. change varied considerably.*

TABLE 2. Increases in intraocular pressure (mm Hg) recorded after stroking the iris, or 'spontaneously' (*S*); results obtained under different anaesthetic conditions, and with unequal stimuli. Details in text

Atropine			Atropine and mepyramine		
0–10	**10–20**	**>20**	**0–10**	**10–20**	**>20**
0	10·4	22·8	0	11·4	21·6
0	12·4 (*S*)	26·4 (*S*)	3	12	22·4
6·4	14	—	3·6	12·4	22·4
8·4	16	—	4	13·6	>25·2
8·8	18	—	4	13·8	25·6
			6	15 (*S*)	26·4
			—	15·2	—
			—	17·2	—
			—	18	—

The time course of the I.O.P. change is shown in Fig. 3. A hitherto undescribed feature of this response, which we have frequently observed and which appears to be one of the signs of vasodilatation, is the broadening of the I.O.P. trace as the pressure rises to its maximum and thereafter; this was associated with the appearance of oscillations on the corresponding pressure-monitoring meter. The frequency of these oscillations on the meter coincided with that of the animal's respiration; this was confirmed by records obtained at greater paper-speeds, e.g. Fig. 3*A* (inset), where these oscillations recurred at a frequency of 50/min. In most experiments these 'respiratory' pulsations were virtually absent or barely detectable on the meter or on the tracing before the iris was stimulated, though occasionally, as in Fig. 3*A*, they were present before deliberate stimulation but increased in amplitude (to 1·2–1·6 mm Hg) after the iris was stroked. As it was possible that a minute movement of the strapped head relative to the eye-needles in their clamps, which was sometimes seen with each breath, could accentuate this effect, the needles were taken out of their

* Further research is needed to establish to what extent this variation is due to such variables as the depth of anaesthesia in each animal or its body temperature, which could affect the tone of the vessels in the eye, or merely to differences in stimulus intensities. With the iris floating freely in the anterior chamber, it is hard to deliver mechanical stimuli of constant intensities; even when it is stroked only once on two different occasions the second stimulus cannot be regarded as equivalent to the first.

clamps in four experiments and left hanging down from the eyes. The respiratory waves in the I.O.P. were recorded under these conditions, as usual, and also in experiments (e.g. Fig. 4) in which the animals were laid in the supine position, with and without a head-holder, and in which it was also observed that no movement of the head or eyes relative to the needles was taking place. Records taken at still higher paper speeds (Fig. 4)

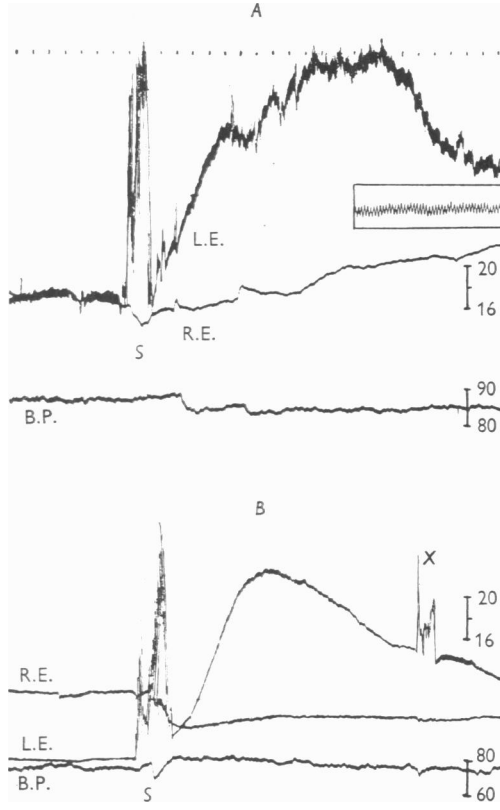

Fig. 3. Irritation reaction in two atropinesterase-free rabbits treated with atropine ± mepyramine; albino irides. Left and right intraocular (I.O.P.) and femoral blood pressures as labelled, with respective calibrations in mm Hg; time in min (dots above).

A. (♂, 2·5 kg). At *S*, left iris stroked 16 ×, 15 min after intravenous administration of atropine sulphate 1 mg/kg and 9 min after mepyramine maleate 2 mg/kg. Subsequent broadening of I.O.P. trace due to respiratory pulsations shown in the inset, recorded at a faster speed for 1 min.

B. (♂, 3·2 kg). At *S*, left iris stroked 20 ×, 73 min after atropine sulphate 1 mg/kg I.V.; paper speed as in *A*. The temporary disturbance at *X* is due to an adjustment of the needle in the anterior chamber.

The artifacts at *S* in *A* and *B* are due to the temporary changes in the I.O.P. caused by the mechanical disturbances in the process of stroking. The irritation reaction is the subsequent slow change.

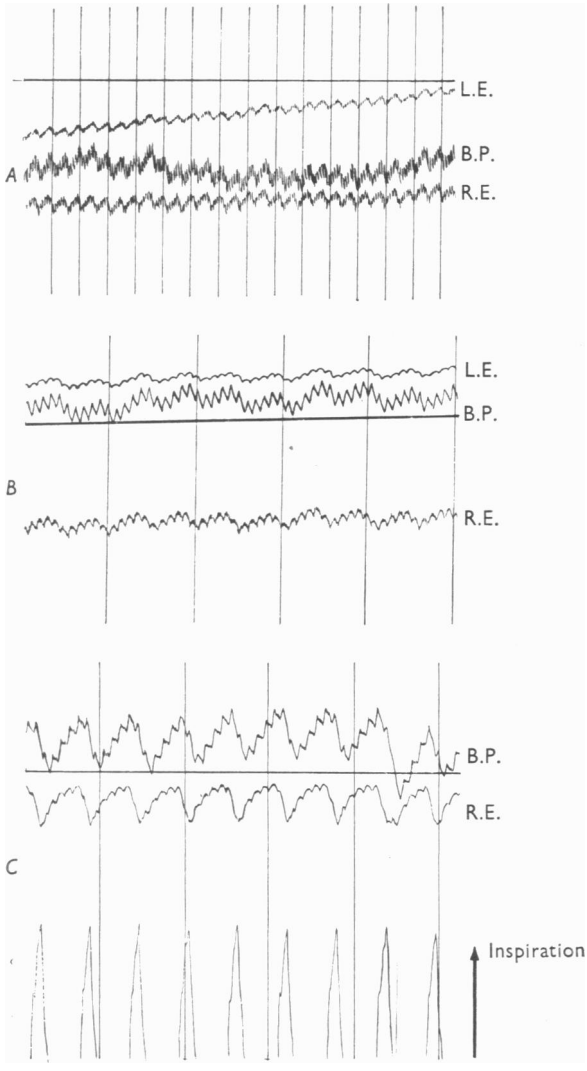

Fig. 4. Examples of respiratory waves in the intraocular pressure, recorded during normal respiration in the supine position, with needles unclamped, at various paper speeds; vertical lines indicate 2 sec intervals.

Atropinesterase-free rabbit, 2 kg; urethane 2·1 g/kg; atropine 1 mg/kg and mepyramine 2 mg/kg I.V.

A. From above downwards, simultaneous pressure records from left eye (29·2–29·8 mm Hg initially), femoral artery (80–81 mm Hg) and right eye (23–24·8 mm Hg).

