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FURTHER STUDIES ON THE PREPARATION, PURIFICATION AND NATURE OF IRIN

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Distilled water extracts of rabbit iris contain an acid which is physiologically active on certain smooth muscles (Ambache, 1957*a*). Improvements in the purification and assay of this substance are now described and also an investigation of its chemical nature. First, a number of acids of biological origin have been screened for activity. Although representative of a variety of structure-types, the acids examined are nearly all inactive or can be otherwise distinguished from irin. Direct evidence is then produced that irin is a long-chain unsaturated hydroxy-fatty acid. Further structure–action studies on available hydroxy-acids and lactones have shown that (+)-ricinoleic acid is a powerful stimulant of the smooth muscle in the hamster colon, whereas oleic acid, which lacks the hydroxyl group of ricinoleic but is otherwise identical with it, is relatively inert. These and other findings suggest that an OH group, a double bond, and the spatial relation between them, determine the irin-like activity of fatty acids.

METHODS

Irin. The source of active material has been the rabbit's iris throughout; although ox irides are larger and would have been more plentiful they contain less than $\frac{1}{10}$–$\frac{1}{20}$ the activity found in rabbits' irides. The procedure for making iris extracts described in the previous paper was modified slightly as follows: (i) unless otherwise stated all extracts were made in distilled water (1 ml./100 mg tissue) neutralized when necessary with 0·02% $NaHCO_3$; (ii) as the pharmacological activity of extracts is reduced by boiling, the heating was omitted—instead, the centrifuged extracts were left for some days at −15° C, which precipitates out further impurities removable by a second or third centrifugation; (iii) the irides were excised more rapidly, by direct removal through a slit in the cornea without prior enucleation of the eyes. I am indebted to Dr B. C. Whaler for four extracts of several hundred irides prepared in this way at Porton. For these, the dried irides were ground, unweighed, in 0·4 ml. of distilled water per iris, i.e. assuming an average weight of 40 mg per iris; the dosage of these extracts is expressed in arbitrary units of '≡ mg'; (iv) some extracts were prepared from irides excised a few hours after death. Through the courtesy of Dr M. Sterne the severed heads from rabbits killed at the Wellcome Research Laboratories in the morning were sent here, where the eyes were excised and the irides were extracted in the early afternoon. Such extracts were rather less active than those made from fresh irides.

Extracted iris tissue has yielded a further supply of irin when homogenized in 1 ml. of fluid per 10 irides ('≡ 400 mg/ml.').

Further purification of crude extracts was achieved in one of two ways. In the older acetone process (Ambache, 1957*a*) the irin-containing acetone supernatant was evaporated to a small volume in a current of cold air, dispensed into ampoules, and vacuum-dried in a $CaCl_2$ desiccator. Large volumes of extracts have been more conveniently processed to a residue in a rotary film evaporator (Quickfit and Quartz model 1 LVE) evacuated by a ballast pump. This first ('Type 1') residue was re-extracted with absolute acetone (1 ml./≡ 100 mg) leaving behind much visible impurity and, in particular, a material which had stained violet with fuchsin-sulphurous acid + $HgCl_2$. This impurity appears to be insoluble in absolute acetone, but is soluble in CCl_4, an alternative solvent which was therefore abandoned. The acetone solution was evaporated to give 'Type 2' residue.

Purification by partition with ether. The above procedure may take 1–2 days. It has now been superseded by the quicker method of extracting the irin from crude extracts into ether, by partition at acid pH (Ambache, 1957*b*). In this procedure equal volumes (usually 0·5 or 1 ml.) of iris extract and of ether are dispensed into a stoppered tube. A measured amount of N-HCl, between 0·002 and a maximum of 0·012 ml. varying with the extract and according to the pH desired, is then added with a Warburg pipette graduated to 0·001 ml., and the tube is shaken (after filling with nitrogen in a few experiments). After allowing usually a standard time of 5 min for the layers to separate (Fig. 1, ▲) the clear part of the ethereal layer is drawn off into an all-glass syringe, leaving a greyish sludgy material at the interface. When separation has to be assisted by centrifugation, the time is necessarily longer (Fig. 1, ○). After measuring the volume collected thus this ethereal phase, which has a faint brownish tinge (due probably to acid haematin), is evaporated at once in a stream of air (or in nitrogen) to a solid ('Type 3') residue and re-constituted by dissolving in de Jalon's rat colon solution (Gaddum, Peart & Vogt, 1949), 1 or 2 ml./≡ 100 mg. A small sample of the aqueous phase is then taken from below the interface at once, or later, for pH measurements made at room temperature in a glass capillary electrode system of the type described by Joels & MacNaughton (1957). The reconstituted ether phase is assayed on the hamster colon against an unpartitioned sample of the original extract. Percentage recovery of the activity is best expressed per millilitre of the ether phase, because water is much less soluble in ether than vice versa and HCl increases the solubility of ether in the water phase enough to affect the amount of irin in that phase. Satisfactory recoveries have been obtained at pH's 2·8–4, as is shown in Fig. 1 from a series of nineteen consecutive experiments. When HCl was not added recovery was almost nil (3% at pH 7·45). Using the maximum amount of 0·012 ml. N-HCl/ml. extract, recoveries were 90% or more in several further experiments (without pH measurement), not included in Fig. 1, but in a later series recoveries became unexpectedly low (50% or less). At the time of these later experiments the glassware had been washed in a chromic acid cleaning mixture. On reverting to glassware cleaned by boiling in nitric acid yields of 55–95% were obtained in nine experiments. It is well known that glassware cleaned in chromic acid requires further treatment with alkali to remove traces of chromates before it can be used for oxidizable substances, for instance in lactic acid determinations (Colowick & Kaplan, 1957); the losses in the above experiments with irin were thus attributable to oxidation. Since irin is inactivated by oxidation (see p. 274), it is desirable to use ether as free as possible from peroxides and other by-products; the ether used throughout these experiments has been of anaesthetic grade. Through the courtesy of May and Baker Ltd., some of the batches of ether were re-tested for peroxides a day or two before delivery and to one batch Na wire was added. The ether was stored at room temperature (when over Na) or at −15° C in its amber-coloured bottle with the black paper wrapping intact; protection from light alone should reduce peroxide formation to mere traces even after many weeks (Bailey & Roy, 1946). The high recoveries in Fig. 1 showed that the batches of anaesthetic ether then in use were quite adequate for these experiments. In a few experiments the ether was passed before use through a column of activated alumina, which is said to remove peroxides (Dasler & Bauer, 1946).

Most residues obtained by other methods have contained a white opalescent material which has been absent from these Type 3 residues. Ether-purified irin can be considered reasonably free from bases, which, in partitions at acid pH, should be retained in the aqueous phase. Table 1 gives the yields of some Type 3 residues, all active in doses of 1–1·5 μg and weighed on a balance graduated to 0·01 mg.

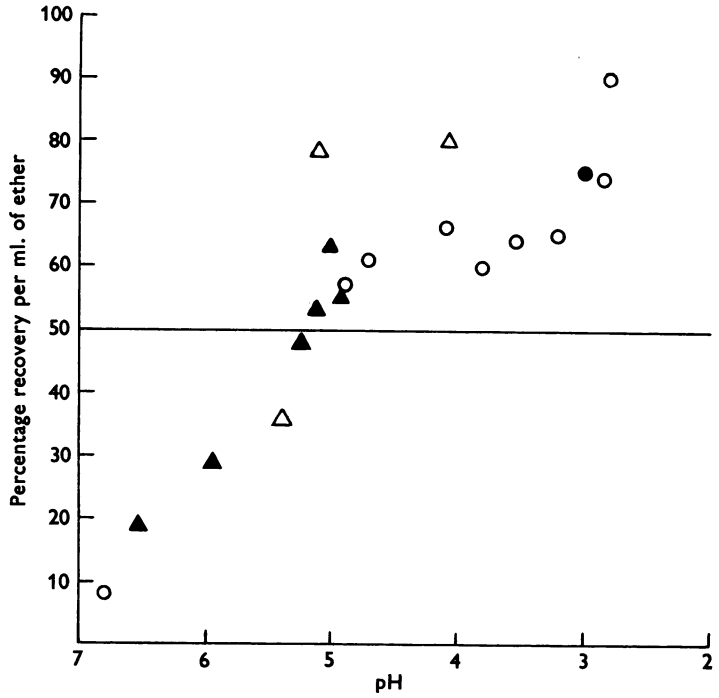


Fig. 1. Recovery of irin after partitioning extracts with equal volumes of ether at various pH's, expressed as percentages of the original activity found per millilitre of ether phase. In experiments ▲ a standard time of 5 min was allowed for the separation of the phases; in Δ this time exceeded 30 min and in ○ the time was not noted but probably did not exceed 15 min. ●, see Appendix, p. 289.

TABLE 1. Yields of irin purified by partition

Initial quantity of extract (≡ mg of tissue)	pH of the partition	Weight of active residue (μg)	Purification (from the original weight of tissue)
233	2·8	70	3328 ×
839	3·19	417–420	2000 ×
336	2·98	330	1018 ×
136	2·98	50*	2720 ×

* After one re-extraction with acetone and two with ether.

Fig. 1 resembles the dissociation curve of a weak acid. Attempts to derive the pK'_a of irin from it have been unsuccessful. To do this it would be necessary to know the partition coefficient (K_2) of undissociated irin between ether and water, when it can be shown mathematically that

$$pK'_a = pH - \log\left[\frac{K_2}{[\text{irin in ether}]} - K_2 - 1\right].$$

By taking pairs of points from the graph in Fig. 1, values of K_a could be deduced, but these increased with pH and varied between 1 and 9; pK'_a thus varied between 4·3 and 5·9. A factor which may have to be taken into consideration is the possibility of lactone formation in the presence of HCl, since evidence will be given below that irin is a hydroxy-acid. A secondary reaction of this kind would naturally affect the equilibrium and raise the 'pK' unduly. The separation time may therefore be important. In the partitions marked ▲ a standard time of 5 min was allowed, to make the experiments more comparable with one another.

When *n*-pentane (A.R.) was substituted for ether in the above type of partitions <5% of the irin activity was recovered from the pentane, even with 0·012 ml. of N-HCl/ml. extract. Recovery was also low with benzene, cyclohexane, and methylene dichloride, but was 75% in one experiment with 2 vol. of ethyl methyl ketone.

Assays and activity tests. In the initial experiments of this paper (pp. 261–267), irin assays were carried out on the rat colon in 10^{-7} g/ml. atropine and 10^{-7} g/ml. lysergic acid diethylamide (LSD), and a number of acidic substances were screened for activity on this muscle. This test object was later superseded by the hamster colon (±atropine 10^{-7} or 10^{-6} g/ml.), the advantages of which have already been enumerated (Ambache, 1957*b*). For this preparation, the first 4 cm of the ascending colon was suspended at 30° C in a 5 ml. bath of atropinized de Jalon's fluid as used for the rat colon. LSD 10^{-7} g/ml. was added in a few experiments only. In its absence some preparations were insensitive to 20–100 μg of 5-hydroxytryptamine (5-HT), as illustrated previously (Ambache, 1957*b*), but other preparations have given small contractions to 1–2 μg of 5-HT. In some of the later experiments of this series the hamster colon preparations showed a decrease in sensitivity to irin occurring soon after assays were begun. This has now been overcome by making the de Jalon's solution, as a routine, with glass-distilled water purified further by passing through a small, well washed, de-ionizer (Elgastat Minor, portable type C 403).

The acids tested were mostly of commercial origin. I am indebted to Dr R. E. Bowman, Parke Davis Laboratories, Hounslow, for keto acids; to Dr F. D. Gunstone, St Andrew's University, for 9-hydroxy-octadec-12-enoic and 12:13-dihydroxy-oleic acids; to Dr H. Laser, Cambridge, for *cis*-11:12-octadecenoic acid; to Dr J. W. Cornforth, Mill Hill, Dr J. Elvidge, Imperial College, London, Dr P. N. Williams, Unilever Research Laboratories, Port Sunlight, and Dr Frank G. Young, Union Carbide Chemical Cy., South Charleston, Virginia, for various lactones; to Dr W. Vogt, Göttingen, for Darmstoff; to Dr V. R. Wheatley, St Bartholomew's Hospital, for a specimen of rabbit sebum, prepared as described by James & Wheatley (1956, 1957). $\Delta\alpha\beta$- and $\Delta\gamma\delta$-δ-hexeno-lactones were synthesized in Mainz through the courtesy of Professor D. Jerchel.

Oleic acid (at least 99%; density 0·89–0·895) was supplied by May and Baker Ltd., and 'pure' but orange-coloured ricinoleic acid (density 0·94) by Messrs Hopkin and Williams; 10^{-4} solutions (v/v) of these were prepared either in de Jalon's fluid without further addition of alkali, or in 0·9% NaCl with an amount of NaOH approximately equal to the calculated equivalent of the acids. Such solutions had pH's of 6·55–7·13. It was not possible to dissolve ricinoleic acid in more concentrated solutions without the addition of almost its full equivalent amount of NaOH. Such solutions were then alkaline, as is well known, and for some experiments had to be adjusted with HCl to pH 7–8. With concentrations exceeding $1–2 \times 10^{-3}$ this process of back-neutralization tended to throw the fatty acid out of solution in microscopic droplets.

For the intraocular injections of Na ricinoleate, albino rabbits of unknown atropinesterase serum-titre were used. They were injected intravenously immediately before the experiments, with at least 10 mg/kg atropine sulphate, 1000–2500 i.u. heparin, and with *ca.* 24–30 mg/kg pentobarbitone Na. Anaesthetic ether was then administered for 10–15 min before injecting the eyes. Injections into the anterior chamber were made through 4–6 mm self-sealing corneal tracks, one eye receiving 2×10^{-3} Na ricinoleate dissolved in 0·9% NaCl solution, the other an equal volume of 0·9% NaCl solution alone. Because of the limited solubility of Na ricinoleate, and the need for 100–200 μg doses (see Results, p. 284), injection volumes of 0·05–0·1 ml. were necessary. Such volumes are bound to cause a considerable rise in intraocular pressure (I.O.P.). However, this temporary rise in I.O.P. helps to seal the valvular tracks in the cornea after the injections, and

does not by itself induce pupillary constriction, as is shown by the control eyes injected with the 0·9% NaCl. Horizontal pupillary diameters were measured with calipers.

Incubation with enzymes. The irin for these experiments was a Type 1 residue dissolved in de Jalon's fluid (pH 6·8–7·9). Samples of this solution were incubated at 37° C for 70–90 min with 1/21–4/10 vol. of a concentrated solution of intestinal alkaline phosphatase (2226 u./ml.), kindly supplied by Dr A. L. Greenbaum, University College London; controls consisted of parallel samples kept at 37° C for the same times but without enzyme. After incubation the pharmacological activities of the two samples were compared on the rat colon and the phosphatase activity of the enzyme-treated sample was determined by the 15 min procedure described by King (1946, p. 56) using disodium phenyl phosphate as substrate and de Jalon's fluid instead of the buffer.

Clostridium welchii α-toxin was kindly supplied by Dr M. Sterne.

