Biochemical Pharmacology, 1963, Vol. 12, pp. 421–428. Pergamon Press Ltd., Printed in Great Britain.

IRIN AND A HYDROXY-ACID FROM BRAIN

N. AMBACHE

Medical Research Council, Royal College of Surgeons, London

(*Received* 21 *December* 1962)

Abstract—Intraocular injections of irin mimic the prolonged atropine-resistant miosis elicited by "antidromic" mechanical stimulation of the trigeminal nerve in rabbits. Procedures for extraction, assay and purification of irin are described, and the evidence that it is a lipid-soluble unsaturated hydroxy-acid. A similar substance is present in aqueous extracts of blood-free rabbit brain tissue. Both acids have been further purified by chromatography on silica. Structure–action studies on hydroxy-acids suggest that their spasmogenic activity on certain muscles depends upon free —OH and —COOH groups, and is enhanced by unsaturation.

A. IRIN

THE search for a long-acting atropine-resistant active substance in the rabbit's iris was prompted by the repeated observation of a phenomenon first described in 1824 by Magendie[1] and studied by Claude Bernard[2] in 1858 and by some sixty authors since; namely, that mechanical or other stimulation of the trigeminal nerve in the middle cranial fossa produces, it is supposed "antidromically", a miosis beginning within 1–2 min and which may last for 1 hr or more. It is now known that this miosis is frequently associated with a rise in intraocular pressure, in other words with vasodilatation in the eye. This response can be elicited from any part of the trigeminal nerve, both outside and inside the orbit and even within the eyeball, and so is seen on injury of the eyeball, or of the iris itself; for instance, after paracentesis (collapse of the anterior chamber), which causes the lens to come forward and hit the iris.

So far as we know, this trigeminal effect does not occur in any other laboratory species, though local injury produces iris spasm which is also a feature of human iritis. Last year several extracts were made in this laboratory (N. Ambache and M. Reynolds, unpublished data) of human irides and of some ciliary bodies obtained from autopsy material and from enucleated eyes; these extracts contained a substance very similar to rabbit irin, about which most is known and to which the first part of this communication is confined.

The phenomenon of antidromic miosis is illustrated in Fig. 1. For these experiments rabbits were chosen which were known to be completely free of the enzyme atropinesterase in their serum (Ambache[3], pp. 472–474). The selection of these rabbits was carried out on the basis of previous manometric determinations of the enzyme level by incubation of their serum with atropine sulphate and $NaHCO_3$ in a Warburg vessel (courtesy of Dr. F. Hobbiger). For the actual experiments of this series, the rabbits were then heavily dosed with atropine sulphate 3–12 mg/kg. The experiment shown in Fig. 1 was carried out in two stages 2 days apart. On the first day the animal was anaesthetized with intravenous nembutal and was injected intravenously both with the atropine and with 500–1000 i.u. of heparin. It then received an intraocular injection into the anterior chamber of each eye. These injections were made

through long self-sealing valvular tracks in the cornea, one of which is visible in Fig. 1A. Each eye received 0·1 ml of de Jalon's solution plus a tiny bubble of air, the purpose of which is to give warning of the onset of clotting in the aqueous humour; when the head is tilted, the bubble remains free-moving in the anterior chamber unless clotting has occurred, in which case the animal is discarded. The left eye (A) served as a control, whilst the right eye (B) received the same volume of de Jalon's solution containing acetone-purified rabbit irin in a dose of ≡35 mg of iris tissue. The two injections were made within 1 min of each other; B was photographed 7·5 and A 9·5 min after the injections. Two days were allowed for the animal to recover from this first experiment. It was then re-anaesthetized with nembutal and decerebrated. After waiting for about 1 hr to allow for recovery from the decerebration, and administering the same dose of atropine as before, the two photographs C and D were taken of the right eye, C 2 min before, and D 2 min after, gentle stroking of the trigeminal nerve in the middle cranial fossa. This figure, then, illustrates the "antidromic" pupillary constriction in a fully atropinized atropinesterase-free rabbit and the mimicry of this effect by an intra-cameral injection of irin.