B. Sequence as in *A*: left eye (37–37·8 mm Hg), femoral artery (87–89 mm Hg), and right eye (24–25·4 mm Hg).

C. Femoral artery (70–74 mm Hg), right eye (13·6–15·4) and intratracheal pressure (inspiratory peaks upwards).

showed the presence of small notches on the I.O.P. trace at a frequency of *ca.* 300/min, which was that of the heart rate. As to the origin of these respiratory oscillations in the I.O.P., vasodilatation of the small vessels in the eye, arterial and perhaps also venous, would appear to increase the transmission of respiratory fluctuations which occur in the arterial and venous pressures. Simultaneous records, such as those in Fig. 4, have shown that in some rabbits the femoral blood pressure manifests the same type of respiratory fluctuations as the I.O.P., but occurring 0·06–0·13 sec later, presumably due partly to the greater distance from the heart. On the other hand, these arterial fluctuations were absent for no obvious postural reason in some rabbits in which the respiratory oscillations were nevertheless present in the I.O.P., in this case perhaps due to transmission of venous pulsations.

Two of these experiments are illustrated in Fig. 3 and will now be considered in detail. In the selection test which preceded these two experiments, the intravenous injection of atropine sulphate 1 mg/kg to the conscious animals produced a mydriasis and a loss of reaction to light which lasted for 4·5 hr in rabbit *A* and 5 hr in rabbit *B*, indicating the absence of atropinesterase in both.

On the day of the experiment the animals were anaesthetized, *B* with urethane 1·7 g/kg I.V. and *A* with a mixture of Na pentobarbitone (40 mg/kg intraperitoneally and 20 mg/kg I.V.) followed by urethane 1·1 g/kg I.V.; in both the corneal reflex was not completely abolished and there was no need for artificial respiration at any time. The rabbits were injected with heparin: *A*, with 900 and *B*, with 750 i.u./kg I.V. In *A*, one needle was shot into the left eye, horizontally from the temporal side, 30 min before the stimulus at *S*, and the other needle into the right eye, horizontally from the nasal side, 23 min before *S*; both needles were fixed in their crocodile clips. Record *A*, which begins 8 and 2 min, respectively, after the intravenous injections of atropine and mepyramine, shows the presence of respiratory pulsations in the left eye. At *S*, the needle was unclamped on the left side and the iris was stroked 16 times between '11 and 7 o'clock', i.e. on the nasal side; the needle was moved like the paddle of an oar (the cornea serving as a row-lock) with its smooth side over the anterior surface of the iris, after which it was replaced in its clip. The irritation induced a miosis, as usual. Whereas 10 and 3 min before *S* both pupils measured 8 mm, after *S* there was no change on the right but a constriction occurred on the left, as shown by the following measurements (time in min after *S* given in brackets): 4·5 mm (2); 3–3·5 mm (6); 3 mm (20); 4 mm (47). Associated with this there was an increase in I.O.P. on the left of 24 mm Hg during the 14 min after *S*, reaching a maximum of 41–42 mm Hg. At the same time there was a gradual broadening of the

respiratory oscillations on the tracing, the amplitude of which was now 1·6 mm Hg. Some broadening also occurred in Expt. *B*, in which, however, the respiratory pulsations were relatively insignificant before stimulation at *S*. The respiratory pulsations in *A* persisted after the I.O.P. returned to 21 mm Hg and were recorded for 1 min at a faster speed about 1 hr after *S* (Fig. 3, inset), when their amplitude was 1·2 mm Hg and their frequency 50/min.

In Expt. *B* (with atropine alone) both needles were shot horizontally from the nasal side about 2 hr before *S*, when the record shows that the I.O.P. was still higher on the right side owing to previous stimulations. At *S* the left iris was stroked 20 times on its temporal side. As in Expt. *A*, this produced a slight fall in I.O.P. on the contralateral side, but on the left the I.O.P. rose in the next 6 min by 18 mm Hg, and there was a 3 mm constriction of the pupil (6·5 → 3·5).

Appearance of an active substance in the plasmoid aqueous humour after paracentesis

Collapse of the anterior chamber by withdrawal of aqueous humour is one of the stimuli known to induce prolonged miosis, uveal vasodilatation and rise in I.O.P. The hypothesis that an active substance other than acetylcholine or histamine is implicated in the irritation phenomena of rabbits' eyes was to some extent supported by the finding that the stimulus set up by paracentesis (emptying of the anterior chamber) was followed by the appearance in subsequent samples of the aqueous humour of a substance producing contractions in atropinized, histamine-insensitive muscle preparations. Aqueous extracts of rabbit iris have powerful pharmacological properties which have been related to their relatively high content of ether-soluble unsaturated hydroxy-fatty acid(s), irin. The present investigations suggest that it is small amounts of this substance which appear in the aqueous humour.

The effect of paracentesis is illustrated in Fig. 5. The activity was here detected by a rat-colon preparation, rendered insensitive to acetylcholine and to 5-hydroxytryptamine (5-HT) by means of atropine 10^{-7} g/l. and lysergic acid diethylamide (LSD) 10^{-7} g/l. (see *A–E*). This experiment showed that the first draught of aqueous humour, taken from a normal rabbit eye under Na pentobarbitone anaesthesia, was inactive (*F*). The same volume of the now plasmoid (containing more plasma proteins, including fibrin) aqueous humour, taken from the same eye, now in a state of miosis, 15 min after the initial paracentesis, contained distinct activity (*G*), which could be matched at *H* by ≡ 1·25 mg of an acetone-purified (polypeptide- and protein-free) rabbit iris extract. Blood drawn from the heart of the same rabbit was inactive (*I* and *J*, 0·05 and 0·1 ml.).

This type of experiment was repeated (Fig. 6) using another irin-sensitive test muscle, the ascending colon of the jird *Meriones libycus*. The whole of the 0·35 ml. of normal aqueous humour of the first draught taken from the left eye was virtually inactive at *A*. The second draught of aqueous humour, now plasmoid, obtained 12 min after *A* was lost through clotting. A third draught of 0·17 ml. plasmoid aqueous humour, taken from the left eye 21 min after *A*, was tested 30 sec later at *B*, and was active, though less than an irin standard in a dose of ≡ 0·25 mg at *C*.