Paper chromatography. Ascending chromatograms of irin and of ricinoleic acid were run in the organic, upper, phase of the following mixture: ethyl methyl ketone–water–diethylamine in the ratios 60:20:3 (Vogt, 1955, 1957*c*). The Whatman no. 1 papers were suspended over this solvent in a small sealed tank and left to equilibrate in air ½–4 hr before being lowered into contact with it. Solvent ascent was allowed for ⅔–17 hr, and was quite rapid at first (12 cm in 40 min). Recovery of irin was <10% in a run lasting throughout the night, presumably because of inactivation at the alkaline pH of this solvent or by oxidation. Shorter runs are therefore advisable in this medium, and better yields might be obtained by running the chromatograms in N_2. In later experiments, when it was realized that irin was an unsaturated acid, its application to chromatogram origins was carried out in a stream of N_2. As with ricinoleic acid, the irin 'spots' exhibited a peripheral ring of fatty appearance.

As the R_F of irin in this solvent is 0·9 and therefore very close to the solvent front, various attempts were made to lower the R_F, but unsatisfactory results were given by other solvent mixtures. For instance, the substitution of propyl methyl, for ethyl methyl, ketone in the above solvent lowered the R_F excessively to 0–0·2. When *iso*butyl methyl ketone was used and the diethylamine was omitted, the irin was diffusely located on the chromatogram at R_F 0·05–0·4. Lastly, the R_F was 0–0·15 in a solvent consisting of the organic phase of the following mixture: ethyl methyl ketone 30, isobutyl methyl ketone 60, distilled water 30, and diethylamine 0·45. Irin and ricinoleic acid were also chromatographed in 50, 60 and 70% methanol.

Paper electrophoresis was conducted as described previously, for at least 4 hr at 250–270 V in buffered 0·1% NaCl. Irin was applied to the origins in acetone or ether solution. The buffer was usually 0·01 M phosphate, as for instance in the experiments of Fig. 3, but for some experiments it was 0·05–0·005 M tris(hydroxymethyl)amino methane. The pH's were kept between 8·2 and 8·7. In one experiment the electrophoresis tank was filled with N_2; otherwise, with air. Irin could not be eluted with ether from electrophoresis papers unless the ether was previously acidified by shaking over an equal volume of approx. 0·01 N-HCl.

Chemical tests

Chemical tests on irin were carried out in solutions or on chromatograms or electrophoresis papers.

Amino acid test. Papers were sprayed with 0·1% ninhydrin in water-saturated butanol and left in an oven at 100° C for 5–10 min.

Test for –NH groups. The method of Rydon & Smith (1952) proved less satisfactory than the procedure of Reindel & Hoppe (1953) modified slightly as follows. ClO_2 was generated in a lidded 1 l. cylinder by adding a few millilitres of 10 N-HCl to $KClO_4$ crystals. Before exposure to this gas, the dry electrophoresis papers were sprayed very lightly with a mixture consisting of equal volumes of acetone and of 95% ethanol +5 drops glacial acetic acid; Reindel & Hoppe recommend that the papers should be dipped into this mixture, but this would have eluted the soluble irin. The moistened papers were then left hanging in the ClO_2 cylinder for 2 min. and were immediately sprayed with 1% (saturated) benzidine solution in 10% acetic acid. The extent of the blue staining zone on the papers was noted at once and before washing off the excess of reagent by dipping into 95% ethanol, as this led to some loss of colour.

Fuchsin-sulphurous acid was made up according to the British Pharmacopoeia (1948, p. 659). For tests on solutions, 0·1–0·2 ml. of the reagent and 1–2 drops of 0·27% $HgCl_2$ were added to 0·025–0·2 ml. of iris extracts or of purified irin redissolved in de Jalon's fluid. Chromatograms sprayed with the reagent (± a little 0·27% $HgCl_2$) have at first a white background which turns pink when the SO_2 is lost. They are therefore best placed for viewing between two narrow sheets of Perspex held apart by a 3 mm air-tight surround of Plasticine, and can be kept thus during the night. Aldehydes stain a deep violet at once, even in the absence of $HgCl_2$; acetals, only in the presence of $HgCl_2$.

Test for glycosides. Aniline hydrogen phthalate was made up according to the procedure described by Partridge (1949); 3% anisidine HCl in butanol, as described by Hofman (1953) and by Hough, Jones & Wadman (1950). The sprayed papers were left in an oven at 100° C for 10–15 min.

Colour test for higher fatty acids. Feigl's (1956, p. 461) procedure was modified as follows. To 0·1 ml. of a saturated, centrifuged, solution of Rhodamine B in benzene were added 0·05–0·1 ml. of a 1% aqueous solution of uranyl acetate, and 0·2 ml. of a benzene solution of Type 3 irin ('≡ 400–500 mg'/ml.). A control tube containing the reagents and 0·2 ml. benzene without irin was prepared simultaneously, and both tubes were shaken.

Colour test for hydroxy-fatty acid. The red colour reaction of hydroxy-acids with *sym* diphenylcarbazide used by Korpáczy (1934) and quoted by Feigl (1956, p. 483) was modified as follows. A 0·5% solution of *sym* diphenylcarbazide A.R. in tetrachloro-ethane was freshly prepared as in Feigl (1956); 0·1–0·2 ml. of this reagent, which should be colourless or pale yellow when fresh, was dispensed into a small Wassermann tube, usually with 1–4 vol. of irin dissolved in de Jalon's solution; a control tube with the same volumes of reagent and of de Jalon's solution without irin was prepared simultaneously. The tubes were shaken intermittently, and the colour in the lower, tetrachloro-ethane, layer was noted. A distinct colour difference between the two tubes could be detected within 7–35 min, usually preceded by a slight milkiness of the watery layer. The test was carried out (*a*) on a 'Type 2' acetone residue purified by a further re-extraction with acetone and (*b*) on several batches of irin purified by ether partition, either alone or combined with subsequent paper electrophoresis at pH 8·2 in 0·01 M phosphate buffer. After electrophoresis successive transverse 1 cm slices of the dry paper (of 2 cm width) were eluted with 1 ml. of de Jalon's fluid and the test was performed on these eluates. It was convenient in these experiments to dissolve the irin, for its initial application to the paper, in ether previously saturated with the electrophoresis buffer. This permitted a rapid application of relatively large amounts of irin (≡ 120 mg/cm) to the paper without a fan; recovery of activity from the paper was about 50%.

I am indebted to Dr C. N. Graymore for a lactic acid estimation by the method of Barker & Summerson.

Chemical inactivations

Phenyl isocyanate. 0·01–0·03 ml. of phenyl isocyanate was added to 1 ml. of iris extract in a weighing bottle which was shaken by hand at intervals or mechanically. After 1 hr at room temperature the characteristic pungent smell of phenyl isocyanate had almost disappeared and the solution contained needles of diphenyl urea, its hydrolysis product, which is almost insoluble in water (0·15 mg/ml.). Centrifugation removed the precipitate of diphenyl urea, and probably with it some phenyl isocyanate (density 1·095). The supernatant was decanted and recentrifuged once or twice until free from the smell of phenyl isocyanate; it was then diluted before assay with an equal volume of glucose-containing de Jalon's fluid, the pentahydroxylic glucose probably removing the last traces of the OH-binding reagent.

N,N'-carbo-di-p-tolyl-imide is virtually insoluble in water and the experiments were conducted in an organic solvent, mostly in acetone but some in ether. Acetone-purified Type 2 iris residues were dissolved in acetone (≡ 100 mg/ml.), and divided into two 1 ml. samples. One of these received 1 mg of the reagent, either as a crystal or as 0·1 ml. of a 1% solution in acetone. The other sample served as an untreated control solution or irin. A second 'reagent control' sample was also prepared consisting of 1 ml. of acetone (without irin) plus 1 mg of reagent. All three

samples stood at room temperature for 15–30 min and were then evaporated to dryness under a fan, and taken up each in 1 ml. of de Jalon's fluid. In the two samples containing the reagent a lining of insoluble crystals was seen on the sides of the vessels; this was scraped off the sides with a needle and stirred into the fluid. After sedimentation or centrifugation, these solutions were assayed on the hamster colon. Although there was no evidence of any interference with irin contractions by the traces of this insoluble reagent, treated irin was assayed against doses of untreated control irin administered into the organ bath together with appropriate volumes of the 'reagent control'.

$KMnO_4$. Aqueous extracts stood for 5 min at room temperature with 1/40th vol. of 2% $KMnO_4$ A.R., the pH not exceeding 7·6. Two vol. of ether and 0·0125 ml. N-HCl per millilitre of extract were then added for partition (pH 3·8) as already described, allowing 5 min for the separation of the phases.

Catalytic hydrogenation. Aqueous extracts were dispensed into glass-stoppered tubes with gum acacia and $PdCl_2$, which were added either separately or as a colloidal mixture, to a final concentration of 0·02–0·05%; a brown precipitate appeared, and the pH was about 6·4. Hydrogen was then bubbled through samples of these extracts for 1–2 hr either continuously or, in order to reduce evaporation losses, intermittently with shaking of the gas-filled tubes. The precipitate blackened on hydrogenation. Controls consisted of unhydrogenated samples of the same extracts with added catalyst.

IBr. Crystalline IBr failed to dissolve completely when shaken in distilled water 1 ml./4·15 mg ('0·02 M'). The dark brown solution was centrifuged and 0·2 ml. (or less) of it was added to 1 ml. of iris extract. After standing at room temperature for 10–30 min, the reaction was stopped by adding an excess of $Na_2S_2O_3$ (0·22 ml., or less, of 0·04 M solution per millilitre of extract). The extract was then partitioned with 1, or more usually, 2 vol. of ether and 0·012 ml. of N-HCl per millilitre, as described above. The control consisted of a parallel tube with the same amounts of IBr inactivated with the $Na_2S_2O_3$ *before* the addition of extract and its subsequent partition under identical conditions.

Chemical nomenclature. Two systems exist for the numbering of carbon atoms in fatty acids. Both systems have been used interchangeably in the text and are therefore shown here:

$$\begin{array}{cccccccc} 6 & 5 & 4 & 3 & 2 & 1 & & \\ C & -C & -C & -C & -C & -COOH & & \\ \epsilon & \delta & \gamma & \beta & \alpha & & & \end{array}$$

RESULTS

Distinction of irin from various acids of natural occurrence

As reported previously (Ambache, 1957*a*), irin is an acid, since it migrates towards the anode on electrophoresis and its distribution on partition between water and chloroform is influenced by pH. Considering the nature of the acidic group in irin which would account for these properties, the following possibilities have been examined.

Sulphonates and organic phosphates

In a recent investigation of the acid–base balance in squid axoplasm Koechlin (1955) found that, of the 345 μequiv/g of organic acids present, 220 μequiv/g consisted of isethionic (2-hydroxy-ethane sulphonic) acid. This sulphonate would therefore appear to be a major anionic constituent of some excitable tissues. It is, however, a strong acid with a pK near 2, whereas irin behaves as a weak acid. It can be omitted from further consideration since

it was inactive on the rat colon in doses of 100–200 μg of the Na salt. De Jalón & de Jalón (1945) have already reported on the low activity of the detergent Na oleyl isethionate on other intestinal preparations.

It is also unlikely that irin is a non-lipid phosphate. Thus, irin was not inactivated by intestinal alkaline phosphatase. Solutions of irin ($\equiv$10–20 mg/ml.) in de Jalon's fluid (pH 6·8–7·9) were incubated for 1·5 hr at 37° C with 111 units/ml. of phosphatase. This treatment did not reduce the pharmacological activity of the irin, nor did incubation for 1·1 hr at 41° C with 8 times the above concentration of enzyme. A confirmatory test at the end of each experiment showed that the phosphatase was still highly active. For instance, at the end of the first experiment, when the incubated mixture of irin and phosphatase was itself tested for phosphatase activity, using disodium phenyl phosphate as a substrate and without altering the pH, it liberated in 15 min 111·3 mg/ml. of phenol by hydrolysis. This corresponds to an ability to split off 36·3 mg P/ml., and shows that alkaline phosphatase was used in gross excess in these experiments.

Irin was also treated with phospholipase C in the form of a preparation of *Cl. welchii* α-toxin which had a toxicity of 0·78 α units/mg. Incubation of irin for 2 hr with 16·6 mg/ml. of this toxin did not result in any loss of pharmacological activity.

Most phosphates are precipitated by lead acetate. After treating iris extracts or solutions of purified irin with Pb acetate to a final concentration of 0·16–1% and centrifuging, irin could still be extracted from the supernatant with ether (50–60% at pH 4); the precipitate was virtually inactive even after it had been treated with H_2S. Likewise, the application of Pb acetate to chromatogram origins did not prevent irin ascent to its usual R_F (see p. 263) and the irin on the developed chromatograms was active even without exposure of the papers to H_2S, showing that irin had not formed an insoluble Pb salt.

The fact that irin behaves as a weak acid also excludes certain types of phosphate ester. Thus there was virtually no migration in an electrophoresis experiment at pH 5·2 (Ambache, 1957*a*). The 'apparent' pK′ of the three dissociations of phosphoric acid are: pK'_1, 2; pK'_2, 6·8; pK'_3, 11·7. In mono-esters of phosphoric acid two dissociations remain, with pK'_1 and pK'_2 and in di-esters only one, with pK'_1; substitution lowers both pK'_1 and pK'_2 (Kumler & Eiler, 1943). For instance, in nucleotides and in sugar phosphates pK'_1 varies between 0·7 and 1·5, whereas pK'_2 lies usually between 5·9 and 6·6 (Chargaff & Davidson, 1955, pp. 227, 269–270 and 458–461) except for the pK'_2 of 4·5 in arginine and creatine phosphates (Lipmann, 1941, p. 112–113). Now, when irin was partitioned between equal volumes of de Jalon's fluid and of chloroform, at pH 8·2 more than 80% of it, presumably in its dissociated form, was found in the water phase, whereas at pH 3·5 more than 80% of the irin, presumably now in the undissociated form, moved into the chloroform (Ambache, 1957*a*, fig. 7). Similar results have been obtained in water-ether partitions (see Fig. 1). Clearly the pK′ of irin, which is the pH at which 50% of the dissociation occurs, must lie between these two extremes, from which it can be deduced that irin is not a *di-ester* of phosphoric acid, as it would then be strongly acidic and have the pK'_1 of 0·7–1·5, which is well below the limits just

given. It is also unlikely that irin is a *mono-ester* of phosphoric acid, since it is not inactivated by alkaline phosphatase; most phosphoric mono-esters have their pK_2' in the region of 6, whereas the results which are summarized in Fig. 1 suggest that the apparent pK of irin is probably lower than that.

In the infra-red alkyl phosphates have extremely strong and broad absorption bands at 1000–1030 cm^{-1}, whereas with irin residues absorption in this region has been weak or absent (see p. 290).

The following individual, pharmacologically active, phosphate esters have been distinguished from irin, as follows:

Adenosine triphosphate (*ATP*). Unlike ATP, irin is soluble in acetone, chloroform and ether. It contracts the rabbit ileum, which is relaxed by ATP, and the guinea-pig ileum, which is contracted only by large doses of ATP. The rat colon is also far less sensitive to ATP-Na (0·6–1 mg) than to irin ($\equiv$ 0·5 mg tissue, which, from Table 1, corresponds to $<0{\cdot}5\,\mu g$ of active substance).