Figure 2 shows the long duration of the irin-induced miosis in a series of five experiments, all on atropinesterase-free rabbits heavily dosed with atropine. The effects lasted

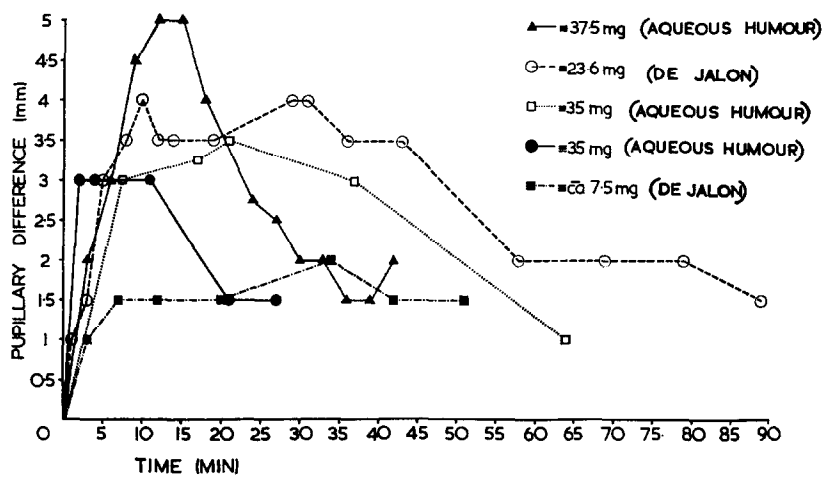

FIG. 2. Prolonged pupillary constriction after sealed intracameral injections of acetone-purified irin dissolved either in pooled aqueous humour or in de Jalon's solution. Ordinate, difference in horizontal pupillary diameter (mm) between the eye injected with these irin solutions and the control eye injected with an equal volume of solvent (aqueous humour or de Jalon's solution) alone. Irin doses as shown. Abscissa, minutes after injection.

30–90 min. It is necessary to comment on the large doses of irin which had to be used in these experiments. Whereas some muscles such as the rat colon are able to detect as little as ≡0·5 mg of iris tissue in a 5 ml bath, the rabbit iris bathed in ca. 0·2 ml of aqueous humour requires ≡10–35 mg, i.e. virtually the whole of the activity extractable from a single iris, to produce a full miosis. Possibly this is due to mopping up of most of the injected irin by the proteins in the aqueous humour of this species. Rabbits' eyes are exceptional in that their aqueous humour contains 0·02 per cent or more of albumin. Irin is bound by some albumins (Ambache[4], Fig. 13).