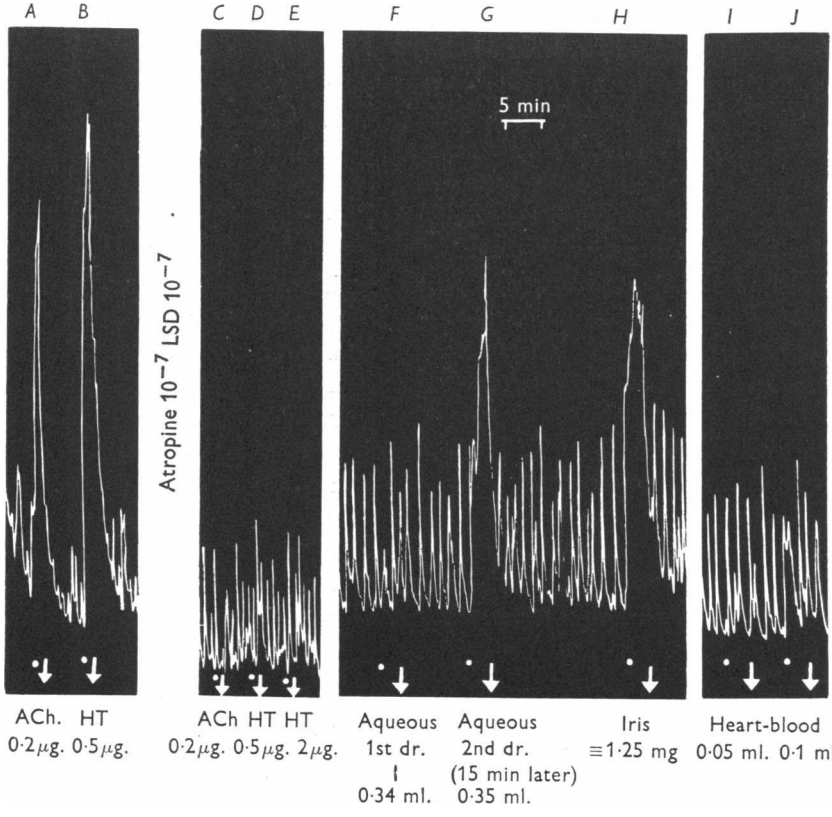

Fig. 5. Activity of rabbit plasmoid aqueous humour. Rat-colon preparation, 22° C; 5 ml. bath. *A–E*, acetylcholine and 5-HT in doses indicated (1 min contacts) before and after respective block with atropine and lysergic acid diethylamide (LSD) 10^{-7} g/l., present in the organ bath after *B*. All other contacts 3 min: *F*, 0·34 ml. normal aqueous humour; *G*, 0·35 ml. plasmoid aqueous humour drawn 15 min after the paracentesis which yielded sample *F*; *H*, ≡1·25 mg acetone-purified rabbit iris extract; *I*, 0·05 ml. and *J*, 0·1 ml. heart-blood from the same rabbit. The rabbit (albino) was unselected and therefore of unknown atropinesterase-content; it received heparin 526 i.u./kg I.V. and, 6 min before draught (*F*), atropine sulphate 1 mg/kg; 5 min after draught (*F*) was taken the pupil on that side was 3 mm smaller than that of the control eye.

Thirty minutes after *A*, 0·32 ml. of aqueous humour was obtained for the first time from the contralateral eye, hitherto untouched, and was tested 1 min later at *D*. In contrast to the initial sample from the left eye (*A*), detectable activity was present in this sample though less than in *B*, which had about half that volume. This would indicate the presence of some contralateral effect due to the earlier paracentesis on the opposite side; the occurrence of contralateral effects in a proportion of rabbits on mechanical stimulation of one eye is well known and is mentioned by Sears (1960).

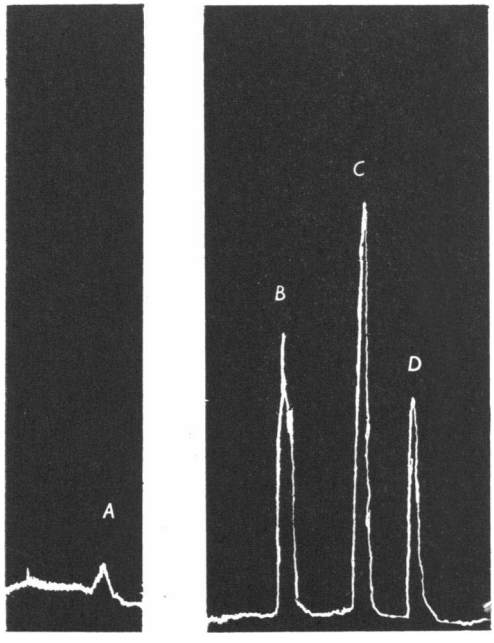

Fig. 6. Activity in samples of rabbit aqueous humour after paracentesis under Na pentobarbitone anaesthesia. *Meriones libycus* colon preparation, 5 ml. bath, 30° C, atropine and LSD 10^{-7} g/l. throughout; 1 min contacts. *A*, 0·35 ml. left aqueous humour of the first draught; *B*, 0·17 ml. left, now plasmoid, aqueous humour of the third draught, taken 21 min after *A*; *C*, ≡ 0·25 mg standard rabbit iris extract. *D*, 0·32 ml. aqueous humour from the contralateral eye (first draught), taken 30 min after *A*. Further details in text.

Since the weight of an adult rabbit iris is of the order of 50–60 mg, it is clear from these results that the activity found in the aqueous humour is low and probably represents a mere 1–2 %, or less, of the irin-activity extractable from the whole iris when the tissue is ground in water.

Detection of a physiologically active substance in perfusates of rabbit anterior chambers (Meriones *colon preparations*)

The evidence that an active substance appears under certain conditions in the aqueous humour was further strengthened by the finding that during perfusion of the anterior chamber a similar substance, with irin-like

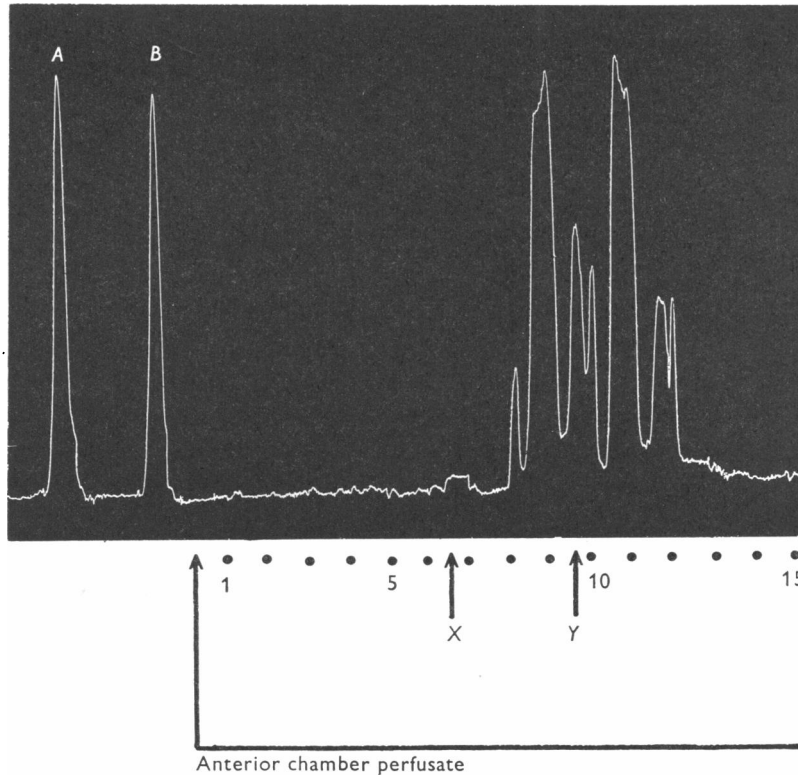

Fig. 7. Appearance of activity in anterior chamber perfusate following collapse. Rabbit, 2·2 kg; Na pentobarbitone; blue irides. Perfusate (de Jalon's solution) monitored by *Meriones trystiami* colon preparation in 1·4 ml. bath, 30° C; atropine 10^{-7} g/l. and LSD 10^{-8} g/l. At *A* and *B*, ≡0·25 mg irin standard; 1 min contacts. Nos. 1–15 refer to organ-bath washes. Outflow from eye connected to organ bath 2 min before no. 1; all subsequent washes at 3 min intervals (dots). At *X*, first collapse of anterior chamber with miosis, due to perfusion needles escaping out of the eye; both needles replaced soon after. At *Y*, replacement of one needle after a second collapse.

properties, was released when miosis was induced by a variety of mechanical stimuli.