Darmstoff. The lipid-soluble Darmstoff (Vogt, 1957*a*, *c*) consists mostly of acetal or semi-acetal phosphatidic acid, that is, of a mono-ester of phosphoric acid. Unlike irin, it is stable in alkali and stains with fuchsin-sulphurous acid. Parallel assays on the rabbit ileum and rat colon preparations show that their index of discrimination between Darmstoff and irin is low. Irin can, however, be further distinguished from Darmstoff by paper chromatography in ethyl methyl ketone–diethylamine–water (60:3:20 by vol.). In this solvent Darmstoff has an R_F of 0·4–0·45 on Whatman No. 1 paper (Vogt, 1957*c*), whereas in a dozen chromatograms on this paper irin was found just behind the solvent front (visible as a faint, pharmacologically inactive, pink band) at R_F values of 0·8–0·97. This is illustrated in Fig. 2, taken from two experiments. The first panel shows the location of irin in one of these chromatograms by assay of successive transverse slices of the paper on a rat colon in atropine and LSD. In this experiment $\equiv$ 20 mg of irin purified by acetone treatment (Type 1 residue) was applied to the paper in 0·05 ml. of distilled water. After 3 hr equilibration and a $3\frac{1}{2}$ hr run the solvent front had reached 15 cm. From Fig. 2 it can be seen that the irin activity was confined to two slices of this paper, i.e. 13–13·5 cm and rather more in 13·5–14·0, giving an R_F value of 0·87 to 0·93.

In the other experiment a larger amount of acetone-purified irin, $\equiv$ 80 mg, had been applied to the paper as a line and after 6 hr run the solvent front had reached 17·2 cm. The assay of the active end of this paper is shown in the second panel of Fig. 2. The topmost slice, 16–17·2 cm, was inactive; the next slice, between 15 and 16 cm, elicited a spasm which kept the lever off scale for 4–5 min, although the slice was removed and the bath fluid was changed after 2 min; the preparation recovered fully only after 20 min. Some activity was present in the next slice, 14–15 cm, and none below that, giving an R_F of 0·81–0·93 with a maximum at 0·87 to 0·93.

The recovery of irin was about 10% in the first experiment of Fig. 2 and appeared to be low in most of the other chromatograms. This is probably due

to inactivation of the irin by oxidation or by the base in this solvent. Nevertheless, by applying an excess of irin, enough of the active substance remained on the papers at the end of all these experiments to permit its accurate location and to obtain a consistent R_F. The high R_F of irin suggests that it is considerably less polar than Darmstoff and is probably of high chain length, comparable perhaps to that of (+)-ricinoleic acid which has a similar R_F in this solvent (see, p. 279).

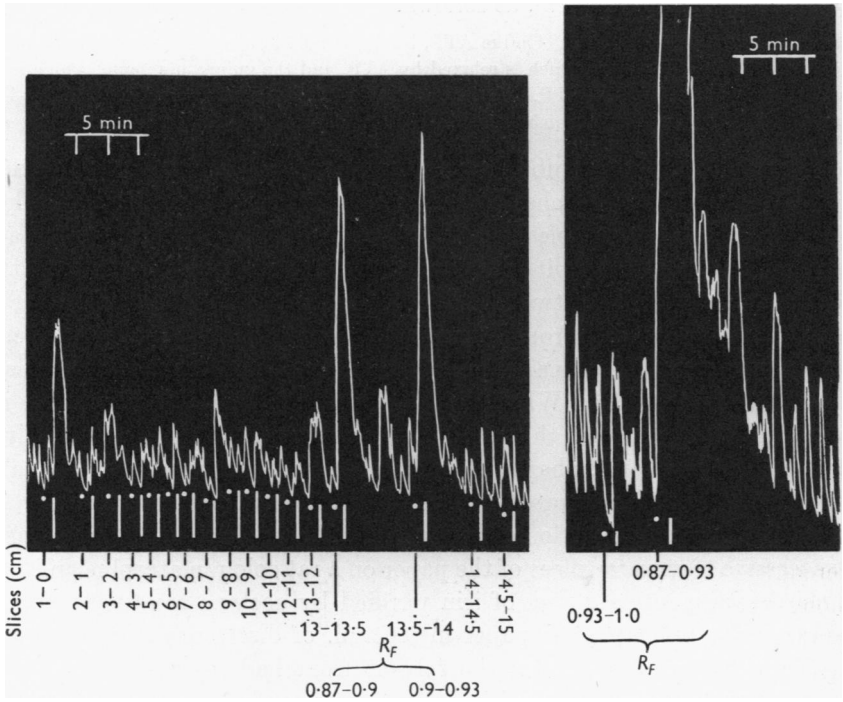


Fig. 2. R_F of irin chromatographed ascendingly in ethyl methyl ketone:diethylamine:water (60:3:20). Irin was located on the papers at R_F 0·87–0·93 by assay on rat colon preparations in 10^{-7} g/ml. atropine and LSD. First panel: successive transverse slices of a whole 15 cm chromatogram; activity confined to slices 13–13·5 and 13·5–14 cm. Second panel: uppermost two slices of another chromatogram showing activity again at R_F 0·87–0·93. For details see text.

Carboxylic acids

The apparent pK of irin suggested by Fig. 1 would fall into the range of pK of carboxylic acids, which is 2·5–6 (5·77 for stearic; Ralston 1948, p. 379). The following classes of carboxylic compounds were therefore examined.

Amino acids. Crude iris extracts, spotted on filter paper, stained with ninhydrin, but after purification by chromatography or by electrophoresis on paper the irin-containing regions of the papers did not stain with ninhydrin. Thus irin is not an amino acid.

The following amino acids or derivatives were tested on the rat colon, as 10^{-3} solutions in

de Jalon's fluid, and were all inactive in doses of 100–200 μg: γ-aminobutyric acid, DL-norleucine, DL-isoleucine, δ-hydroxylysine HCl, DL-glutamic acid, Na pantothenate, and L-tyrosine.

In addition, activity tests were carried out on a few imino (NH) acids (100–200 μg) as these might have escaped detection with ninhydrin. The heterocyclic L-proline, hydroxy-L-proline, neutralized DL-pyroglutamic acid, and ϵ-caprolactam were all inactive on the rat colon and did not affect irin-responses.

Saturated fatty acids. The extractability of irin from water into ether at acid pH resembles a well known property of fatty acids. Purified irin does in fact give a colour test characteristic of the higher fatty acids (see p. 269). However, it is mentioned by Gabr (1956) that the C_2–C_{18} saturated fatty acids are pharmacologically inactive, and Vogt (1957*b*) has reported that stearic acid is inert on the guinea-pig ileum. In the present investigation two acids of this type were tested on the rat colon: Na acetate (50–200 μg) and neutralized *n*-caproic acid (100 μg). Both were inactive.

The pharmacological properties of 'branched-chain' fatty acids are unknown; their biological occurrence has been demonstrated in sebum by James & Wheatley (1956, 1957) using the method of gas–liquid chromatography. Rabbit sebum, which contains a wide range of such acids, was therefore tested on the rat colon. A 19 mg specimen of sebum failed to dissolve completely in 1·9 ml. of de Jalon's fluid, even when NaOH was added to pH >10. It was diluted to 9·5 ml., neutralized and tested. The suspension was inactive on the colon in 0·1 and 0·2 ml. doses.

Unsaturated fatty acids. The evidence that irin is unsaturated is given below. A number of naturally occurring unsaturated fatty acids were therefore considered, but could all be distinguished from irin on pharmacological grounds. Among the *mono-ethenoid acids* oleic (*cis*-octadec-9-enoic) acid could be excluded because it does not contract the guinea-pig ileum (Feldberg, Holden & Kellaway, 1938; Vogt, 1957*b*) or the hamster colon (see Fig. 8), and its isomers octadec-3-enoic and *trans*-octadec-2-enoic, because they are inactive, respectively, on the rat colon and on the guinea-pig ileum (Gabr, 1954, p. 79; 1956). In the present experiments *cis*-octadec-11-enoic (Laser, 1950) could likewise be excluded; this acid had to be rendered soluble at pH 7·6–8·5 by the addition of alkali and by warming, and was then inactive on the rat colon in doses of 250 μg. Undecylenic (hendec-10-enoic) acid was also insoluble in de Jalon's fluid and a 1 in 1000 emulsion was inactive on the rat colon in a dose of 0·05 ml.

The *diethenoid* linoleic (octadeca-9:12-dienoic) acid has some stimulating effect on the guinea-pig's intestine, as was shown by Gabr (1956), but according to Vogt (1957*b*) the effect is only weak and cannot be regularly obtained, thus differing from that due to irin. On the hamster colon linoleic acid was inactive in doses of 70–100 μg. The *triethenoid* linolenic acid renders the guinea-pig gut insensitive to stimulants (Gabr, 1954).

The unsaturated fatty acid SRS, which is split off from lecithin by cobra venom and which has been studied by Vogt (1957*b*), can be excluded because its stimulating effect on the guinea-pig ileum decreases with successive applications and finally disappears, whereas no such tachyphylaxis was seen with irin on the guinea-pig ileum in the presence of atropine and mepyramine (Ambache, 1957*a*) or on other preparations.

Prostaglandin, a muscle-contracting substance described by von Euler (1936), is now known to be an unsaturated fatty acid (Bergström, 1949). It differs from irin in its great stability, since it can be boiled for 20 min at pH 1–7 without loss of activity (von Euler, 1936). According to Eliasson's (1957) results its R_F (0·4–0·45 in the ethyl methyl ketone–diethylamine–water mixture described above) is about half that of irin and corresponds exactly with that of Darmstoff.

Keto acids. The α-keto Na pyruvate (50–200 μg) and the γ-keto Na laevulinate (0·2–1 mg) and sodium ketoglutarate (50–200 μg) were all inactive on the rat colon.

The following long-chain keto-acids failed to dissolve completely even in the presence of just less than their calculated equivalents of NaOH, and were inactive in doses of 0·1–0·2 ml. of such '10^{-3}' and '5×10^{-4}' (g/ml.) suspensions: 5-keto-undecanoic; 5-keto-tetradecanoic; 9-keto-tetradecanoic; 5-keto-octadecanoic; and 5-keto-docosanoic.

Dicarboxylic acids. Na succinate (100–200 μg) and neutralized glutaconic acid (m.p. 135–136° C) were inactive; Na adipate was inhibitory in doses of 0·6–1·2 mg. A saturated solution of the mono-ester, ethyl hydrogen sebacate, $HOOC(CH_2)_8COO.Et$ was inactive in 0·1–0·2 ml. doses.

Carboxylic and other steroids. The sodium salts of pure cholic and glycocholic acids and of taurocholic acid (a sulphonate), and a commercial sample of mixed bile salts, all failed to contract the rat colon in doses of 200 μg/5 ml.

Other acids

L-*Ascorbic acid.* Ascorbic acid was tested because it is known to be concentrated into the aqueous humour in rabbits. It could be excluded because it was inactive on the rat colon in concentrations of up to 4×10^{-3} g/ml.

'*SRS-A*' is a slow reacting substance released from lung tissue during anaphylactic shock (Brocklehurst, 1956). It is acidic and contracts the guinea-pig ileum, but differs from irin in lacking the stimulating effect on the rat colon, even when tested in concentrations >20 times greater than those which contract the guinea-pig ileum (Brocklehurst, 1956; and see Vogt, 1958).

An unsaturated acid which contracts the atropinized rat colon and tortoise jejunum has been found in extracts of nasal mucosa from dogs and sheep by Toh & Mohiuddin (1958). This substance is thermostable at pH 10·8, whereas irin is inactivated by alkali even at room temperature and is thermolabile.

Amphoteric purines and pyrimidines. The replacement of hydrogen in the purine ring by oxygen in the 2-, 6-, and 8-positions is known to confer weakly acidic properties upon the purine molecule, as for instance in xanthine (2,6-dioxypurine) and its derivatives. Thus caffeine, which is 1,3,7-trimethylxanthine, resembles irin in being extracted by chloroform from acid aqueous solution.

However, on the atropinized rat colon xanthine (6·9 and 34·5 μg) was inactive, as also were caffeine and inosine (hypoxanthine riboside) in amounts of 200 μg. The dioxypyrimidine uracil was inactive in doses of 5–10 μg.

Interaction of irin with drugs

In the course of the activity tests just outlined a number of drugs were examined as possible antagonists to irin. None of the following interfered with irin contractions of the rat colon preparation in atropine $\pm$LSD: morphine SO_4 (2×10^{-5} g/ml.), pethidine (2×10^{-6}), neutralized salicylic acid (2×10^{-4}), dihydroergotamine methane sulphonate (4×10^{-7}), eserine SO_4 (10^{-5}), strychnine HCl (10^{-7}), cocaine HCl (10^{-7}), and dibenamine HCl (4×10^{-7} applied for 9 min).

The chemical nature of irin

Preliminary exclusion of various radicals

Tests for nitrogen. An elementary analysis of the relatively crude 'Type 2' iris residue, purified by a further re-extraction with absolute acetone, showed that 6·37% of nitrogen was still present after purification by this method. I am indebted to Dr C. R. Owen, Imperial College, for this analysis, which was carried out on 2 mg of material obtained from 1400 mg of iris tissue. It is, however, unlikely from the results below that irin is itself a nitrogenous compound.

The absence of a strongly basic quaternary NH_4^+ group in the irin molecule can be inferred from the fact that irin does not behave as an ampholyte in partitions and on electrophoresis at various pH's. The absence of an $-NH_2$ group can also be deduced from the negative ninhydrin test reported above. The ninhydrin reaction would, however, fail to detect the presence of a secondary –NH (imino) group. To reveal such imino-groups a sensitive colour reaction can be used, which has been described by Reindel & Hoppe (1953) and which is, for instance, capable of detecting on paper 1 μg/cm^2 of protein, i.e. 0·15 μg of N_2. In this test papers are exposed to chlorine dioxide, which converts RNH groups to RNCl; the papers are then sprayed with benzidine, which at once reveals the RNCl by forming a blue colour. Electrophoresis papers treated in this way all showed two distinct blue bands due to nitrogenous impurities (a narrow band at the 'origins' and a wide band on the negative side) but there was no trace of blue colour in the pharmacologically active areas of these papers (Fig. 3*A*). In the experiment of Fig. 3*A* the extract applied to the electrophoresis paper had been purified to a 'Type 1' residue by acetone treatment as described in Methods; for application to the paper this residue was taken up in 90% acetone. In another experiment in which a parallel sample of the same residue was taken up in undiluted acetone, the blue bands on the sprayed paper were much fainter initially, and faded after the paper was dipped in 95% ethanol (see Methods). This experiment showed that although less nitrogenous material was taken up by re-extracting residues with undiluted acetone, its presence on the electrophoresis papers could still be detected, but always outside the pharmacologically active area.

This test excludes small peptides (e.g. ophthalmic acid, Waley, 1957), carbamyl phosphates and sphingosides, in which fatty acids are linked to sphingosine by a peptide bond, and lastly, lactams; this result is relevant to the interpretation of the infra-red spectra of irin since some lactams absorb at 1740 cm^{-1}, where all irin residues examined so far have an intense carbonyl absorption.