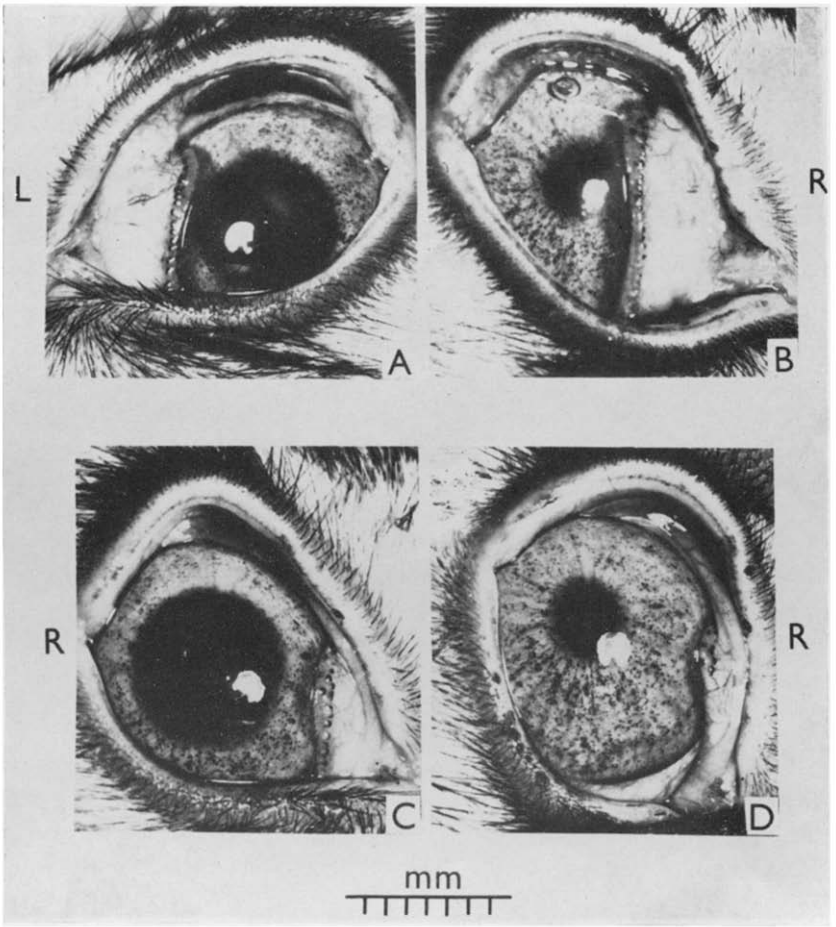

FIG. 1. Atropinesterase-free rabbit; pentobarbital sodium 38 mg/kg (supplemented later by 14 mg/kg), atropine sulphate 12 mg/kg and 340 i.u./kg heparin, all i.v. B, miotic effect of irin; A, control; C and D, 2 days later, after decerebration under nembutal and further atropine. Antidromic trigeminal constriction shown at D. Details in text.

facing page 422

Method of extraction

The irides, both pigmented and albino, are pulled out through a slit in the cornea. This, of course, sets up the injury reaction within them. They are dried between Whatman no. 1 filter paper, weighed (30–50 mg), and then ground in distilled water of pH 7·6–8·2, using 1 ml of water per 100 mg of tissue. This method avoids the use of strong acids or alkalis and of heat. Many extracts have also been made of irides which were blood-free, i.e. taken after the heads had been perfused with 0·5–1 l. of Locke's solution. These were all as active as usual. Hence the activity is not due to a substance derived from the blood normally trapped in the tissue, nor is it produced by possible interactions of blood with tissues. The extracts are then centrifuged to remove all cell debris and a clear supernatant solution is obtained which contracts various smooth muscles, including the irides of three species. The activity in this solution is most conveniently assayed either on the rat colon preparation suspended in de Jalon's solution containing atropine and lysergic acid diethylamide, or, more conveniently, on atropinized preparations of the diagonally banded ascending colon of the golden hamster (*Mesocricetus auratus*) or of its mutants as now available commercially. This preparation has far less rhythmic activity and is insensitive to histamine and sometimes also to 5-hydroxytryptamine. These is no tachyphylaxis with irin on either preparation.

Figure 3 illustrates the effect of ≡1–5 mg of irin on an atropinized hamster colon preparation, and also the assay of an extract purified by partition with ether at pH 2·81.

The activity present in these extracts was easily distinguished from all known bases, either by means of antagonists or by incubation with various enzymes, e.g. chymotrypsin, as fully described elsewhere.[4, 5] The activity was then shown to be due to an acid, both by electrophoresis on paper, in which irin migrated towards the anode, and by partition either with chloroform or with peroxide-free ether, when 60–90 per cent of the activity was recovered from the organic phase at pH near 3, and little or none at pH 7–8. In some recent partitions with ether in the presence of ascorbic acid 70 per cent of the activity was recovered at pH 3.

Purification

The following methods were used:

(1) The addition of 20 vols. of acetone. A copious precipitate, consisting probably of proteins and polypeptides, is removed by centrifugation. This is inactive. The clear supernatant solution is evaporated to an active residue, and the activity is redissolved in undiluted acetone, and evaporated to a second active residue.

(2) Partition with peroxide-free ether; this is a more rapid method of purification. One volume of extract is shaken with 1 volume of ether after the addition of HCl to pH 3. It is as well, at this point, to bear in mind the possibility that, as soon as HCl is added, a change might occur in the molecule of the substance under investigation. Thus, acidification is known to produce lactonization in hydroxy-acids and to split plasmalogenic acids at their acetal (or vinyl ether) group.

(3) Combination of these two methods with chromatography on silica.

Figure 4 shows the use of the hamster preparation for detecting the activity of various fractions obtained after chromatography of irin on a silica column. The reconstituted peak Fraction 11 obtained in this experiment was used to study the vasodilator action of irin; 0·15–0·2 ml injected subcutaneously in human subjects produced an erythema lasting for 1–2 hr.[6]

Chemical nature of irin

We have seen that irin is a lipid acid. That it is unsaturated is shown by the reduction of activity upon hydrogenation and by its destruction by iodine monobromide. This halogen is known to attack double bonds unless they are in the $\alpha\beta$ position, i.e. right next to the carboxyl group. Further evidence that irin is a hydroxy-acid was obtained as follows:

(1) The red colour test with sym-diphenylcarbazide. This coloration is given also by prostaglandin, the menstrual component "A", and by a whole series of unsaturated lactones examined in this laboratory.

(2) Inactivation by phenyl isocyanate, which is a hydroxyl-binding reagent.

(3) Inactivation by N,N'-carbo-di-*p*-tolyl-imide, a carboxyl-binding reagent.