In four experiments on unatropinized rabbits of unknown atropinesterase content, anaesthetized with Na pentobarbitone, the anterior chamber was perfused with drug-free unoxygenated de Jalon's solution. The needles

were left unsupported and the outflow was connected to a 1·4 ml. micro-bath so that the activity could be monitored continuously by the response of jird ascending-colon preparations at 30° C (mostly from *Meriones trystiami*), rendered insensitive to acetylcholine by atropine 10^{-7} g/l. and to 5-HT by LSD 10^{-8} g/l. Tests have shown that this type of muscle is

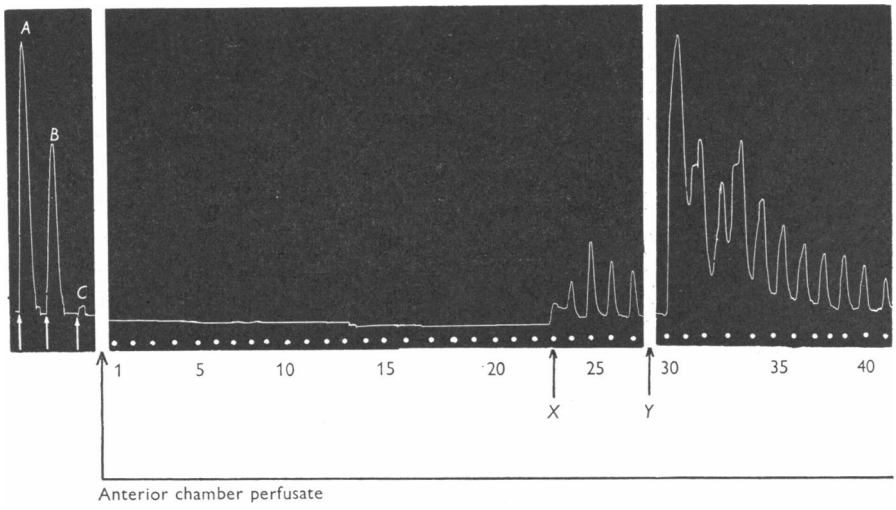

Fig. 8. Activity in anterior chamber perfusate from 3·15 kg rabbit (Na pento-barbitone), monitored by *Meriones trystiami* colon preparation, 1·4 ml. bath, 30° C; atropine 10^{-7} g/l. and LSD 10^{-8} g/l. Irin standards at *A* (≡ 0·2 mg), *B* (≡ 0·03 mg) and *C* (≡ 0·01 mg, threshold); 1 min contacts. Nos. 1–40 refer to organ-bath washes every 3 min (dots). Outflow from anterior chamber (de Jalon's solution) connected to organ bath 3 min before wash no. 1. At *X*, 'spontaneous' activity (see text). At *Y*, collapse of the anterior chamber. Needles replaced and perfusion resumed 3·5 min before wash no. 30. Latency due partly to refilling of anterior chamber and to dead-space lag.

normally insensitive to histamine (2–5 μg of the acid phosphate) and, because of the virtual absence of rhythmic activity, the preparations were capable of detecting $< \equiv$ 0·05 mg of activity in rabbit iris extracts, both crude and after ether-purification, which removes polypeptides. Activity appeared in these perfusates after collapse of the anterior chamber (Figs. 7, 8) or stroking of the iris.

One of these perfusions (Fig. 8) will be considered in greater detail, as it illustrates several features of this type of experiment. The inflow needle was shot into the anterior chamber at 12.04 p.m. and the outflow needle at 12.22 p.m., both without iris contact; the needles were left unsupported. The perfusion-reservoir was raised 41·7 cm above organ-bath level, thus locking the I.O.P. at 20 mm Hg and producing a flow of 0·165 ml./min. The dead-space between the eye and the organ bath was 0·26 ml. in this

experiment, representing a time-lag of 1·5 min. Although the organ-bath fluid was renewed at 3 min test-intervals, no activity was detected for the next 66 min (Fig. 8, nos. 1–22). At 1.30–1.42, when the depth of the anaesthesia had decreased somewhat, as indicated by blinks and small whisker movements (showing some return of corneal sensation), slight activity appeared 'spontaneously' in each of the 3 min test-intervals (Fig. 8, nos. 23–27). When the conjunctival sac had been flushed with 10 ml. of 0·9 % NaCl solution at 12.53 p.m., this had produced no effect, but now at 1.43 p.m. it initiated a slight head movement, which resulted in the unsupported inflow needle escaping from the anterior chamber and in iris contact by the outflow needle which then also escaped. Both needles were finally firmly re-inserted through their original corneal tracks at 1.50½ p.m. Figure 8 (nos. 30–40) shows the great and prolonged increase in activity detected in the blood-free perfusate produced by the combination of these stimuli (collapse of the anterior chamber and rubbing of the iris); this started at 1.54 p.m. There was also a pronounced miosis and the pupil, which measured 7 mm at 1.43 p.m., was down to 2 mm at 1.52 p.m. At 2.11 p.m. examination of the iris under a dissecting microscope with a 40 × magnification showed the absence of visible bleeding from any part of it, although engorgement of the vessels was seen. Later in this experiment the activity increased whenever the iris was stroked (three tests).

Conditions under which releases of active substance are demonstrable

Rat-colon preparation. For reasons already mentioned the use of *Meriones* preparations would be the method of choice for irin assays, and is still the best for some types of experiment. However, we have found that some *Meriones* colon preparations are contracted by bradykinin, e.g. < 35 ng/ml. in the experiment of Fig. 8 and 2 ng/ml. in others. It is known that in some species kinins may be formed from blood proteins by contact activation with glass and, although there was no visible bleeding into the perfusates, the plasmoid aqueous humour may contain 0·02 % of plasma proteins. It is well established, however, that rabbit blood is exceptional in that its proteins do not give rise to spontaneous kinin-formation on dilution or on activation by contact with glass (Armstrong, Jepson, Keele & Stewart, 1955; Schachter, 1956). Hence it is unlikely that bradykinin was formed in these experiments. That the activity described above was not, in fact, due to bradykinin was shown by the remaining twenty-two experiments in which activity was recorded as before, although bradykinin-insensitive rat-colon preparations were used for its detection. Moreover, partitions at pH 3 of eight samples of perfusate showed the presence of an active acidic lipid; bradykinin and other polypeptides are insoluble in ether.

The rat colon, suspended in atropine and LSD at 23 ± 2° C, frequently displays rhythmic activity of such amplitude, for instance, in Figs. 2 and 5, as would interfere with the detection of the minute amounts of active substance liberated into the perfusates. This was largely overcome by working at room temperature, and using 1-methyl lysergic acid butanolamide tartrate $1–4 \times 10^{-7}$ g/l. to block 5-HT, instead of LSD which accentuates the rhythmic activity (Fig. 2). The concentration of atropine was 10^{-7} g/l. in three of these experiments, but was raised to 10^{-6} g/l. in the rest, in order to accelerate the sensitization of these preparations to irin, which is known to occur after atropine (for example, in Fig. 1, Ambache, 1957). Under these conditions ≡ 0·05–0·1 mg of ether-purified irin hydroxy-acid could be detected. All but one of the preparations were tested with bradykinin (50–100 ng) before use; only those, a total of 21, which relaxed to bradykinin or did not respond to it were used for perfusate-assays.