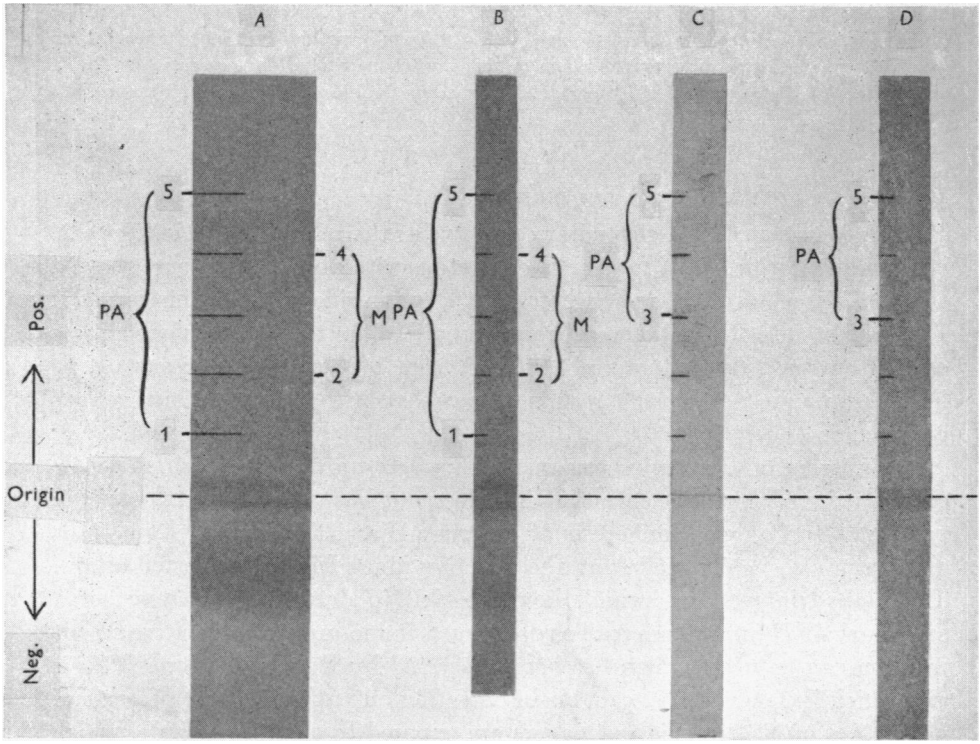


Fig. 3. Colour tests on electrophoresis papers (details in text). Acetone-treated extracts were applied as a line at the origins ($\equiv$ 15 mg/cm in *A* and *B*; $\equiv$ 6·6 mg/cm in *C* and *D*). The brackets PA show the location of the pharmacological activity after electrophoresis (M = maximum); assays on rat colons. These irin-containing areas remained unstained by *A*, ClO_2 and benzidine, a test for NH_2 and NH; *B*, anisidine HCl, test for sugars; *C*, Schiff's reagent alone, test for aldehydes; *D*, Schiff's reagent + $HgCl_2$, test for acetals.

Exclusion of aldehyde and acetal groups. Iris extracts contain an acetal-like impurity. In the presence of a trace of $HgCl_2$, which is known to catalyse the hydrolysis of acetals with the liberation of free aldehydes (Hack, 1953), this impurity stains violet with fuchsin-sulphurous acid (Schiff's reagent). The violet colour was obtained with solutions of Type 1 iris residues, i.e. after the first stage of purification with 20 vol. of acetone, but not after the next stage of purification by re-extraction with absolute acetone (Type 2 residues). When crude iris extracts were purified by partition into ether, $\equiv$ 5 and $\equiv$ 10 mg of purified, pharmacologically active material did not give the violet coloration,

whereas ≡ 2·5 mg of the original extract did. Lastly, paper electrophoresis showed that the Schiff-staining impurity remained adsorbed at the origin. When papers, on which irin had migrated towards the anode, were sprayed with the fuchsin-sulphurous acid reagent, a faint band was seen only at the origin (Fig. 3, paper *C*). On re-spraying the parallel paper of Fig. 3*D* with the same reagent, now with added $HgCl_2$, the colour of this band deepened rapidly but still remained confined to the origin. The pharmacologically active area of the paper did not stain at any time. Since glucose 'spots' applied as neutral markers to the origins of electrophoresis papers are shifted endosmotically some 0·6–2·25 cm towards the negative pole (Ambache, 1957*a*), the location of the present violet bands exactly at the origin suggests an adsorption of the Schiff-staining material to the paper. It is therefore unlikely, from these experiments, that irin is a fatty aldehyde, either free or condensed as an acetal.

Exclusion of a glycoside. The pharmacologically active areas of electrophoresis papers did not stain when sprayed either with aniline hydrogen phthalate (Partridge, 1949) or with anisidine hydrochloride (Hough *et al.* 1950; Hofman, 1953) although these reagents revealed the presence of carbohydrate elsewhere on the papers, as brown bands at the origin and on the negative side (the latter probably due to endosmotic shift).

Evidence that irin is an unsaturated hydroxy-fatty acid

The evidence that irin is an unsaturated hydroxy-fatty acid is based upon the following properties and colour reactions which are given by extracts purified in various ways, and upon the destruction of the pharmacological activity by reagents which have the capacity to bind either with alcoholic hydroxyl or with carboxyl groups, or which attack double bonds.

Colour test for carboxylic acids. In the presence of uranyl ions, the higher fatty acids form benzene-soluble red addition compounds with the dye Rhodamine B which, now in its quinone-form, becomes fluorescent (Feigl, 1956, p. 461). This test was performed, as described in Methods, on several batches of irin purified into ether at acid pH and by two or three further re-extractions of the residues with ether. With ≡ 45–100 mg of such Type 3 iris residues, dissolved in benzene, a pink colour, seen better as a distinct orange fluorescence in ultra-violet light, appeared in the benzene layer.

Colour test for hydroxy-fatty acids. Hydroxy-fatty acids react with freshly prepared 0·5% solutions of *sym*-diphenylcarbazide in tetrachloro-ethane to form a red colour (Feigl, 1956, p. 483); in the present experiments $<100\,\mu g$ of Na ricinoleate and of δ-dodecalactone (alkali-treated) could be detected by this test, modified as described in Methods. This colour reaction was given by the following types of active irin residues, dissolved in de Jalon's fluid: (*a*) a Type 2, acetone-purified residue; (*b*) several different batches of irin purified by ether-partition (Type 3 residues), some purified further by re-extractions with ether; (*c*) two Type 3 residues, further purified by dissolving in de Jalon's

fluid and repartitioning with ether at acid pH. A noticeable colour difference between the irin tubes and the blanks, containing reagent and de Jalon's fluid alone, could, in one experiment, be detected after 35 min with as little as ≡ 4 mg (about 2 μg) of material. Eventually, however, some colour develops in the blank, and it is therefore easier to carry out this test with larger amounts of irin. Thus with ≡ 20–30 mg the intensity of this pink, faintly purple, colour in the irin tube was greater and its onset could usually be detected within 10 min.

This colour was not due to lactic acid since a parallel test on one of the above batches of ether-purified irin failed to detect any lactic acid ($< 3\mu$g in ≡ 100 mg residue); it is known that in water–ether partitions lactic acid is 10–12 times more soluble in water than in ether, even in the presence of 0·5 N-H_2SO_4 (Seidell, 1941), and would therefore be left behind in this purification.

The colour tests with *sym*-diphenylcarbazide were repeated on eluates from a heavily loaded electrophoresis paper (see Methods). After electrophoresis in phosphate buffer and location of the pharmacological activity on the dry paper, a 2 cm width of the paper was cut into successive transverse 1 cm slices; each slice was then eluted with 1 ml. of de Jalon's fluid. The colour tests were carried out in a row of tubes each receiving equal 0·5 or 0·8 ml. samples of these successive eluates and 0·1 ml. of 0·5 % *sym*-diphenylcarbazide solution in tetrachloro-ethane. The maximum colour (in the tetrachloro-ethane layer) corresponded to the tube containing the maximum pharmacological activity (≡ 26–33·6 mg by assay).

The relative insolubility of irin in petroleum ether. Hydroxy-fatty acids and lactones are known to be much less soluble in petroleum ether than in other organic solvents. In fact, when petroleum ether was used for partitions the recovery of irin was ≪25 %. In an experiment in which the same iris extract was partitioned successively at pH 3·30, first with 1 vol. of petroleum ether A.R. (b.p. 40–60° C), and then again with 1 vol. of diethyl ether, the recovery of activity was 5 % from the petroleum ether and 50–55 % from the diethyl ether. Irin was therefore at least 10 times more soluble in diethyl than in petroleum ether.

Type 3 residues of irin were also relatively insoluble in petroleum ether. When, for instance, such a residue was extracted with 1 ml. of petroleum ether (b.p. 40–60° C) per '≡ 100 mg' the activity of the petroleum ether-insoluble fraction was 10 times greater than that of the petroleum ether-soluble fraction. However, with a Type 2 residue the reverse result was obtained, 10/11th of the activity being found in the petroleum ether-soluble fraction; this may be due either to a true difference between the two types of irin or to a solvent effect of the impurities in the Type 2 residue.

Inactivation of iris extracts by phenyl isocyanate (OH-binding reagent). Phenyl isocyanate reacts with free alcoholic (or phenolic) hydroxyl groups to form phenyl urethanes as follows:

$$C_6H_5.N{:}C{:}O + ROH \longrightarrow C_6H_5NH.COOR.$$

(It can also react with amine groups, but these appear to be absent from the irin molecule.) Since in colour tests irin behaves as a hydroxy-acid, it should be possible to attack its OH group with phenyl isocyanate and to produce its phenyl urethane derivative, the latter possibly inactive. This expectation has been fulfilled and irin is in fact easily inactivated by exposure to phenyl isocyanate at room temperature. These experiments have been performed on unpurified aqueous extracts of iris; as is shown in Fig. 4, after such extracts are

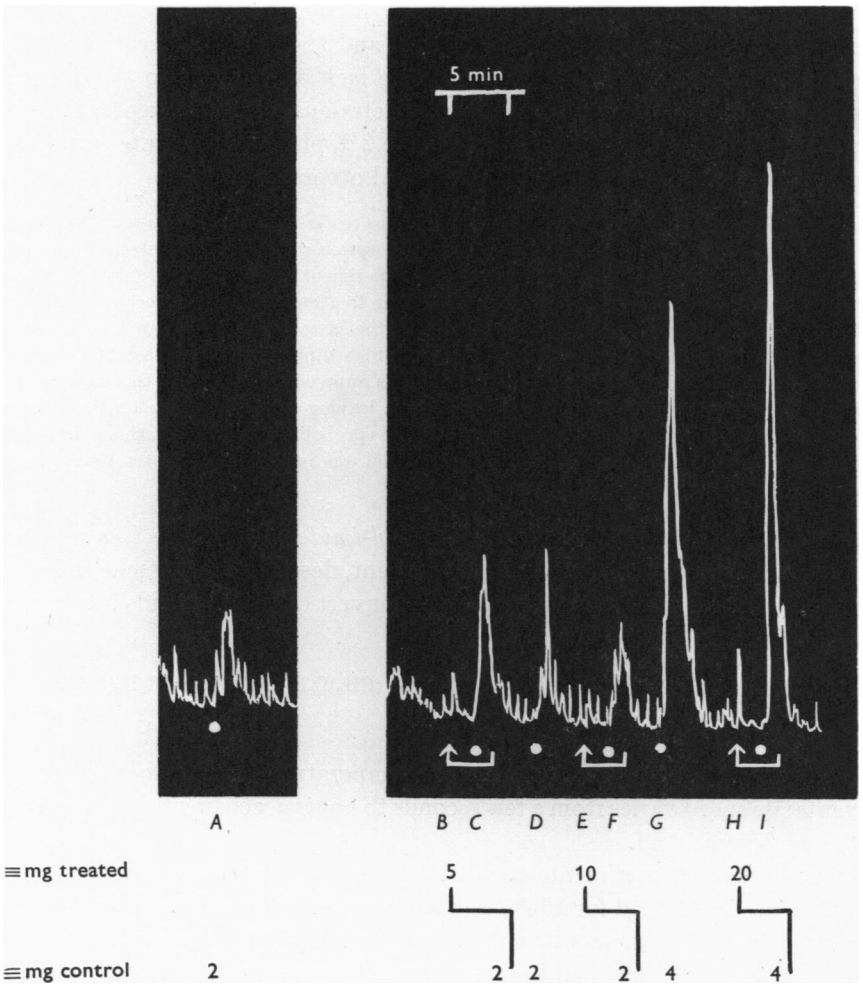


Fig. 4. Inactivation of iris extract by 2% phenyl isocyanate: assay on atropinized hamster colon. At the dots, doses of control sample of untreated extract administered by itself at *A*, *D* and *G*; contact periods 75 sec. At the arrows *B*, *E* and *H*, doses of treated extract, ineffective by themselves but not preventing the response to challenge doses of the control administered 90 sec later at *C*, *F* and *I* without changing the bath fluid for another 75 sec.

treated with 0·01–0·03 ml. of phenyl isocyanate per millilitre for about 1 hr, they can be assayed on the hamster colon without any adverse effect of the reagent on the preparation. This is because phenyl isocyanate is gradually hydrolysed to the almost insoluble diphenyl urea, which can be removed by centrifugation. In the experiment of Fig. 4, the preparation responded to ≡ 2 mg of control, untreated, extract at *A* and *D* and to ≡ 4 mg at *G*, but not to ≡ 5, 10 and 20 mg of the treated extract at *B*, *E* and *H* respectively. After failing to obtain any response for 90 sec with these doses of treated extract a challenge dose of the control was introduced into the organ bath. The appearance of a response now, at *C*, *F* and *I*, whilst the treated extract was still in the organ bath, showed that the lack of response to the doses of treated extract had not been due to any deleterious effect on the preparation. The loss of activity after this treatment with 2% phenyl isocyanate was therefore >90% in this experiment, as in several others.

In order to examine whether the loss of activity was due to a combination of irin with the reagent and not merely to its removal out of solution by adsorption on to the precipitate of insoluble diphenyl urea, the precipitate was emulsified in one experiment and injected into the organ bath without effect. In another experiment in which, after treatment with 3% phenyl isocyanate, more than 95% of the activity had disappeared from the extract, the precipitate was dried and extracted with petroleum ether (b.p. 80–100° C) in which diphenyl urea is insoluble; previous experiments had shown that irin in Type 2 residues is appreciably soluble in this solvent. The petroleum ether was evaporated in a weighing bottle, leaving a visible residue on the sides, of appearance different from that of diphenyl urea; this was probably the phenyl urethane derivative of irin. When reconstituted in 0·9% saline this material was inactive in doses ten times greater than those of standard.

Inactivation of irin by N,N'-carbo-di-p-tolyl-imide (carboxyl-binding reagent). The carboxyl-binding properties of this reagent, described by Zetsche, Lüscher & Meyer (1938), depend upon its ability to convert carboxylic acids into mono-acylated di-ureides as follows:

$$(CH_3)C_6H_5.N{=}C{=}N.C_6H_5(CH_3) + RCOOH \rightarrow (CH_3)C_6H_5.NH.CO.\underset{\displaystyle R.C{=}O}{\overset{\displaystyle |}{N}}.C_6H_5(CH_3).$$

The reaction can be carried out at room temperature in a number of organic solvents; its speed varies from a few seconds to hours according to the reactivity of the carboxylic acid.