(4) The presence in infra-red spectra of absorption bands at 1715 cm^{-1} and at 3300 cm^{-1}, characteristic of carboxyl and hydroxyl groups respectively.

(5) As shown in Fig. 5, irin which had been purified by partition into ether at pH 2·98 was exceptional in that the usual OH band at 3300 cm^{-1} had vanished and the carbonyl band had shifted to 1740 cm^{-1}, as in δ-dodecalactone.

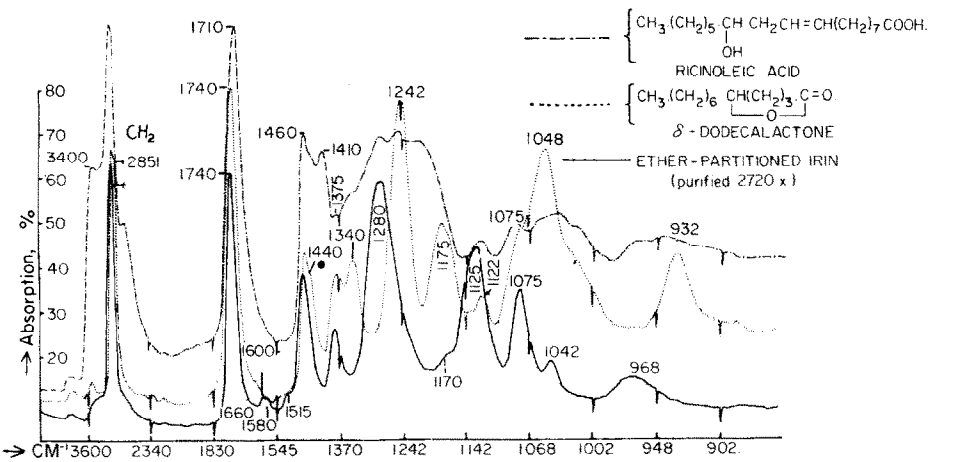

FIG. 5. Infra-red absorption spectra of ether-purified irin compared with (+) ricinoleic acid and δ-dodecalactone. See text.

Before leaving the subject of irin reference should be made to the observation that traces of an irin-like substance appear in the aqueous humour of rabbits after paracentesis. It has been mentioned above that, when the anterior chamber of a rabbit is collapsed, the lens comes forward and irritates the iris with the production of the prolonged miosis. Samples of the reformed, but now plasmoid, aqueous humour taken 15 min later produced contractions of the rat colon preparation previously rendered insensitive to acetylcholine by atropine and to 5-hydroxytryptamine by lysergic acid diethylamide. The amounts detected were small, probably again because of binding by the protein in the anterior chamber (as mentioned above), with which the plasmoid aqueous humour is even more heavily loaded than the normal.

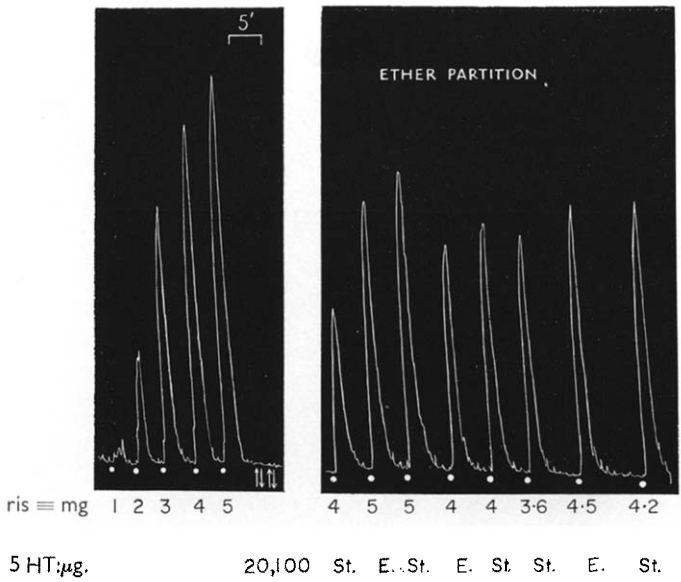

FIG. 3. Preparation of diagonally banded ascending colon of the golden hamster suspended in 5 ml of de Jalon's solution; atropine 10^{-7}.

First panel: Effect of centrifuged aqueous extract of rabbit iris in doses of 0·01–0·05 ml (≡1 to ≡5 mg of iris); 1 min contacts. No response to 5-hydroxytryptamine 20–100 μg.