In eight of the following experiments the rabbits were known to be completely atropinesterase-free. Effective and lasting atropinization with atropine sulphate 1–10 mg/kg I.V. at the beginning of the experiment did not alter the results, mioses and irin outputs being produced as usual upon various forms of stimulation. In some of the experiments, when the rabbits were known to have the enzyme atropinesterase in their sera, atropine was, nevertheless, administered intravenously just before shooting the needles into the eye. This was done only for convenience, as it was easier to avoid iris contact in mydriatic eyes.

Experiments with de Jalon's solution. The anterior chamber was perfused, at constant intraocular pressures, with de Jalon's solution in sixteen experiments, five under Na pentobarbitone and eleven under urethane anaesthesia. With three exceptions the perfusion needles were fixed in crocodile clips. In a proportion of these experiments some activity was present in the perfusates from the start, lasting usually 3–12 min, but in one experiment for > 1 hr. In some instances this was attributable to accidental iris contact, but subsequent experiments showed that initial activity could be present even when care had been taken to avoid the iris or lens, both at the time when the needles were shot into the eyes and during their subsequent clamping (Fig. 9). This effect could possibly be due to a lens movement (see below) resulting from an impact wave in the anterior chamber when the needles are shot, or to the piercing of the cornea. Thus, further experiments might show whether this initial output can be prevented by topical application of a suitable local anaesthetic to the cornea; but trials on the rat-colon preparation showed that it was depressed by the presence of cocaine. In a total of twenty-one perfusates initial activity was absent or small (e.g. Fig. 10*C*, 12*C*) in eight and distinct in

thirteen, of which eight were attributable to known, accidental, stimuli, leaving five which could not be accounted for by iris or lens contact (e.g. Fig. 9 *C*).

Stroking the iris or emptying the anterior chamber led to the appearance of activity in the perfusates, or increased its output, sometimes very markedly. One of these experiments is illustrated in Fig. 10. Although

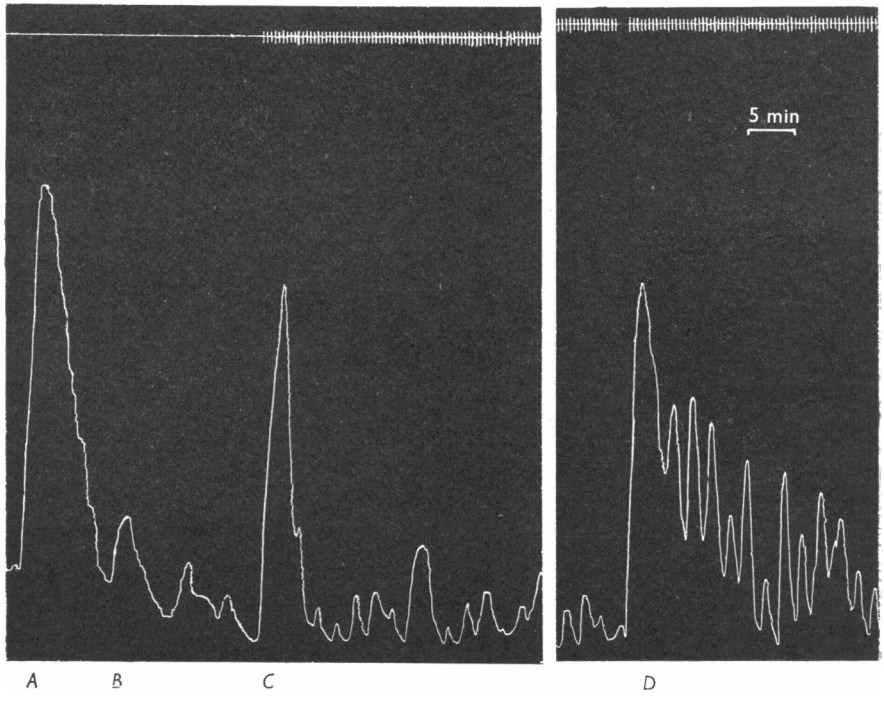

Fig. 9. Initial activity in anterior chamber perfusate (de Jalon's solution) not due to contact with either iris or lens. Atropinesterase-free rabbit (♀, 3·1 kg; albino iris); urethane 1·8 g/kg; atropine 1 mg/kg; heparin 500 i.u./kg.

Rat-colon preparation, 1·4 ml. bath; atropine 10^{-6} g/l.; 1-methyl lysergic acid butanolamide tartrate 2×10^{-7} g/l. At *A* and *B*, ether-purified irin standards ($\equiv 0{\cdot}25$ and $\equiv 0{\cdot}05$ mg, respectively); 2 min contacts. *C*, initial activity in perfusate (flow shown above by counter registering every tenth drop); organ-bath fluid renewed at 2 min intervals thenceforth. *D* (40 min later), appearance of activity after emptying the anterior chamber by lowering the perfusion reservoir (drop-record shows cessation of flow) for 1 min.

this rabbit was atropinesteratic, atropine 2 mg/kg (and heparin 500 i.u./kg) had been injected intravenously 11 min before the first needle was shot. The outflow needle was shot 4 min after that, and its tubing was connected to the organ bath through the drop-chamber $2\frac{1}{4}$ min later. After 2 min a small initial activity was detected (*C*). In this experiment the

dead-space between the eye and the organ bath was 0·23 ml. and, the flow being 0·348 ml./min, the time-lag introduced by the dead-space was only 0·7 min. Figure 10 *D* shows the effect of emptying the anterior chamber; perfusion of the collapsed chamber after replacing the needles was resumed 2 min before *D*. Because the activity which appeared at *D* after this collapse was off the scale, the organ bath was washed out every minute for the next 50 min. After the contraction subsided the more usual procedure of washing out the organ bath at 2 min intervals was resumed

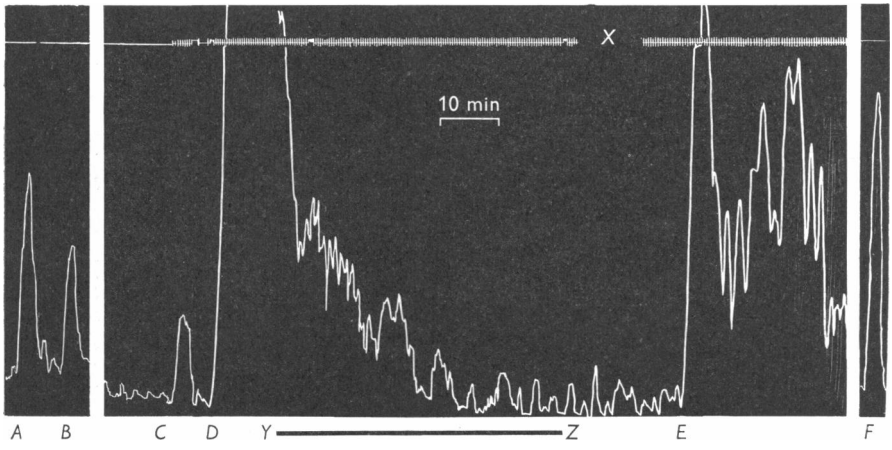

Fig. 10. Slight initial activity (*C*) in anterior chamber perfusate (de Jalon's solution) and subsequent greater output of activity following collapse (*D*) and stroking of the iris (*E*).