In the present experiments parallel samples of Type 2 irin residues, dissolved in acetone, stood for 15–30 min at room temperature, some with added reagent (1 mg/ml.), some without. When the samples were assayed on the hamster colon after they had been evaporated to dryness and reconstituted in 1 ml./ ≡ 100 mg of de Jalon's fluid, it was found that the reagent had inactivated the treated samples to the extent of 45–60%. One of these experiments is illustrated in Fig. 5. The response at *A* to 0·12 ml. of the treated irin (≡ 12 mg) was matched by half that dose of the untreated irin, administered

at *C* by itself and at *B* together with 0·12 ml. of 'reagent control solution' prepared as described in Methods. A comparison of *B* and *C*, and later of *E* and *G*, showed that any traces of this water-insoluble reagent which might have been present in these solutions did not interfere with the assays.

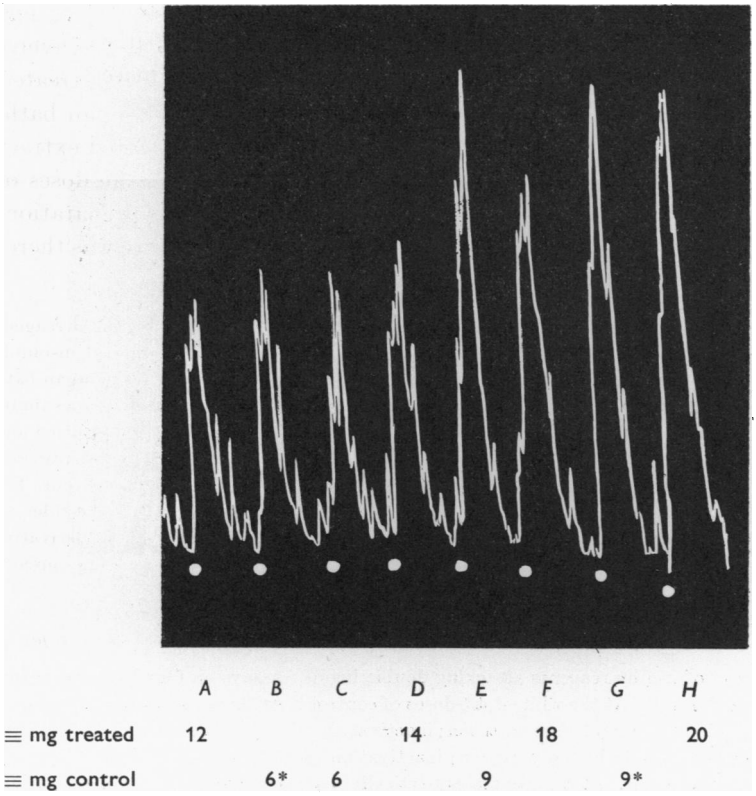


Fig. 5. Inactivation of irin by 0·1% *N,N'*-carbo-di-*p*-tolyl-imide (30 min at room temperature in acetone); assay on atropinized hamster colon. Treated irin at *A*, *D*, *F* and *H*, doses as indicated. Untreated irin administered alone at *C* and *E*, and together with the 'reagent control solution' (for details see text) at *B* and *G* (asterisks).

Repeating the assay at a higher dose level, the response to 0·18 ml. of treated irin (≡ 18 mg) at *F* was less than that to ≡ 9 mg of the untreated irin administered at *E* by itself and at *G* together with 0·18 ml. of the reagent control solution. The inactivation in this experiment was therefore 55%.

Inactivation was not significantly greater with higher concentrations (1–4%) of the reagent or with longer exposures. Infra-red spectra of acetone-purified irin suggest that it may consist of a mixture of hydroxy-acid and of lactone (see below). In the above reaction with the carbo-di-imide it is to be expected that any irin present in the lactone form, i.e. without a free carboxyl group, would be spared.

Evidence of unsaturation: inactivation by procedures attacking double bonds. The method of staining unsaturated compounds on paper by exposure to OsO_4 vapour (Hack, 1953; Vogt, 1957*b*) was insufficiently sensitive to reveal the minute quantities of irin in the active areas of electrophoresis papers. The evidence that irin is unsaturated is based on its inactivation by oxidation with $KMnO_4$, by catalytic hydrogenation and by halogenation with IBr (or by chlorine gas). When tested on the atropinized hamster colon, iris extracts treated with these reagents were either inactive or possessed a mere fraction of their original activity.

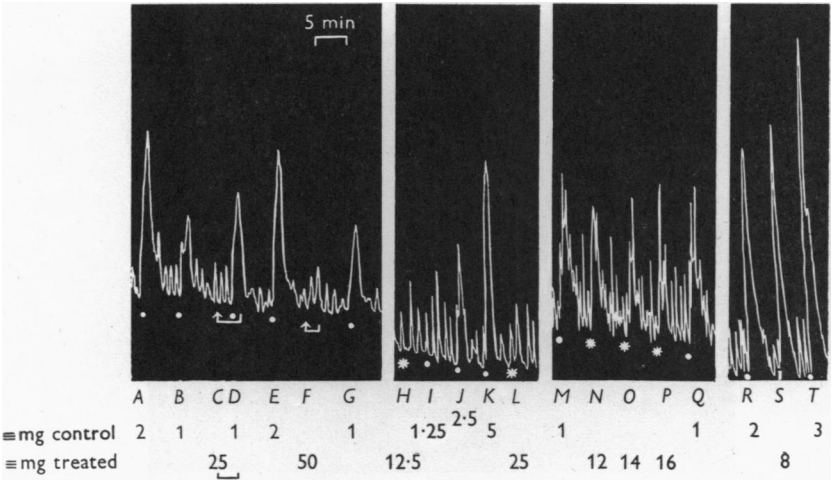


Fig. 6. Inactivation of irin by reagents attacking double bonds. Assays on four hamster colons (±atropine 10^{-7} g./ml.) At the white dots, doses of controls. At the arrows *C* and *F*, sample exposed for 5 min to $KMnO_4$ before partition; inactivation > 98%. At the asterisks, samples exposed to IBr for 30 min before partition; inactivation > 90% in *H–L* and 94% in *M–Q* from another experiment. At *S* a sample catalytically hydrogenated for 1¾ hr; inactivation nearly 75%. For details see text.

The destructive effect of $KMnO_4$ could be shown in two ways: (1) by comparing the activities of duplicate chromatograms of irin, one with and one without 1% $KMnO_4$ applied to the origins before chromatography; although $KMnO_4$ remained at the origins, irin could not be recovered from three such treated chromatograms, whereas in their untreated control duplicates it was found at its usual R_F; (2) by treating aqueous iris extracts with 0·05% neutral $KMnO_4$ for 5 min before partitioning with 2 vol. of ether at acid pH, in order to separate the irin from $KMnO_4$, which stays in the aqueous phase. Whereas normally in partitions at pH 3–4 with only 1 vol. of ether >60% of the activity can be recovered from the ether phase (see Fig. 1), after $KMnO_4$, even on partition with 2 vol. of ether, recovery was virtually nil in three experiments.

one of which is shown in Fig. 6 *A–G*, and 28% when the $KMnO_4$ was reduced to 0·01% and the treatment time to 3 min.

In the experiments on catalytic hydrogenation, extracts were bubbled for 1–2 hr with hydrogen in the presence of a palladium catalyst. It was not necessary to partition before assay since the traces of catalyst did not seem to affect the colon adversely; if anything, they seemed to increase the sensitivity of the colon to control irin solutions. Hydrogenated extracts showed a 50–90% loss of activity when assayed against unhydrogenated controls containing the same amounts of catalyst (Fig. 6 *R–T*).

With iodine monobromide as with $KMnO_4$ it was necessary, before assay, to separate the treated irin from the reagent by partition with 2 vol. of ether. Six extracts treated with 0·0019–0·005 M-IBr (0·03–0·083%) for 10–30 min showed a loss of 56–95% of their activity when compared with that of controls prepared as described in Methods. Two of these experiments are illustrated in Fig. 6 *H–Q*.

The results with these three procedures are fully concordant and provide a strong indication for the presence of at least one essential double bond in the irin molecule. Of the three procedures the reaction with IBr is the most informative, since it serves to exclude one possible double-bond position. It is known that (in 30 min) IBr will not halogenate double bonds in the $\alpha\beta$ position, i.e. when immediately adjacent to carbonyl groups, both in cyclic unsaturated lactones (Cavallito & Haskell, 1946) and in straight-chain $\alpha\beta$-unsaturated acids (Caldwell & Piontkowski, 1934). An $\alpha\beta$-unsaturated acid or lactone is thus excluded by the fact that IBr inactivates irin.

The possibility that there is more than one double bond in irin has to be kept in mind. However, an example will be given below of a singly unsaturated hydroxy-acid, ricinoleic, which has strong muscle-contracting properties. There is, therefore, no need to invoke at present more than one double bond and there has not been any evidence so far, in infra-red and ultra-violet absorption studies, for the presence of conjugation, but these do not exclude the possibility of a

$$CH{=}CH.CH_2.CH{=}CH$$

type of structure. There has also been no evidence of a triple bond, which excludes certain acids found in some vegetable oils such as hydroxy-ximenynic acid (Ligthelm, 1954); in fact, acetylenic acids are unknown in animal tissues, but for their activity see Ambache & Reynolds (1959).

Inactivation of irin by alkali. Irin is inactivated by standing in NaOH at room temperature. Both with crude, distilled-water extracts of rabbit iris and with solutions of acetone-purified irin (Type 2 residues), the loss of pharmacological activity after treatment with $\frac{1}{10}$th vol. of N-NaOH for about 3 hr amounted to 66–80%. Parallel samples of the same extracts or irin solutions treated with $\frac{1}{10}$th volume of 0·8–1 N-HCl for $3\frac{1}{4}$ hr lost only 20–30% of their activity. In the experiment of Fig. 7 the samples of extract were treated with acid or with alkali at room temperature for $3\frac{2}{3}$ hr. The inactivation of the acid-treated sample was $<20\%$ (Fig. 7*C*). The inactivation of the alkali-treated sample was determined twice; it was 83% when assayed on the rat colon

preparation of Fig. 7 and 66–75% in a second assay on a hamster colon. In another experiment the control, untreated, irin was dispensed into a weighing bottle containing the same volumes of NaOH and of neutralizing HCl as had been added to the treated sample, and both samples were assayed on a hamster colon preparation; the destruction by NaOH was 62% in 4 hr.

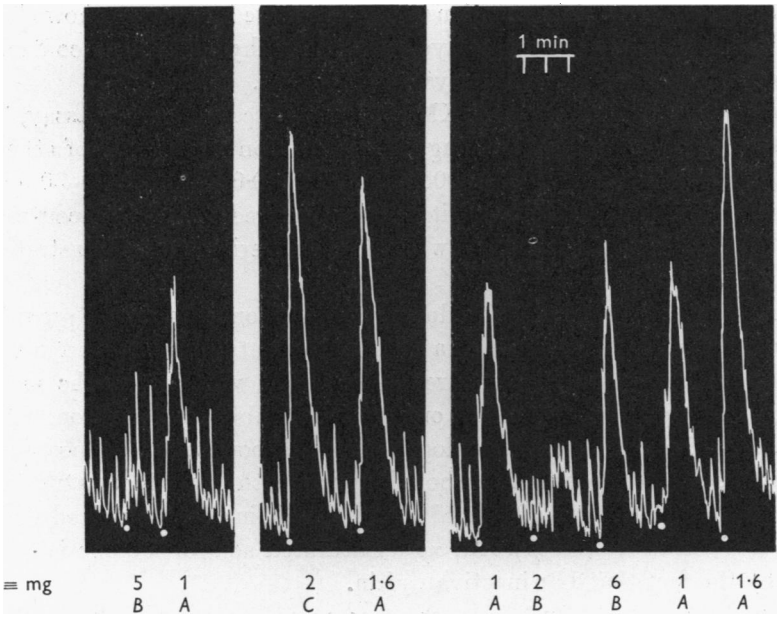


Fig. 7. Inactivation of irin by alkali at room temperature: assay on rat colon (atropine + LSD). Solution *A*: untreated control sample of rabbit iris extract (≡ 100 mg/ml.). Solution *B*: parallel sample, after treatment with 1/10th vol. of N-NaOH for 3¾ hr and neutralization. Solution *C*: sample treated with 1/10th vol of 0·8N-HCl (pH 1) for 3¾ hr and neutralized. Before testing, solutions *A*, *B*, and *C* were diluted 1 in 5 in de Jalon's fluid. Doses in ≡ mg given below. Contacts 90 sec.

In several experiments samples of extracts or of irin solutions were sealed in glass ampoules with or without added NaOH or HCl. The ampoules were left in a boiling water-bath for 10–30 min. In all these experiments there was a loss of activity even without addition of acid or alkali (pH 6–7), amounting to 40% in 10 min and 60–70% in 30 min; this was increased to 85–96% both by acid and by alkali. Since we know that irin is an unsaturated compound, these destructions could have been due partly to oxidation.

It is a well known property of some unsaturated fatty acids that in alkali they undergo isomerization at double bonds, which shift into positions nearer the carboxyl groups. For instance Gabr (1954) found that the smooth-muscle-contracting activity of octadec-3-enoic acid was considerably reduced after heating with alkali, probably as a result of isomerization. The inactivation of irin by alkali might perhaps be explained by isomerization (or by racemization) to an inactive derivative.

Pharmacological actions of some hydroxy-acids and lactones

Rat colon preparation

Before it was realized that irin was unsaturated, the following types of saturated hydroxy-acids were tested on the atropinized rat colon preparation and failed to elicit contractions in the doses stated in brackets: (1) as a prototype of α-hydroxy-acids, sodium lactate (70–140 μg) (α-hydroxy-stearic was inactive in a test on the hamster colon); (2) as a β-hydroxy-acid, sodium β-hydroxybutyrate (50–200 μg); (3) as a γ-lactone, butyrolactone (200 μg); 4) as a δ-lactone, β-hydroxy-β-methyl-δ-valerolactone (approx. 2·5–8 mg). A 50 μg dose of sodium citrate was inactive and 200 μg produced a slight relaxation.

Interaction with irin. Irin contractions were neither depressed nor potentiated by γ-butyrolactone or by α-hydroxy-ββ-dimethyl-γ-butyrolactone in 200 μg doses.

Hamster colon preparation

The aromatic *p*-hydroxybenzoic (20–400 μg neutralized) and 3,4-dihydroxybenzoic (20–200 μg) acids were inactive.

Unsaturated hydroxy-fatty acids. It has been shown on p. 274 that irin is an unsaturated hydroxy-acid, probably of high-chain length. Acetylenic hydroxy-acids aside, only three naturally occurring hydroxy-fatty acids of known constitution have been described so far, all of vegetable origin. They are (+)-ricinoleic acid and the *iso*-ricinoleic (+)-9-hydroxy-*cis*-octadec-12-enoic acid from *Strophanthus sarmentosus* seed oil (Gunstone, 1952), both of which have one double bond, and kamlolenic acid, which has three; kamlolenic acid was unobtainable.

The activity of (+)-*ricinoleic* (12-*hydroxy-cis-octadec*-9-*enoic*) *acid.* The oldest known and best characterized of these vegetable acids is (+)-ricinoleic acid, $CH_3(CH_2)_5CHOH.CH_2.CH{=}CH(CH_2)_7COOH$. Although considerably less active than irin, it is capable of producing graded contractions of the atropinized hamster colon and can be assayed satisfactorily on this muscle, as shown in Fig. 8. The figure also illustrates in two ways the fact that the hydroxyl group in the molecule of ricinoleic acid is essential to its pharmacological activity. First, although the threshold dose of sodium ricinoleate was 6·6 μg in the second experiment of Fig. 8 (see *S*) and less than 9·4 μg in the first (*F*), there was no response in either of these experiments to 44·5–89 μg of sodium oleate (see *B* and *C*) nor, in a third experiment, to 100 μg but 200 μg produced a minute contraction. Ricinoleic acid is therefore at least ten times more active than oleic. Now in their chain length and in the position of their *cis* double bond, oleic and (+)-ricinoleic acids are identical; the only difference between them is the hydroxyl group on carbon atom 12 in (+)-ricinoleic acid.