Second panel: Assay of irin purified by partition with 1 vol. ether at pH 2·81. Ether phase collected, evaporated to residue, and reconstituted in de Jalon's solution (≡ 100 mg/ml). E = reconstituted ether phase; St = sample of the original unpartitioned extract.

facing page 424

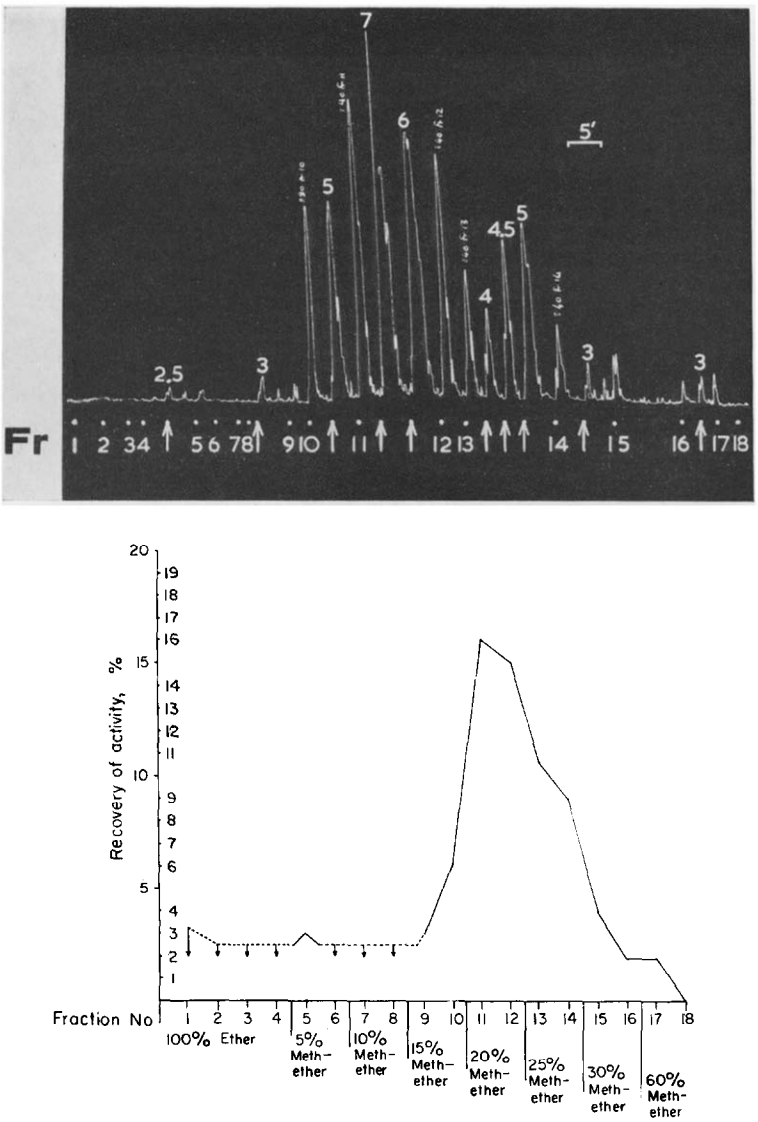

FIG. 4. Column chromatography, on silica, of irin residue obtained by acetone purification followed by partition with ether at pH near 3.

Upper panel: hamster colon; 5 ml bath, 10^{-7} atropine. At the arrows, responses to ≒ 2·5–7 mg of unchromatographed irin standard (individual doses shown above the respective responses) interspersed between the eighteen chromatographic fractions at the dots, as numbered below. The column was successively eluted with 100% ether (fr. 1–4), then 5% methanol–ether (fr. 5–6), 10% methanol–ether (fr. 7–8), 15% methanol–ether (fr. 9–10), 20% methanol–ether (fr. 11–12) 25% methanol–ether (fr. 13–14), 30% methanol–ether (fr. 15–16) and 60% methanol–ether (fr. 17–18); all fraction volumes 3 ml except fr. 1–4, which were 1·5 ml. After evaporation, all fractions were reconstituted in de Jalon's solution to ≡ 200 mg tissue/ml and were tested in doses of 0·4 ml (fr. 2–10 and 15) or 0·8 ml (fr. 16–18), except for the most active fractions, which were tested in doses of 0·2 ml. (fr. 11–14) and for fr. 1 (0·25 ml). Most of the activity appeared in fr. 10–15.