Beverens rabbit, ♀, 3·6 kg; blue irides; urethane; further details in text. Perfusate, connected at *C*, monitored by rat-colon preparation in atropine 10^{-6} g/l. and 1-methyl lysergic acid butanolamide tartrate 2×10^{-7} g/l.; 1·4 ml. bath, washed out at 1 min intervals during the 50 min indicated by the lower black line (*Y–Z*), otherwise every 2 min. Ether-purified standards at *A* and *F* (≡0·1 mg) and *B* (≡0·05 mg).

Top signal: drop-counter registering every 10th drop and showing cessation of flow at *D*, due to escape of perfusing needle; the counter was stopped for 10 min at *X*, during which time the flow rate did not change.

21 min before *E*. The outflow needle was unclamped 2·5 min before *E* and the temporal half of the iris was stroked during the next minute; the needle was then replaced in its clip. *E* shows the reappearance of activity after 1·5 min and its persistence for the next 25 min.

The mioses recorded in this experiment were only 1·5 mm after *D* and after *E*. This was noticed in some other experiments and may have been due either to the low calcium content of this perfusion solution or to delayed effects of urethane, of the kind mentioned by Dale & Laidlaw (1910). They observed the depressing effect of this anaesthetic upon smooth

muscles and state that it may even abolish the response to directly acting drugs such as muscarine.

At the end of this experiment the following temperature readings were taken with a thermocouple: centre of the cornea (outer surface), 29·5° C in the perfused and 32·5° C in the control eye; perfusate emerging from the outflow needle, 30·5–31° C; perfusate entering the drop-chamber, 24·5° C, which was only 0·5° C above room temperature on that day, showing that adequate cooling was achieved in the outflow circuit well before the arrival of the perfusate at the organ bath.

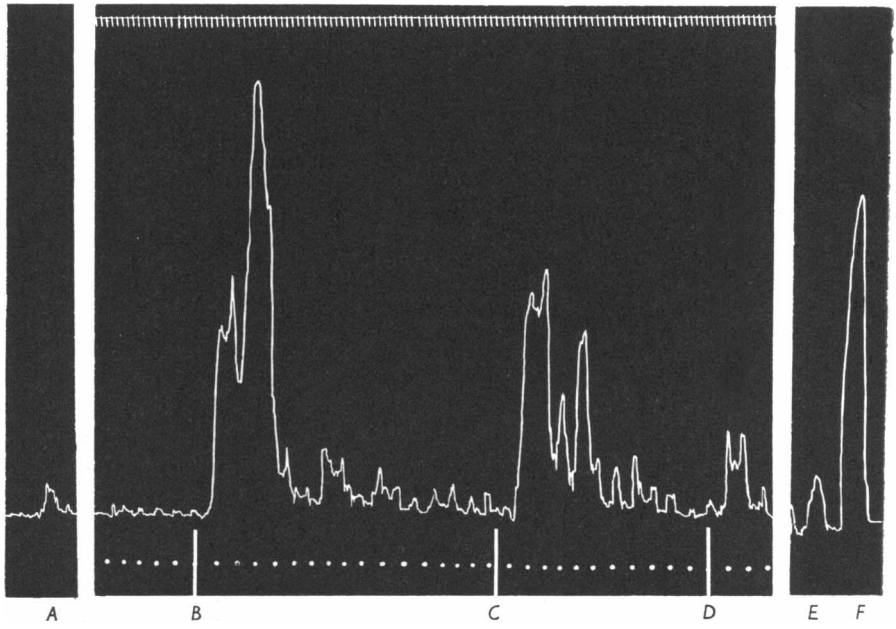

Fig. 11. Output of active substance following irritation of the iris at *B*, *C* and *D*. Atropinesterase-free 2·8 kg rabbit, ♂ Chinchilla; slate-grey iris; urethane 1·6 g/kg; atropine 1 mg/kg. Further details in text.

Anterior chamber perfusate (de Jalon's solution; drop-counter registers every tenth drop) monitored by rat-colon preparation in 1·4 ml. bath containing atropine 10^{-6} g/l. and 1-methyl lysergic acid butanolamide tartrate 3×10^{-7} g/l.; organ bath washed at 2 min intervals throughout (dots). Responses to ether-purified irin standards: *A* and *E*, ≡0·1 mg and *F*, ≡0·25 mg; 2 min contacts.

Another of these experiments is illustrated in Fig. 11. The rabbit was atropinesterase-free and received atropine sulphate 1 mg/kg and heparin 300 i.u./kg intravenously about 2 hr before *B*. The rat-colon preparation was less sensitive than in the preceding experiment, as shown by the responses (*A*, *E* and *F*) to the irin standard, which was the same in both experiments. When the anterior chamber perfusion was started, the I.O.P.

was set at 16·5 mm Hg by fixing the reservoir 28 cm above the level of the eye. When the outflow (0·22 ml./min) was connected 2 min later to the organ bath through the drop-chamber, the initial activity was barely detectable. Twenty minutes later the I.O.P. was lowered for 1 min by bringing

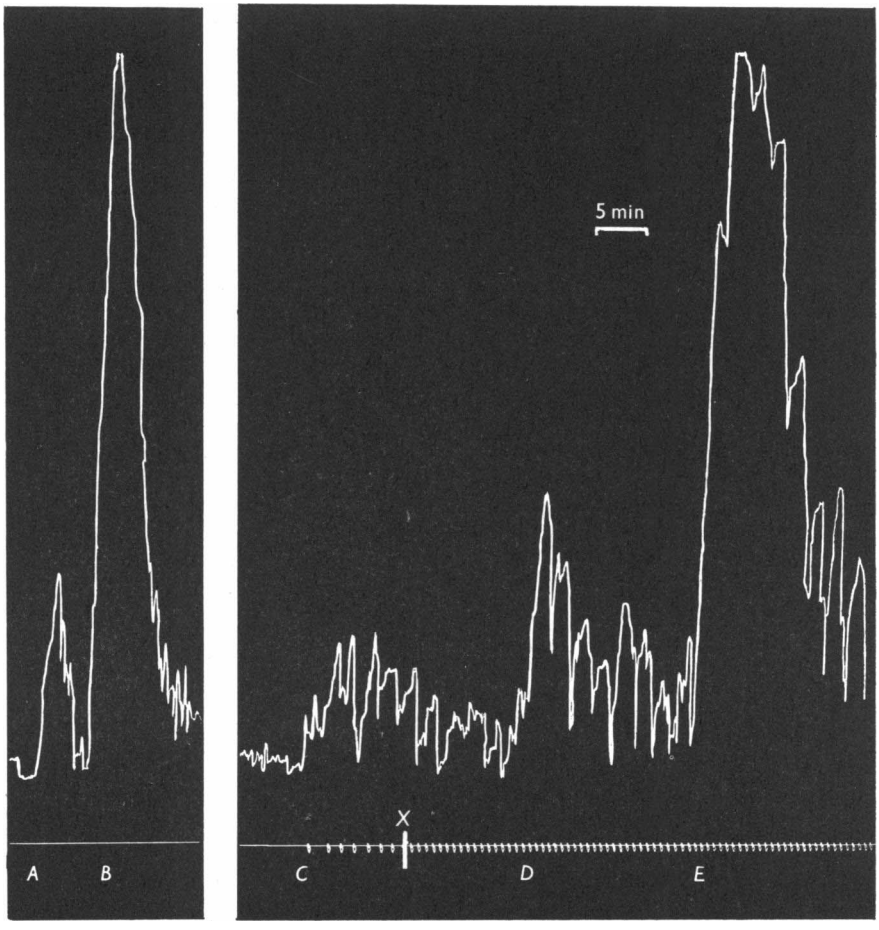

Fig. 12. Small initial activity (at *C*) of anterior chamber perfusate (de Jalon's solution) and subsequent outputs on stroking the iris (*D* and *E*).