This confers optical activity upon the ricinoleic molecule, which has recently been shown to have the D-configuration (as in D-glyceraldehyde). Secondly, as with irin, sodium ricinoleate was inactivated by the hydroxyl-binding reagent phenyl isocyanate (*L* and *N*), showing again the dependence of activity upon the presence of the hydroxyl group in this fatty acid. An experiment on a preparation of guinea-pig ileum taken 35 cm above the ileo-caecal valve showed that this muscle was at least ten times less sensitive than the hamster colon to the same solution of Na ricinoleate.

The following properties distinguish irin from (+)-ricinoleic acid. The activity of irin on the hamster colon preparation is at least 6–18 times greater than that of Na ricinoleate. Thus in Fig. 8 the effect of 18·8 μg of ricinoleate at *Q* was matched in height at *R* by ≡ 3 mg of iris extract, a dose which, according to the data given in Table 1, would correspond to 1–3 μg, or less, of

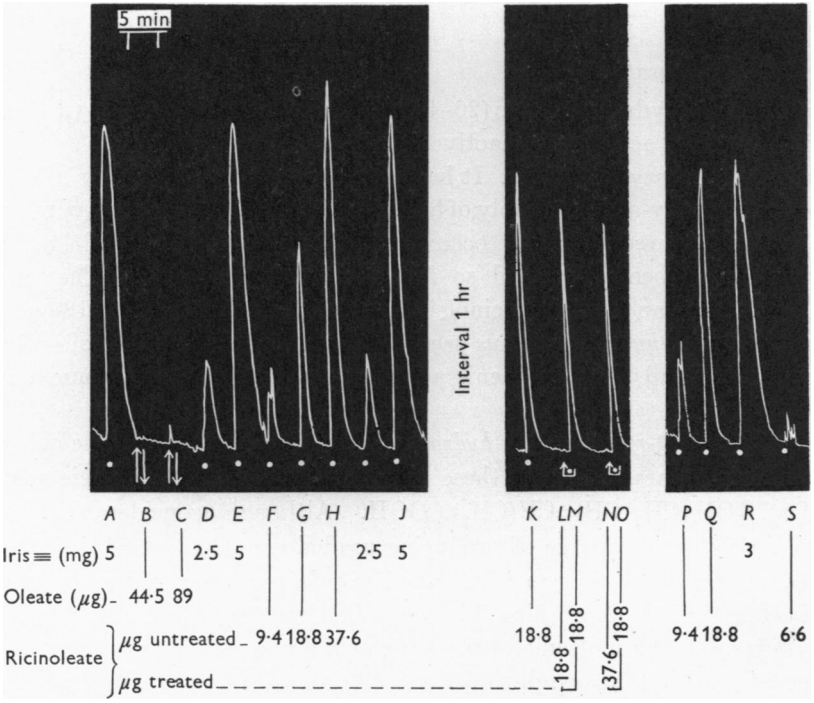


Fig. 8. Activity of Na ricinoleate and its assay on atropinized hamster colons. *A*, *D*, *E*, *I*, *J* and *R*, rabbit iris extract. Although 44·5–89 μg Na oleate was inactive (*B* and *C*), 9·4–37·6 μg Na ricinoleate produced graded contractions (*F*, *G*, *H*, *K*; and *P*, *Q*, *S*). Second panel: inactivation of ricinoleate by phenyl isocyanate. At the arrows: *L*, 18·8 μg and *N*, 37·6 μg of treated ricinoleate, now inactive but not preventing the response to 18·8 μg of untreated ricinoleate 60 sec later at *M* and *O*. Third panel: another preparation, showing broader response to iris extract (*R*) than to ricinoleate (*Q*); ricinoleate threshold at *S*. Contacts 90 sec in the first and third panels and 60 sec in the second.

purified irin. Although the contact period was 90 sec in both cases, relaxation after the irin contraction was slower than after the ricinoleate, giving rise to a larger contraction area on the tracing (compare also *H* with *A* and *E*). The first panel of Fig. 8 also shows that the slope of the dose–response curve for the two substances is different, since doubling the dose of irin produces the same increase in height of contraction as quadrupling the dose of ricinoleate.

Ricinoleic acid is a liquid at room temperature, whereas Type 3 ether-purified irin (i.e. in the acid or 'lactone' form) is a soft solid, which suggests a difference in double bond position (Δ). In ricinoleic as in oleic acid the double bond is exactly in the middle of the 18C chain (Δ9:10) and the uninterrupted sequence of seven consecutive methylene groups on either side of the double bond is inadequate to confer solidity. The melting points of these two *cis* acids are: oleic, α-form, 13·4° C and β-form, 16·3° C; ricinoleic, three forms melting at 5·0, 7·7 and 16° C (Hilditch, 1956). The effect of double-bond position upon the melting point is well established in the series of 'positional' *cis* isomers of oleic acid, in which the melting point increases as the double bond is nearer the carboxyl group. Thus with Δ6:7 octadecenoic the melting point is 30° C; with Δ4:5, it is 52·5° C; with Δ3:4, 56·5° C and with Δ2:3, 59° C (Markley, 1947, table 6; Deuel, 1951, table 14). This would suggest that in irin, which is a solid, the double bond may be nearer the carboxylic end of the molecule.

Chromatography. Ricinoleic acid was chromatographed for 2–2½ hr on Whatman No. 1 paper in ethyl methyl ketone-diethylamine-water (60:3:20 v/v), the solvent already used for the chromatography of irin (p. 263). As with irin, ricinoleic was located on the dried chromatograms by assay of successive slices of the paper. The activity was found at R_F 0·875–1 in one experiment (in nitrogen) and at R_F 0·73–0·89 in another (Fig. 9, in air). These values are within the R_F limits found for irin in the same solvent (0·8–0·97). In the experiment of Fig. 9 a spot of 235 μg of ricinoleic acid was applied to the origin in 0·0025 ml. acetone; the paper was dipped after ¾ hr equilibration and the solvent was allowed to ascend for 2½ hr in an atmosphere of air.

The use of various methanol-water mixtures as solvents for the chromatography of fatty acids on Whatman No. 1 paper has been described by Savary (1954). In 70% methanol the R_F of ricinoleic acid was 0·74–0·86. In 60% methanol its R_F was 0·58–0·77; irin was found slightly higher, at R_F 0·79–0·92. In 50% methanol the R_F of irin was 0·82–0·89; ricinoleic acid was not recovered.

Stability in NaOH. Samples of sodium ricinoleate solutions were left in 0·1 N-NaOH for 2–5 hr at room temperature; they were then neutralized with an equivalent volume of N-HCl, and assayed on atropinized hamster colons against untreated samples of the same solutions. Even after 4 and 5 hr standing in alkali the recovery of activity was between 90 and 100%. Thus ricinoleic acid appears to be much more stable than irin in alkali. Again, this may be due to a difference in double-bond position; the tendency to isomerize is greater when the double bond is close to the carboxyl group, and is particularly marked in the Δβγ position.

Threo-12:13-*dihydroxy-oleic acid* (*m.p.* 52° C). Derived from vernolic acid, a naturally occurring epoxy-acid (Gunstone, 1954), 12:13 dihydroxy-oleic differs from ricinoleic acid only by having an extra hydroxyl group on carbon atom 13. A solution of its sodium salt contracted the hamster colon but its activity was less than $\frac{1}{2}$–$\frac{1}{3}$ that of Na ricinoleate. In subthreshold doses it potentiated irin contractions.

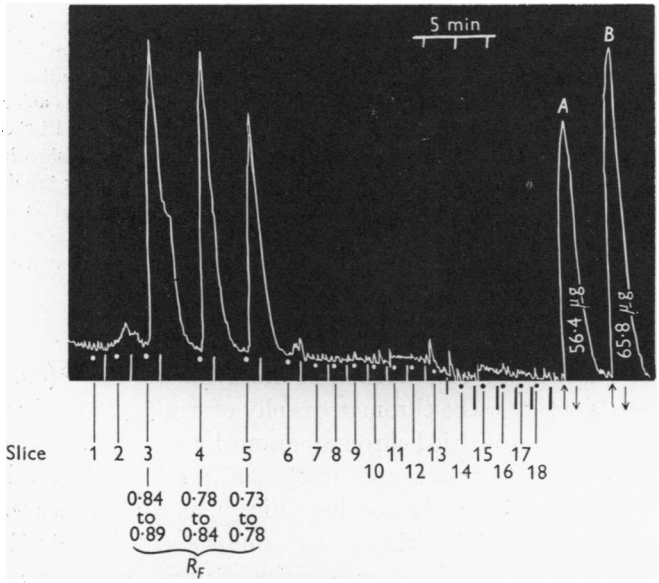


Fig. 9. R_F of ricinoleic acid chromatographed ascendingly in ethyl methyl ketone:diethylamine: water (60:3:20). Location of activity at R_F 0·73–0·89 by assay of successive transverse slices of a chromatogram on a hamster colon in atropine. Starting from the solvent front (17·9 cm), slice 1 corresponds to cm 17·9–17; all subsequent slices (at the dots) 1 cm downwards; each slice was left in the organ bath for 2 min. At *A* 56·4 µg and at *B* 65·8 µg of Na ricinoleate administered for 2 min.

(+)-9-*hydroxy-cis-octadec*-12-*enoic* (*iso-ricinoleic*) *acid*. Isolated from *Strophanthus sarmentosus* seeds by Gunstone (1952), this acid can be considered as ricinoleic acid with the hydroxyl and double-bond positions transposed. Its activity was $\frac{1}{5}$th or less that of ricinoleic.

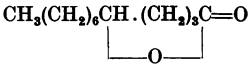
Lactones. To the effect of lactones and their interaction with irin particular attention was paid when it was suspected that irin might be capable of forming a lactone when acidified with HCl for partition. Infra-red spectra suggest that irin is then in the form of a long-chain unsaturated δ-lactone. The following δ-lactones were therefore examined, and some unsaturated γ-lactones as well (Ambache, 1957*c*). Most of these compounds (see Table 2) had negligible side chains, but were nevertheless useful for testing the activity of various types of unsaturated lactone rings.

A. *Saturated δ-lactones.* (1) *δ-dodecalactone.* This fatty lactone differs from irin in being a liquid; its structure is given in Table 2 (formula 1). As shown in Fig. 10, δ-dodecalactone failed to stimulate the atropinized hamster colon in doses of 20–100 μg. Moreover, although the dodecalactone did not relax the preparation or inhibit its rhythmic activity when administered by itself,

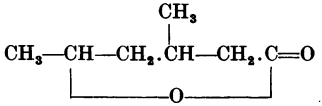
TABLE 2. Types of lactone tested

A. *Saturated*

1. δ-Dodecalactone

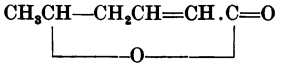


2. β-Methyl-δ-hexanolactone

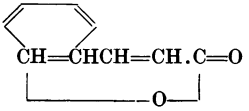


B. *αβ-Unsaturated*

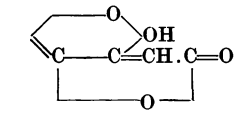
3. Δαβ-δ-Hexenolactone



4. Coumarin

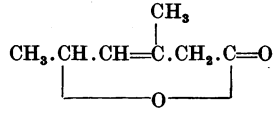


5. Patulin

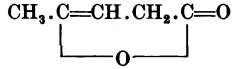


C. *βγ-Unsaturated*

6. β-Methyl-Δβγ-δ-hexenolactone

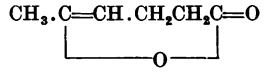


7. Δβγ-Angelicalactone



D. *γδ-Unsaturated*

8. Δγδ-δ-Hexenolactone



it clearly interfered with the response to challenge doses of irin administered 1 min later, whilst the lactone was still in the organ bath. The irin responses were reduced by 20 μg of the dodecalactone and abolished by 60–100 μg, but, as is shown in Fig. 10, recovered fully within 3–4 min after the lactone was removed by a change of bath fluid. On unatropinized hamster colon preparations 100 μg of dodecalactone halved the response to 0·04 μg of acetylcholine but completely blocked equivalent doses of irin; 200 μg blocked acetylcholine as well.

These results show that δ-dodecalactone does not satisfy certain structural requirements possessed by the irin molecule. The presence in dodecalactone of a long, lipid-soluble methylene chain together with a saturated 6-membered *delta*-lactone ring is evidently not enough to confer upon it muscle-contracting properties. Since, as we have seen, irin is unsaturated, the presence of a double bond, either inside the potential lactone ring (see Appendix) or outside it, would appear to be essential to its activity. Nevertheless, there appears to be sufficient resemblance between dodecalactone and irin to explain the antagonism described above.

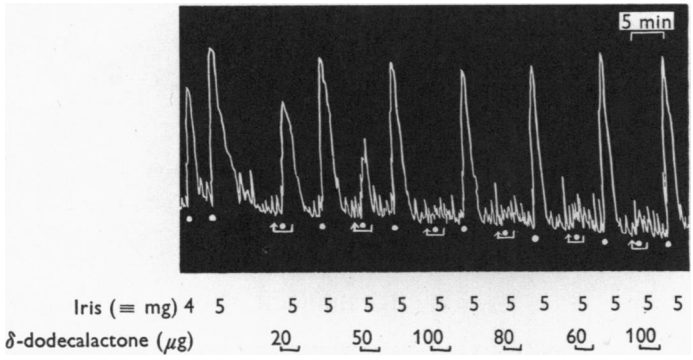


Fig. 10. Hamster colon in 10^{-7} atropine (5 ml. bath). Suppression of irin contractions by δ-dodecalactone. At the white dots, iris extract ≡ 5 mg except for the first contraction (≡ 4); 90 sec contacts. At the arrows, δ-dodecalactone administered 1 min before the extract; doses as shown.

(2) *β-methyl-δ-hexanolactone.* This compound (Table 2, formula 2) failed to stimulate and did not interfere with irin contractions when it was present in the organ bath.

B. *αβ-unsaturated lactones.* It has been mentioned already that the essential double bond in irin cannot be in an αβ position, because irin is inactivated by iodine monobromide. As was to be expected, αβ-unsaturated lactones, whether *delta* or *gamma*, failed to stimulate the atropinized hamster colon preparation.

The simplest available αβ-unsaturated *delta*-lactone was L-Δαβ-δ-hexenolactone (Table 2, formula 3). In doses of up to 400 μg this was inactive by itself; 100–400 μg had little effect upon irin contractions in one experiment, but 400 μg potentiated slightly in another. Racemic Δαβ-δ-hexenolactone had no effect in three experiments.

Coumarin (Table 2, formula 4) produced some block of irin contractions; Na coumalate had no effect.

The αβ-unsaturated *gamma*-lactone patulin (Table 2, formula 5), inactive by itself, also reduced irin contractions not only when it was present in the bath, but also for some time afterwards (see also Eliasson, 1958).