Lower panel: graph showing percentage recovery in each fraction. ↓ = <.

B. AN ACTIVE UNSATURATED HYDROXY-ACID FROM RABBIT CEREBRAL HEMISPHERES

Aqueous extracts have been made of the cerebral hemispheres, or sometimes of the whole brain, taken from rabbits after perfusion of the heads with 0·5–1 l. of Locke's solution until the venous return from the head showed no traces of blood. The blood-free brain tissue was ground in distilled water of pH 7·7–8·2, using 1 ml/100 mg of tissue. The centrifuged, clear supernatant solution from these extracts produced contractions of the atropinized hamster colon, but was 10–20 times less active per ≡mg of tissue than corresponding iris extracts.

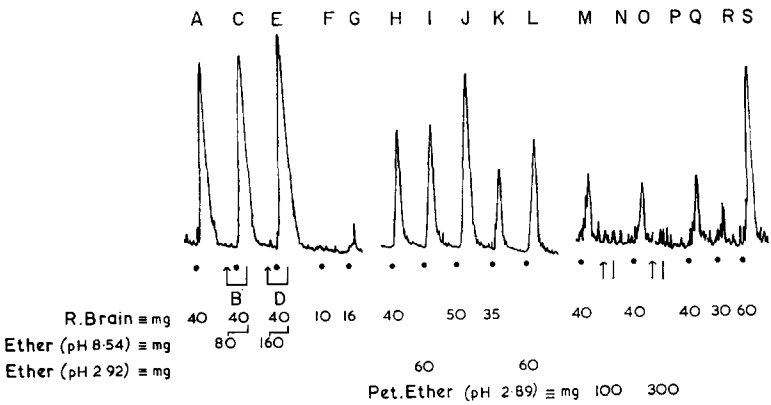

FIG. 6. Purification of an aqueous cerebral hemisphere extract by partition with diethyl ether at acid pH. Hamster colon, 5 ml bath, 10^{-7} atropine; 1 min contacts. Responses to unpurified extract at A, C, E and H, ≡40 mg tissue; at F, ≡10; G, ≡16; J, ≡50; K, ≡35 mg. At the arrows, no response to ≡80 mg at B nor to ≡160 mg at D, of reconstituted ether phase from partition at pH 8·54. After partition with 1 vol. ether at pH 2·92 the reconstituted ether phase is active in doses of ≡60 mg at I and L, matching ≡40 mg of unpurified extract at H, and bracketed by ≡50 mg of it at J and ≡35 mg at K. Between G–H, interval of $1\frac{3}{4}$ hr during which sensitivity of preparation altered.

M–S, from another preparation. Partition of the same extract at pH 2·89 with petroleum ether (b.p. 40–60°C) fails to extract the activity. No response to ≡100 and ≡300 mg of reconstituted petroleum ether phase at arrows N and P. Unpartitioned extract ≡40 mg at M, O, Q; ≡30 mg at R and ≡60 mg at S.

The active material could be purified through acetone with from 50 to >66 per cent recovery. It was, however, most frequently purified by partition with peroxide-free ether at pH near 3. This is illustrated in Fig. 6, where the activity of ≡40 mg of the initial extract is shown at A, C and E. When a sample of the extract was adjusted to pH 8·54 and partitioned with 1 vol. ether, the reconstituted ether phase was inactive in doses of ≡80 mg at B and ≡160 mg at D, though not preventing the response to the control standard doses co-administered 1 min later at C and E. Since the preparation could detect ≡16 mg at G, <10 per cent entered the ether phase in this alkaline partition. When, however, another sample of the same extract was adjusted to pH 2·92 and partitioned with 1 vol. ether, the reconstituted ether phase was active in ≡60 mg doses, as shown by the contractions at I and L, which matched ≡40 mg of the original extract at H and were bracketed by ≡50 mg and ≡35 mg at J and K, respectively. Thus at pH 2·92 some 66 per cent of the activity had entered the ether phase.

When, however, the same extract was partitioned with 1 vol. petroleum ether (b.p. 40–60°C) at pH 2·89, the reconstituted organic phase was inactive in doses of ≡100–300 mg (M–S).

Like irin, this active material was destroyed by treatment with phenyl isocyanate (Fig. 7), by N,N′-carbo-di-*p*-tolyl-imide, and by iodine monobromide. The active substance, therefore, appears to be an unsaturated hydroxy-acid.