Unatropinized rabbit of unknown atropinesterase content (4·1 kg, albino iris); urethane 1·8 g/kg; no heparin. Rat-colon preparation, 1·4 ml. bath; atropine 10^{-6} g/l. and 1-methyl lysergic acid butanolamide tartrate 3×10^{-7} g/l. At *A* and *B*, ether-purified irin standards ($\equiv$ 0·1 and $\equiv$ 0·25 mg, respectively); 2 min contacts. At *C*, outflow from anterior chamber connected to organ bath; counter registers every twelfth drop (below). Organ-bath fluid renewed every 2 min thenceforth. At *X*, perfusion rate increased by raising the reservoir. At *D*, iris stroked 8 times over a small area close to pupillary margin. At *E*, iris stroked 10 times between '7 and 10 o'clock'.

the perfusion reservoir down to bench level. This, however, did not empty the anterior chamber. In the absence of true collapse the low I.O.P. by itself did not seem to provide an adequate stimulus and the subsequent output of active substance was barely detectable. Likewise, the first time the iris was stroked gently (10 ×) 33 min later over a quarter of its anterior surface, there was a miosis of 1 mm but no detectable output of the rat-colon-contracting substance. The outflow was now disconnected for a few minutes in order to test the sensitivity of the rat-colon preparation (≡ 0·1 mg dose at *A*). After this, the outflow was reconnected to the drop-chamber 12 min before the iris was stroked for the second time at *B*, when the temporal half of the iris was stroked some 25 times, mostly on the anterior but partly on the posterior surface. This induced a miosis of 1·75 mm and the output of activity shown, which lasted for 8 min; the latency of this effect, corrected for the delay created by the 0·29 ml. dead-space, was 0·5 min. Subsequently, two smaller outputs with corrected latencies of 0·7 min were recorded when a quarter of the iris was stroked 5 times, on the front surface at *C* (miosis 1 mm) and on the back at *D* (no further miosis).

The variability of the outputs observed after stroking was noted not only in this experiment but in one or two others, for example, that illustrated in Fig. 12, though here the difference could have been due partly to the different siting of the two stimulations at *D* and *E*; this animal was not heparinized.

Davson & Matchett (1951) have reported that movement of the lens can raise the I.O.P. and induce miosis in rabbits' eyes. In one of the present experiments under Na pentobarbitone, miosis and an output of activity was recorded when a third, solid needle was shot deep into the lens during the perfusion. Two further outputs of activity were recorded later when movements of the lens were induced through the impaled needle, once antero-posteriorly and once sideways. Such movements are communicated to the ciliary body through the suspensory ligaments of the lens.

The fact that increases in the I.O.P. sometimes occur 'spontaneously' was noted on p. 387. In one of the present perfusion experiments initial activity appeared to be absent at the beginning of the perfusion, but 38 min later the iris, which was untouched throughout, contracted 'spontaneously' and rat-colon-contracting activity appeared 2·5 min later. There were, however, several unusual features in this experiment, e.g. (1) heparin 1 i.u./ml. was added to the perfusing solution and to the organ-bath fluid in their respective reservoirs, (2) the conjunctiva had been incised at both canthi, before the perfusion, and (3) a thread was needled through the upper eyelid 0·5 hr before the spontaneous effect occurred.

Experiments with 'eye solution'. A few experiments were carried out

with some other perfusing solutions. The alkalinity of an oxygenated 'mock aqueous humour' (pH 8·3 without CO_2) did not suit the rat-colon preparation. With oxygen alone, a modified phosphate-free Tyrode solution of the same magnesium content as the aqueous humour and pH 8·26 was more satisfactory; in a perfusion of the anterior chamber, outputs of

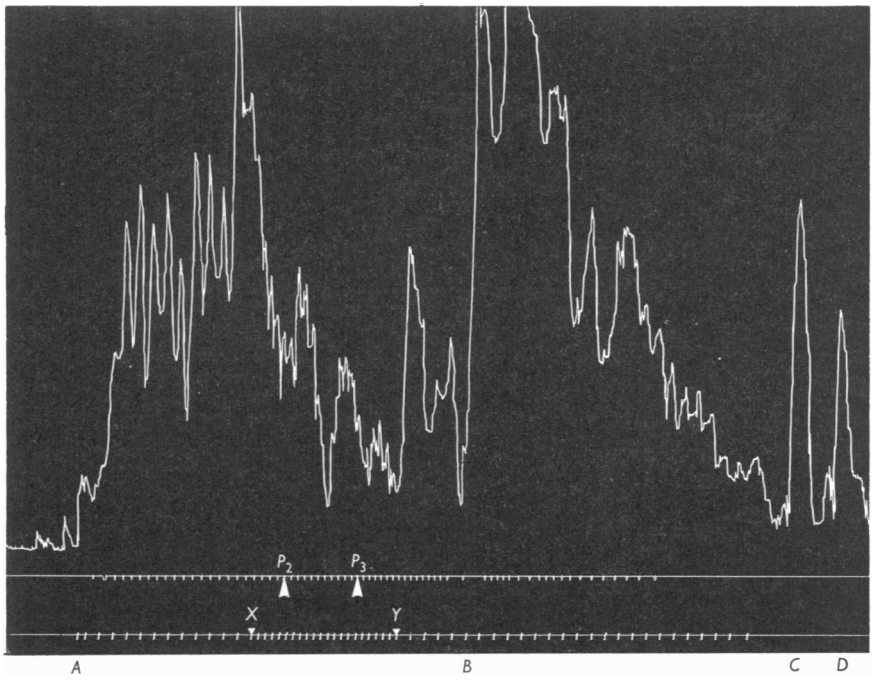

Fig. 13. Perfusion of rabbit anterior chamber with an 'eye solution' (Table 1) bubbled with 95 % O_2–5 % CO_2, showing considerable initial activity (*A*) and effect of collapse (*B*). Unatropinized rabbit (♀, 3·2 kg; albino iris); urethane 1·8 g/kg; heparin 936 i.u./kg.

Effluent monitored by rat-colon preparation suspended in the same solution, 1·4 ml. bath; atropine 10^{-6} g/l. and 1-methyl lysergic acid butanolamide tartrate 4×10^{-7} g/l. At *C* and *D*, ≡0·1 and ≡0·05 mg ether-purified irin standard; 2 min contacts.

Upper signal: counter registers every twelfth drop of effluent, connected at *A*. At P_2 and P_3, reservoir raised to increase the perfusion rate. Note cessation of flow during collapse at *B*, due to escape of inflow needle, replaced 1·5 min later.

Lower signal: organ-bath washes during perfusion, at 2 min intervals except between *X* and *Y* (1 min).

active substance were obtained with this solution, as previously, upon various forms of stimulation.