C. *βγ-unsaturated lactones.* Reasons will be given in the Appendix for

believing that acid-treated irin may be a $\beta\gamma$-unsaturated *delta*-lactone. Unsubstituted prototypes of such a structure are not, however, available. The substituted β-methyl-$\Delta\beta\gamma$-δ-hexenolactone (Table 2, formula 6) was inactive by itself in doses of up to 300 μg, and its potentiation of irin contractions was small and irregular. This may be due to the β-methyl substituent group which, at least in unsaturated $\Delta\beta\gamma$-γ-lactones, suppresses the reactivity

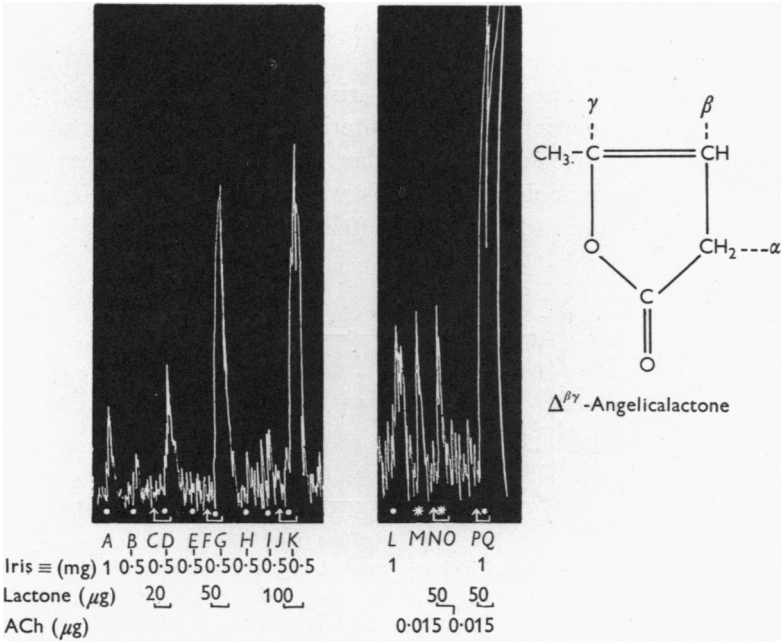


Fig. 11. Hamster colon, suspended in 5 ml. bath. Potentiation or irin by $\Delta\beta\gamma$-angelicalactone. At the white dots, iris extract: ≡ 1 mg at *A*, *L* and *Q*; ≡ 0·5 mg at *B*, *D*, *E*, *G*, *H*, *I* and *K*; contact periods of 90 sec. At the asterisks, acetylcholine 0·015 μg at *M* and *O*; contacts 30 sec. At the arrows, $\Delta\beta\gamma$-angelicalactone, 20 μg at *C*, 50 μg at *F*, *N* and *P*, and 100 μg at *J*, administered 1 min before *D*, *G*, *O*, *Q* and *K* respectively.

of the unsaturated lactone ring. Thus Cavallito & Haskell (1945) have shown that the capacity to react with cysteine, by addition of its thiol group to the double bond, is abolished by β-substitution both in $\Delta\alpha\beta$- and $\Delta\beta\gamma$-γ-lactones.

Although $\Delta\beta\gamma$-angelicalactone (Table 2, formula 7) has the double bond in the $\beta\gamma$ position, it is of course a *gamma*-lactone with a 5-membered ring. By itself this lactone did not contract the hamster colon preparation but in several experiments its presence in the organ bath enhanced, sometimes considerably, the effect of irin administered 1 or 2 min later. One of these experiments is shown in Fig. 11, which illustrates the graded potentiation of irin contractions by increasing doses of the angelicalactone. It also shows that the potentiation is specific to irin since acetylcholine contractions were hardly affected by the

lactone. The effect of the angelicalactone would seem to be in some way associated with the unsaturated lactone ring, since the sodium salt of its isomer, laevulinic acid, of which $\Delta\beta\gamma$-angelicalactone is the enol-lactone, did not potentiate irin.

D. *$\gamma\delta$-unsaturated δ-lactone.* $\Delta\gamma\delta$-δ-hexenolactone (Table 2, formula 8) was inactive by itself in doses of 25–400 μg. It potentiated irin in one experiment but not in three others.

Rabbit iris

Ricinoleic acid. The effect on the iris of injecting Na ricinoleate, dissolved in 0·9% NaCl solution, into the anterior chamber of rabbits anaesthetized with pentobarbitone sodium and ether was studied in four experiments. The pH of the Na ricinoleate solutions was carefully adjusted beforehand to 7·40–7·81. In doses of 100–200 μg (0·05–0·1 ml. of solution) Na ricinoleate

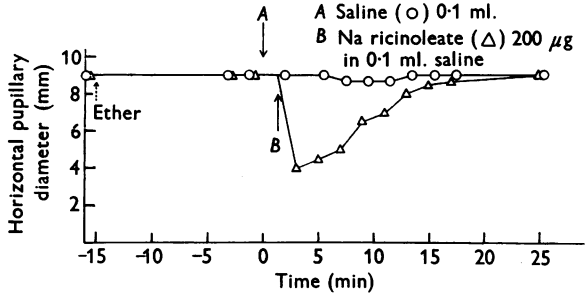


Fig. 12. Miotic effect of Na ricinoleate injected into the anterior chamber. Rabbit, 2·5 kg, premedicated with atropine, etc., and anaesthetized as described in Methods. Ether administered for 15 min before intraocular injections: at *A*, 0·1 ml. of 0·9% NaCl into right eye (○); at *B*, 200 μg of Na ricinoleate in 0·1 ml of 0·9% NaCl into left eye (△).

elicited pupillary constrictions of 2–5 mm which began within a few seconds of the injections. In each experiment the contralateral eye was injected in an identical manner (see Methods) with an equal volume of 0·9% NaCl solution of pH 7·3–7·49; these control injections did not constrict the pupil. One of these experiments is illustrated in Fig. 12, which shows the duration of the effect.

The fact that such large doses of ricinoleate are required for the production of miosis may be due either to a poor entry of this lipid-soluble substance into the tissues of the iris from the aqueous humour, or to the high albumin content (of the order of 0·02% or more) of the aqueous humour in rabbits, especially during ether anaesthesia. The effect of albumin is described below.

Interaction of plasma albumin with irin

It is known that serum albumins have the property of binding fatty acid and other anions by mechanisms which have been discussed extensively in a paper of Luck's (1949); the number of ions bound per mole of albumin is a function of chain length and can be as high as 25, from which it can be calcu-

lated that a given dose of albumin could bind about one-tenth its own weight of a long-chain fatty acid. The following results show that albumin does in fact interfere with the action of irin.

In the experiment of Fig. 13 concentrations as low as 10^{-5} of crystallized bovine plasma albumin, as supplied by Armour Laboratories, antagonized the effect of irin on the unatropinized hamster colon preparation at 30–31° C. Thus, 50 μg of albumin at *B* 'blocked' almost completely the effect of ≡ 3 mg of

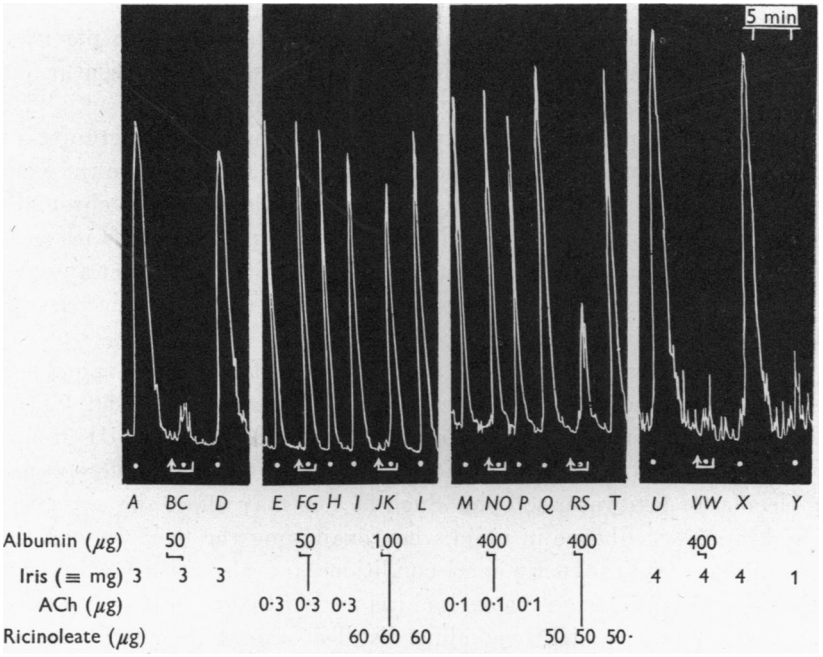


Fig. 13. Antagonism of crystallized plasma albumin (at the arrows) to irin: hamster colon; 5 ml. bath. Responses to iris extracts are reduced by 50 μg albumin at *C*, and by 400 μg at *W*, whereas acetylcholine (ACh) responses are unaffected at *G* and *O*; responses to Na ricinoleate are reduced by 400 μg albumin (*S*), but only slightly by 100 μg (*K*). All contacts 1 min, except for ACh (30 sec); albumin administered 1 min before the test doses. Between *L* and *M*, an interval of ł12 min.

irin (< 1–3 μg of active substance) administered 1 min later at *C*. The 'antagonism' was only temporary and within 3 min of washing out the albumin the response to irin was almost fully restored at *D*. The same 50 μg dose of albumin at *F* and even 400 μg at *N* did not affect the acetylcholine responses at *G* and *O* respectively. Na ricinoleate contractions were less susceptible to the albumin effect than were irin contractions. Thus, 100 μg of albumin only slightly reduced the response at *K*, but 400 μg of albumin did reduce the response to 50 μg of Na ricinoleate considerably, as is shown at *S*. At *W* 400 μg

of albumin virtually abolished the large response to ≡ 4 mg of iris extract; this response was smaller than the contraction at *Y* to ≡ 1 mg in the absence of albumin. In an experiment on a rat colon preparation (23–26° C) larger amounts of albumin (0·2–1 mg) were required to reduce irin contractions. These results suggest that irin would be ineffective if administered parenterally or into albuminous body fluids, such as the aqueous humour when plasmoid, unless it is given in such excess as first to saturate the binding capacity of the albumins.

DISCUSSION

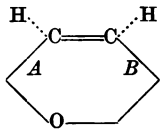
The solubility of irin in various organic solvents, its behaviour on partition, and its R_F, all suggested that it might be some sort of lipid. The present results show that it is an unsaturated hydroxy-fatty acid, and that its activity is dependent upon the intactness not only of hydroxyl and carboxyl groups, but also of at least one double bond. It should be possible to determine the exact location of the double bond(s) in irin by oxidative cleavage and chromatographic examination of the fission products. An attempt to do this with small quantities of irin on paper has been unsuccessful, but if much larger amounts of active material were used the more sensitive method of gas chromatography should yield the required information.

The presence of a hydroxyl group in an unsaturated fatty acid is valuable in increasing its water-solubility. However, should the hydroxy-acid form a lactone, then the internal esterification of its polar OH and COOH groups, both of which render it soluble in water, would result in a completely non-polar molecule far less water-soluble. Such factors may affect the extraction of irin and have to be borne in mind when examining the behaviour of this substance under certain experimental conditions and also its diffusibility in tissues. Irin is obtained from the excised iris merely by grinding the tissue in 10 vol. of neutral, or very slightly alkaline, distilled water at room temperature. This simple extraction procedure, adopted throughout the present work, avoids the disadvantages of other methods involving the use of heat, acids or alkalis, which might, for instance, change the chemical structure of an active substance normally present in the tissue; the possible conversion by alkali of semi-acetal to acetal phosphatidic acids (Vogt, 1957*a*) is a case in point.

It is possible that in the living tissue irin is present as an inert precursor, in which the pre-formed hydroxy-acid might be anchored either by its hydroxyl group, through an ester linkage, or by its carboxyl group, through either an ester or a peptide link. The biosynthesis of such a precursor raises a number of interesting possibilities. Much is already known about the metabolism and formation of unsaturated acids in living tissues by the action of dehydrogenases upon the coenzyme A esters of saturated fatty acids (see Stern, del Campillo & Raw, 1956, reaction scheme on p. 972). Thus enzymes exist for the introduction of double bonds not only in the 9:10 but also in the $\alpha\beta$ posi-

tions, and it is thought that the enzyme crotonase may catalyse the isomerization of $\Delta\alpha\beta$ to $\Delta\beta\gamma$ acids. When the structure of irin is more fully established, further work should be directed towards discovering the enzyme(s) and co-factors concerned in the manufacture and release of this substance in the iris tissues. More certain knowledge of its structure should also help further investigations of the part played by irin in various antidromic and axon reflex phenomena which have been observed in the rabbit's iris, and also in its reaction to injury. In these studies the fact that albumin interferes with the action of irin may be relevant; if irin is released in relatively protein-free tissue spaces, its action should be unhampered at first, but might later be curtailed by the onset of inflammatory processes and the appearance of plasma proteins in the tissue spaces.

Infra-red spectra of ether-purified irin suggest that in acid it may form a δ-lactone and that if a double bond is present inside this lactone ring it can only occupy the $\beta\gamma$ position. (The proximity of a double bond to the carboxyl group would raise the strength of such a fatty acid. According to Karrer (1946, p. 197) $\beta\gamma$-unsaturated acids are more strongly dissociated than $\Delta\alpha\beta$- or $\Delta\gamma\delta$-acids. Inside a lactone ring a double bond could have only the *cis* configuration. As shown in the diagram, each of its carbon atoms has two free valencies, one on either side



of the plane of the double bond. Since the position of two of the valencies (marked *A* and *B*) is fixed by the lactone ring to one side of this plane, the hydrogen atoms satisfying the other two valencies (dotted) have to be in the *cis* position, on the other side of it. Nearly all the acids so far obtained from animal fats have the *cis* configuration.) All available types of lactone ring have therefore been tested for activity and this structure–action study has also served to exclude certain double-bond positions. We have seen that the inactivation of irin by iodine monobromide precludes $\alpha\beta$-unsaturation; this receives corroboration from the fact that the three $\alpha\beta$-unsaturated lactones in Table 2, including $\Delta\alpha\beta$-δ-hexenolactone, did not have stimulant properties. Next, $\gamma\delta$-unsaturation appears to be excluded by the fact that $\Delta\gamma\delta$-δ-hexenolactone was inactive. With regard to the possibility of $\beta\gamma$-unsaturation it is interesting that $\beta\gamma$-angelicalactone potentiated irin, though incapable of eliciting contractions; it is, however, not a *delta* but a *gamma*-lactone. In fact, unsubstituted $\beta\gamma$-unsaturated δ-lactones have never yet been synthesized; the nearest approach to this type of structure is compound No. 6 in Table 2, which was inactive. This lactone, however, has a methyl group on the β carbon atom, which would radically alter its properties. Several instances are known in

which such β-methyl substitution in other βγ-unsaturated compounds (such as fatty acids or γ-lactones) suppresses their reactivity, either in the addition reaction mentioned on p. 283, or as a substrate for the enzyme crotonase.