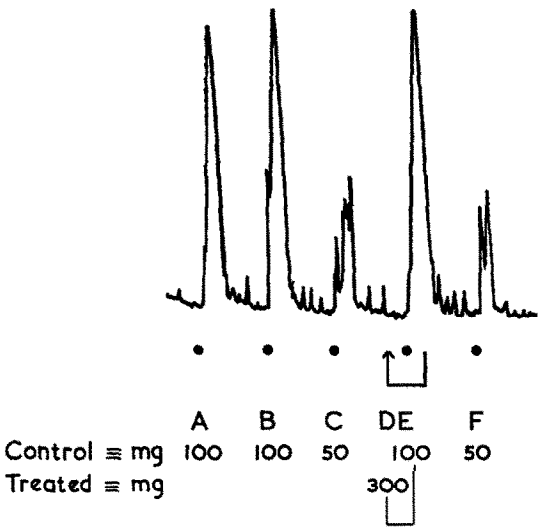

FIG. 7. Destruction of activity by phenyl isocyanate. Atropinized hamster colon. Responses to control untreated cerebral hemisphere extract shown at A and B (≡100 mg tissue) and at C and F (≡50 mg). At the arrow D ≡300 mg of the same extract, after treatment with phenyl isocyanate, is quite inactive but does not prevent the response E to ≡100 mg of control untreated extract, co-administered 1 min later at the dot.

The residues obtained by partitions with ether at pH 3 were dried in a high vacuum, re-extracted with a small volume of peroxide-free ether, and applied to silica columns for chromatography. As shown in Fig. 8, elution was carried out in the following sequence: the first five fractions with 100% ether; then five with 5% methanol–ether; six with 10% methanol–ether; five with 15% methanol–ether; four with 20% methanol–ether; four with 25% methanol–ether; four with 30% methanol–ether; and, lastly, two with 100% methanol.

Figure 8 shows the comparison between chromatograms of the brain substance, of irin purified by partition into ether at pH ca. 3, and of (+)-ricinoleic acid, all eluted under identical conditions in different parallel experiments. Whereas the ricinoleic acid appeared mainly in fractions 2 and 7 (100% ether and 5% methanol–ether), irin appeared in fractions 18–33, i.e. 15–30% methanol–ether. With the brain acid, in six out of eight experiments the peak of activity appeared in the 10% methanol–ether fractions, as typified by fraction 14 in the experiment illustrated by graph C. In the other two experiments (one with ether-purified, and the other with acetone-purified, irin) the peak appeared in the 15% methanol–ether fractions, as shown in graph A.

The activity of these brain extracts was not due to the following two known acidic constituents of brain: α-hydroxy-nervonic and N-acetyl neuraminic acids, which were both inactive on the hamster colon preparation. Likewise, *p*- and *o*-hydroxyphenylacetic acids and homogentisic (2:5-dihydroxyphenylacetic) lactone (all related to homovanillic acid) were also inactive. Lastly, a sample of chromatographically