In further experiments the 'eye solution', of which the composition is given in Table 1, was used buffered with CO_2. In this solution the Na and

HCO_3 contents are those normally present in rabbit plasma whilst the K, Ca, Mg, and glucose are as in the aqueous humour. When equilibrated with a physiological P_{CO_2} by bubbling with $95\%O_2 + 5\%CO_2$ this solution (pH 7·6) provided good assay conditions; in it rhythmic activity of the rat colons was low or absent, their sensitivity to ether-purified irin was satisfactory, and bradykinin was inactive or inhibitory. When the anterior chamber was perfused with this 'eye solution', outputs of active substance into the perfusates were also detected either initially or after stroking the iris or collapse of the anterior chamber, and once, after the iris had contracted 'spontaneously', i.e. in response to some undefined stimulus, the final diameters of the constricted pupils were 2–4 mm.

An experiment in which the initial activity was particularly marked is illustrated in Fig. 13. The perfusion began at a rate of 0·15 ml./min, 8 min after both needles had been shot into the anterior chamber. The great initial activity (after *A*) was not due to iris contact.

DISCUSSION

The use of de Jalon's solution for perfusing the anterior chamber in rabbits may have the advantage of reducing somewhat the natural irritability of the eye in that species. However, even with this low-calcium solution, as later with the 'eye solution', outputs of an active substance, resembling the unsaturated hydroxy-acid(s) more abundantly present in the iris itself, have been recorded after various forms of stimulation which are known to induce the phenomena characteristic of irritation in rabbits' eyes, namely miosis, vasodilatation and increased intraocular pressure.

The fact that the irritation reaction in the rabbit iris involves a contraction of the cholinergically innervated sphincter pupillae muscle focused attention on the possibility that this was due to a release of acetylcholine. This seems unlikely since the miosis still occurs after effective atropinization of the iris, i.e. in rabbits known to be atropinesterase-free. Nevertheless, there are instances of smooth muscles at which neurohumoral transmission is in all probability cholinergic and yet cannot be blocked by atropine. However, the rabbit sphincter pupillae would not appear to belong to this category since atropine is capable of abolishing the light reflex and produces mydriases lasting for 4–5 hr; thus there is good evidence that the action of endogenous acetylcholine on this muscle is completely blocked by atropine. Another reason for excluding the intervention of acetylcholine is the prolonged nature of the miosis; recovery from oculomotor stimulation is rapid, no doubt because of the presence of cholinesterase.

As regards the vasodilatation and raised intraocular pressure, after excluding acetylcholine with atropine and histamine with mepyramine, another substance which has to be considered is bradykinin, since it is an atropine-resistant vasodilator. The experiments with the bradykinin-insensitive rat-colon preparations show that in fact a different substance is released in the eye when these phenomena occur. The use of the rat-colon preparation in the presence of acetylcholine- and 5-hydroxy-tryptamine-antagonists eliminates several other substances besides bradykinin, such as histamine, arachidonic acid (Jaques, 1959; probably hydroperoxidized, Dakhil & Vogt, 1962) and, as shown recently by Cleugh, Gaddum, Mitchell, Smith & Whittaker (1964), purified substance P. Bisset & Lewis (1962) reported the insensitivity of this preparation to other polypeptides, including oxytocin, vasopressin and angiotensin. But under different experimental conditions Regoli & Vane (1964) have found that a synthetic α-hypertensin, α-L-Asp^1. Val^5 Hypertensin II (Ciba), was highly active on this muscle; they have kindly supplied us with some of their material, which did contract our rat-colon preparations and was even more active on *Meriones* colon preparations (0·1 ng/ml.). Until a specific antagonist is found to angiotensin, we cannot, strictly speaking, be certain of its absence from the eye perfusates. However, angiotensin is formed by a renal enzyme and, being a vasoconstrictor, is unlikely to play a part in these iris phenomena in which vasodilatation is so marked; it is known not to affect capillary permeability in the rabbit (Bisset & Lewis, 1962), whereas after irritation capillary permeability is increased in rabbits' eyes.

It is much more likely that the activity in the perfusates is due to the same substance(s) as found previously in much larger amounts in aqueous extracts of the rabbit iris; preliminary experiments suggest that the activity is extractable from the perfusates into ether at pH 3, thus distinguishing it from polypeptides, which are all insoluble in ether. The ether-soluble unsaturated hydroxy-acid irin, which is present in aqueous extracts of rabbit iris, has been purified by chromatography on silica gel and this material was shown to produce contractions of various smooth muscles and prolonged erythema on subcutaneous injection into the human skin (Ambache, 1961). Since the rat and *Meriones* colon preparations are both so sensitive to this acid, it is reasonable to suppose that the activity detected by these muscles in the perfusion experiments was due to irin itself. Its miotic and vasodilator properties would account for most of the irritation phenomena occurring in rabbit eyes; irin may also contribute to the production of pain, since Keele & Armstrong (1964, p. 83) have found that the chromatographically purified material elicits pain in the human skin when applied to a blister base.

In the unperfused eyes traces of the same substance(s) were found in

the plasmoid aqueous humour after paracentesis. The fact that only 1–2 %, or less, of the activity which it is possible to extract by grinding the whole iris in water appears in the plasmoid aqueous humour may be due to a number of factors, such as the presence of protein in the aqueous humour and in the tissues of the iris; as shown previously (Ambache, 1959, Fig. 13) irin, like some other fatty acids, is bound by albumin. Alternatively, it may be due to prolonged binding by receptors, which would be consistent with the great duration of the irritation phenomena. What is detected, then, in the aqueous humour, and in the perfusates, might merely be the excess left over after all these losses have been deducted. Lastly, the smallness of the output could be due, in part, to a poor diffusibility of the fatty acid(s) through the tissue of the iris, and to its retention within the iris (with prolongation of effects) because of a relative impermeability of its epithelium. The ultrastructure of the albino iris, as seen under the electron microscope, would support such a view: the epithelium is two cells thick on both the anterior and posterior surfaces of the iris, and the abundance of desmosomes suggests that these covering cells are tightly bound (G. Causey, 1964, personal communication).

Further work will show whether similar chemical phenomena occur in other tissues on irritation or injury. In the iris this type of substance would seem to be released only in abnormal circumstances and then perhaps extravascularly, i.e. in the tissue spaces, which are normally relatively free from the binding plasma proteins, and where (beyond the albumin barrier, so to speak) irin would be free to exert its action before it is swamped by proteins.

SUMMARY

1. The miosis and rise in intraocular pressure which occur in rabbits' eyes after various forms of mechanical stimulation, such as irritation of the iris or collapse of the anterior chamber, were not prevented by atropine in animals known to be atropinesterase-free; they also occurred after mepyramine.

2. After paracentesis an active substance, which contracted the rat- and *Meriones*-colon preparations, appeared in samples of plasmoid aqueous humour.

3. A method is described for perfusing the anterior chamber at a fixed intraocular pressure, without initial loss of pressure.

4. In experiments in which the anterior chamber was perfused with a de Jalon's solution, the appearance in the effluent of small amounts of an active substance which contracted *Meriones*- and rat-colon preparations could be induced by the following mechanical stimuli: collapse of the anterior chamber, stroking of the iris, or movement of the lens.

5. Similar results were obtained in experiments in which the anterior chamber was perfused with an 'eye solution' (Table 1), which proved satisfactory for rat-colon preparations suspended in the same solution.

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