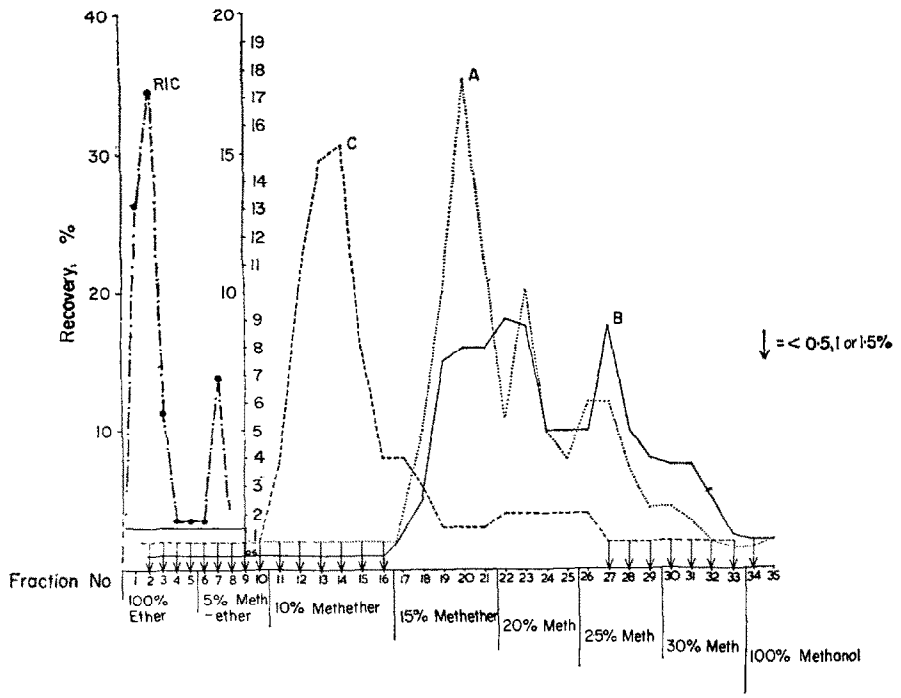

FIG. 8. Chromatography on silica columns of the active substance from rabbit cerebral hemispheres, purified either by partition with ether (A) or by acetone-treatment (C). At B, a chromatogram of ether-purified irin is shown for comparison, and at Ric., the active region of a chromatogram of (+)-ricinoleic acid. Details in text. All fractions were assayed on hamster colon preparations. The scale on the left applies to ricinoleic acid and the other scale to A, B and C.

purified rat brain sulphatide was also inactive. This is of interest because the sulphatide molecule satisfies some of the structural requirements for this type of activity, discussed below; thus, it has a hydroxyl group, a double bond and an acidic group, which, however, is not a carboxyl but a sulphate.

C. STRUCTURE-ACTION STUDIES ON UNSATURATED HYDROXY-ACIDS

A survey of available hydroxy-acids and lactones has shown that (+)-ricinoleic acid, its *trans*-isomer (+)-ricinelaidic acid, and their acetylenic analogue (±)-ricinstearolic acid, were the three most active compounds in tests upon the hamster or rat colon, though all three were considerably weaker than irin. If we consider (+)-ricinoleic acid as the prototype of substances possessing this kind of activity on certain smooth muscles, among which the hamster and the rat colon preparations appear to be the most sensitive (the guinea-pig ileum was insensitive to ricinoleic acid), then

Fig. 9 illustrates the structure-dependence of this type of activity. Since Na oleate and methyl ricinoleate were inactive, it would seem that free hydroxyl and carboxyl groups are essential. Other data suggest that unsaturation enhances the activity and, lastly, since *iso*-ricinoleic was five times weaker than ricinoleic, the spatial arrangement of these three groups within the molecule is important.

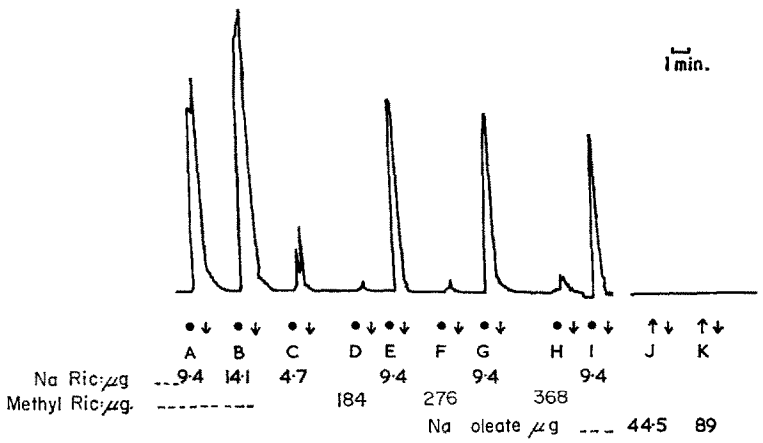

FIG. 9. Dependence of activity upon free —COOH and —OH groups. Atropinized hamster colon, 5 ml bath. Graded contractions produced by Na ricinoleate shown at A (9·4 μg), B (14·1 μg), C (4·7 μg) and at E, G and I (all 9·4 μg). There is virtually no response to emulsified methyl ricinoleate at D (184 μg), F (276 μg) and H (368 μg). Na oleate is also inactive at J (44·5 μg) and K (89 μg), from another preparation.

REFERENCES

1. M. MAGENDIE, *J. Physiol. expér.* **4**, 176 (1824).
2. C. BERNARD, *Leçons sur la Physiologie et la Pathologie du Systéme nerveux* Vol. 2, p. 205. J.-B. Bailliere, Paris (1858).
3. N. AMBACHE, *Pharmacol. Rev.* **7**, 467 (1955).
4. N. AMBACHE, *J. Physiol.* **146**, 255 (1959).
5. N. AMBACHE, *J. Physiol.* **135**, 114 (1957).
6. N. AMBACHE, *J. Physiol.* **160**, 3P (1961).