It is perhaps significant that, whilst all the above lactones fail to stimulate, (+)-ricinoleic acid has fairly pronounced muscle-contracting properties on the hamster colon. The inactivation of (+)-ricinoleic acid by phenyl isocyanate and the fact that oleic acid is relatively inert, though differing from ricinoleic in respect of a single hydroxyl group, show again that this type of pharmacological activity is OH-dependent. Although (+)-ricinoleic acid bears sufficient resemblance to irin to affect probably the same receptors in the hamster colon, its activity is considerably weaker than that of irin, and that of *iso*-ricinoleic acid is weaker still. Thus, since positional rearrangement of the double-bond and OH groups in these fatty acids decreases their activity, it would appear that the exact spacing of these essential groups in the irin molecule is fairly critical.

SUMMARY

1. Rapid methods for the purification of irin, through ether, and for its assay on the hamster colon, are described.

2. In pharmacological tests the action of irin was not reproduced by the sulphonate isethionic acid.

3. Irin was not destroyed by alkaline phosphatase and could be distinguished from adenosine triphosphate by its pharmacological properties and from Darmstoff (*a*) by its high R_F (0·9) on chromatography in ethyl methyl ketone-diethylamine-water (60:3:20), (*b*) by its lability in alkali, and (*c*) by failure to stain with fuchsin-sulphurous acid in the presence of $HgCl_2$.

4. In tests for NH_2 and NH groups electrophoretically purified irin failed to stain with ninhydrin or with ClO_2-benzidine. In pharmacological tests various amino- and imino-acids were inactive, as well as some amphoteric purines.

5. In benzene solution ether-purified irin gave rise to a fluorescence with Rhodamine B in the presence of uranium ions, which is characteristic of higher fatty acids.

6. Smooth-muscle-contracting properties were not found in a survey which included several saturated, unsaturated and keto-fatty acids, a few dicarboxylic acids and some carboxylic steroids.

7. A pink colour, which is given by hydroxy-fatty acids with *sym* diphenylcarbazide, was obtained with irin purified through ether or by electrophoresis.

8. As expected of a hydroxy-acid, irin was inactivated by phenyl isocyanate, a hydroxyl-binding reagent, and by *N*,*N'*-carbo-di-*p*-tolyl-imide, a carboxyl-binding reagent.

9. Irin was inactivated by catalytic hydrogenation, by $KMnO_4$ and by IBr,

and is therefore unsaturated. The inactivation with IBr excludes an $\alpha\beta$ position for the double bond.

10. A number of hydroxy-acids and lactones have been tested for spasmogenic activity. Some interacted with irin, e.g. δ-dodecalactone inhibited, whilst $\Delta\beta\gamma$-angelicalactone potentiated, irin. The only compound with strong muscle-contracting properties was (+)-ricinoleic acid, which is also an unsaturated hydroxy-fatty acid (12-hydroxy-*cis*-octadec-9-enoic). *Iso*-ricinoleic acid and 12:13-dihydroxyoleic acid were considerably weaker.

11. Like irin, ricinoleic acid was inactivated by phenyl isocyanate. Oleic acid, which differs from ricinoleic only by the absence of the hydroxyl group on C12, was inactive. This type of activity is therefore OH-dependent.

12. On the hamster colon ricinoleic acid was 6–18 times less active than irin, and differed from irin both in its stability in alkali at room temperature and in R_F. On the rabbit iris Na ricinoleate acted as a weak miotic.

13. Crystallized bovine plasma albumin interfered with the action of irin on the hamster colon.

I am indebted to Mrs M. Reynolds for valuable assistance, to Drs E. M. Crook, S. P. Datta, C. Long, W. Vogt and V. P. Whittaker for much helpful discussion, and to Dr W. Feldberg for reading the manuscript.

APPENDIX

INFRA-RED SPECTRA OF IRIS EXTRACTS

With E. M. BRADBURY*

Fig. 14 shows the infra-red absorption spectrum of a film of irin (continuous line); superimposed are spectra of δ-dodecalactone and of (+)-ricinoleic acid. The irin was a material concentrated some 2720 times by partition into ether after acidifying with HCl at pH 2·98 (point ● in Fig. 1) and by further re-extractions of the ethereal Type 3 residue once with acetone and twice with ether (see Table 1); in this experiment the time of exposure to HCl during the partition was not measured but was probably longer than usual, as separation of the phases had to be assisted by centrifugation (see Methods, p. 256). For the preparation of the film 86 μg of material (from ≡ 236 mg of iris tissue) was dissolved in 0·85 ml. of ether and then applied drop-wise to a sheet of AgCl melt, forming, as the ether evaporated, a small circular spot of solid irin. The thickest part of this film was examined in an automatically recording double-beam infra-red microspectrometer, suitable for exceedingly small samples of this kind (Ford, Price, Seeds & Wilkinson, 1958).

We have seen that ether-purified irin gives a colour test (p. 269) of a hydroxy-fatty acid. In fact, when a scraping, taken after spectrography from this very specimen of irin, was dissolved in de Jalon's fluid, it gave, with the *sym* diphenylcarbazide reagent, a strong pink colour appearing within 6 min; a parallel blank remained colourless. This implies the presence of a hydroxy-acid in the specimen. Yet the significant feature of its spectrum is that it does not suggest a hydroxy-acid, but a lactone. Thus, the irin spectrum differs from that of ricinoleic, and resembles the dodecalactone spectrum in that the hydroxyl absorption at 3400–3500 cm^{-1} is negligible and the broad 'carboxylic' band at 2700–2500 cm^{-1} is virtually absent. Also, the carbonyl absorption of the irin is at 1740 cm^{-1} as in the δ-dodecalactone, and not at 1710 cm^{-1} as in the ricinoleic acid. These results suggest the formation of a lactone by internal esterification of the –OH and –COOH groups of the hydroxy-acid, most probably when HCl was added for the partition of irin. HCl

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catalyses lactonization and it is known that the hydroxy-acids which form such cyclic internal esters most readily are those with the –OH group in the γ- or δ-position; ϵ-hydroxy-acids, for instance, show little natural tendency to form lactones.

Carbonyl absorption. The 1740 cm^{-1} carbonyl band is characteristic not only of δ-lactones but also of esters and some cyclic lactams. Lactams, however, appear to be excluded both by the ClO_4-benzidine test described above (Fig. 3) and by the fact that in the irin spectrum there is no significant absorption due to NH in the 3100–3500 cm^{-1} region. Moreover, if the 1740 cm^{-1} absorption of irin were due to its being an ester rather than a lactone, then we must look elsewhere in this spectrum for a charge which would account for the former acidic behaviour of this same batch of irin during its partition between water and ether. No evidence of such a charge can be found, as there is no absorption due either to carboxyl or to phosphate. Thus, the presence of a P–O–C linkage would give rise to an extremely strong and broad band at 1000–1030 cm^{-1}, where irin absorbs very weakly, less, for instance, than the phosphate-free δ-dodecalactone in Fig. 14.

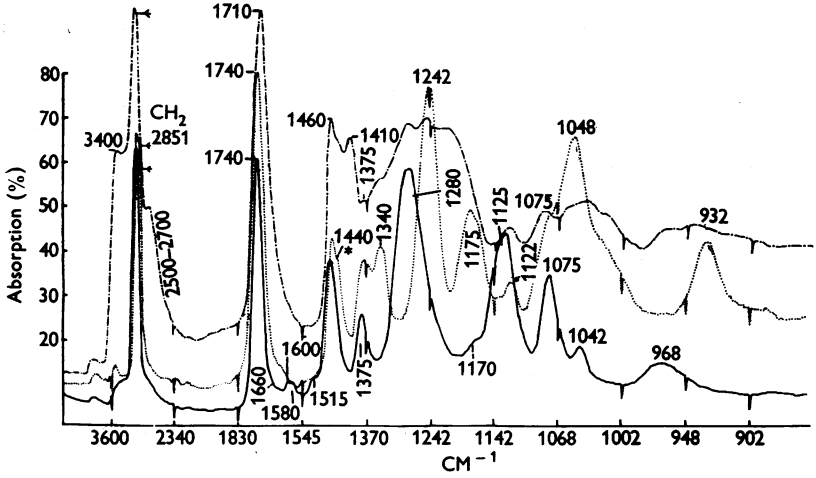

Fig. 14. Infra-red absorption spectra of films of irin, —, of δ-dodecalactone, · · · ·, and of ricinoleic acid, – · – · –: for details see text. The height of the absorption at 2851 cm^{-1}, which after photographic reduction is a notch barely visible on the trace, has been indicated by the horizontal arrows.

This absorption is also insignificant when compared with that of a substance known to contain P–O–C– linkages. For instance, in a spectrum of sphingomyelin the absorption at 1030 cm^{-1} due to the two P–O–C linkages was almost equal in height to the absorption at 2851 cm^{-1} produced by the 37 methylene groups in the sphingomyelin molecule.

In other spectra of irin purified by different methods, including Type 2 residues, the 1740 cm^{-1} lactone band has been weaker and variable. There were, at the same time, additional hydroxylic (3400–3500 cm^{-1}) and carboxylic bands (suggesting a hydroxy-acid), the latter at 2700–2500 cm^{-1}, at 1580 cm^{-1} ($RCOO^-$) and at 1710 cm^{-1} (un-ionized RCOOH). Incidentally, the location of the RCOOH band at 1710 cm^{-1} excludes an $\alpha\beta$-unsaturated acid, which would absorb slightly below 1700 cm^{-1}. The virtual absence of all these bands in the present spectrum would therefore seem to be further evidence of almost complete lactonization in this particular specimen. It is clear, also, that in lactonized irin there is no second —OH group other than the one which disappears on lactonization.

Lactones vary considerably in the exact location of their carbonyl absorption, according to their structure. Since in irin this band occurs at 1740 cm^{-1}, we may proceed to exclude the following possibilities, by making use of the data compiled by Bellamy (1958): (1) a lactide of an α-hydroxy acid, which would absorb at 1757–1767 cm^{-1}; (2) a β-lactone (1820 cm^{-1}); (3) a saturated γ-lactone

(1760–1780 cm^{-1}); (4) a $\beta\gamma$-unsaturated γ-lactone which, by virtue of its enol-lactone, vinyl, type of structure (CO—O—C=C) would absorb at about 1790 cm^{-1}; (5) a saturated ϵ-lactone (1728 cm^{-1}; Rothman, Wall & Eddy, 1954).

An $\alpha\beta$-unsaturated γ-lactone would absorb at 1750 cm^{-1} and is excluded both by the inactivation of irin with IBr (p. 275) and by the absence in this spectrum of the large absorption which an $\alpha\beta$ double bond, conjugated with carbonyl, would produce at 1600–1650 cm^{-1}.

These being excluded, we are left with a δ-lactone as the likeliest possibility. This can be saturated or unsaturated. Since irin is known to be unsaturated, we must consider what effect the presence of an endocyclic double bond, inside the lactone ring, would have upon the carbonyl absorption. Of the three possible positions for such a double bond ($\Delta\alpha\beta$, $\Delta\beta\gamma$, and $\Delta\gamma\delta$) two can be excluded by this spectrum. Thus a $\Delta\alpha\beta$-δ-lactone would absorb at 1720 cm^{-1} and would also show a strong absorption at 1600–1650 cm^{-1} due to the effect of conjugation already mentioned. Likewise a $\Delta\gamma\delta$-δ-lactone, being an enol-lactone, would absorb at 1780–1800 cm^{-1}. If, therefore, the double bond is inside the lactone ring then the only position possible for it is $\Delta\beta\gamma$, i.e. Δ3:4. There are other features of this spectrum which would be not inconsistent with such a possibility. If, however, the double bond should be exocyclic to the δ-lactone ring, its first possible position could only be between carbon atoms Nos. 6 and 7 since a double bond between C5 and 6 ($\Delta\delta\epsilon$) would give rise to a CO—O—C=C vinyl sequence and therefore to absorption at 1780–1800 cm^{-1} as in the enol-lactones.

In summary, these results would appear to exclude the following double-bond positions: Δ2:3; Δ4:5 and Δ5:6. This leaves either Δ3:4 (which would have to be *cis*; see p. 287) if the double bond is endocyclic, or Δ6:7 or beyond, if it is exocyclic.

Methylene absorption. In the spectra of irin, ricinoleic acid and δ-dodecalactone there are absorption bands at 2851 cm^{-1} and at 1460–1465 cm^{-1}, which are characteristic of methylene groups. Although there are 9 CH_2 groups in the dodecalactone molecule for every carbonyl, yet their collective absorption at 2851 cm^{-1} is weaker than that due to the single carbonyl group at 1740 cm^{-1}. Irin differs from dodecalactone in being a solid, and does in fact appear to have more methylene in it than dodecalactone, since its absorption at 2851 cm^{-1} is very nearly equal to the absorption at 1740 cm^{-1}; likewise, in ricinoleic acid (with 13 CH_2 groups to one carbonyl) the bands at 2851 cm^{-1} and at 1710 cm^{-1} are approximately equal.

Studies on long-chain paraffins have shown that the intensity of the CH_2 bands show a steady increment proportional to the number of methylene units in the chain (Bellamy, 1958, p. 18). By taking the ratios of the optical densities (O.D. $= \log I_0/I$) at 2851 cm^{-1} and at 1740 cm^{-1} we can arrive at a very rough estimate of the number of methylene groups in this probably still somewhat impure sample of irin as follows:

For dodecalactone,

$$R_1 = \frac{\text{O.D. at 2851 cm}^{-1}}{\text{O.D. at 1740 cm}^{-1}} = 0{\cdot}6033,$$

representing a known ratio of 9 CH_2 : 1 CO groups.

For irin,

$$R_2 = \frac{\text{O.D. at 2851 cm}^{-1}}{\text{O.D. at 1740 cm}^{-1}} = 0{\cdot}9456.$$

Therefore

$$\text{Ratio } \frac{CH_2}{CO} \text{ for irin } = \frac{R_2}{R_1} \times 9 = \frac{0{\cdot}9456}{0{\cdot}6033} \times 9$$

$$= 14{\cdot}11 \text{ methylene groups.}$$

This would make irin into a C_{19} acid, whereas an even-numbered acid (say C_{18}) is more likely. However, the molecular extinction coefficient for the carbonyl absorption can vary by at least 10%. Thus in simple saturated esters it can range from 569 to 610 and in fatty acids between 502 and 564 (Bellamy, 1958, pp. 180 and 170). With these limits in mind, it is interesting to note that in ricinoleic acid the bands at 2851 cm^{-1} and at 1710 cm^{-1} are equal and that their optical density ratio was 0·9742, which is close to that found above for irin.

Methyl absorption. Both irin and δ-dodecalactone absorb at 1375–1380 cm^{-1}, a band characteristic of CH_3 groups. The following calculation suggests that there is probably only one methyl group in irin.

For dodecalactone

$$R_3 = \frac{\text{O.D. at 1380 cm}^{-1}}{\text{O.D. at 1740 cm}^{-1}} = 0{\cdot}2456,$$

representing a known ratio of 1 CH_3 : 1 CO groups.

For irin,

$$R_4 = \frac{\text{O.D. at 1380 cm}^{-1}}{\text{O.D. at 1740 cm}^{-1}} = 0{\cdot}2792.$$

Therefore

$$\text{Ratio } \frac{CH_3}{CO} \text{ for irin} = \frac{R_2}{R_1} \times 1 = \frac{0{\cdot}2792}{0{\cdot}2456} = 1{\cdot}137 \text{ methyl groups.}$$

Other bands. The dodecalactone spectrum shows absorptions at 1242, 1175, 1122, 1075 and 1048 cm^{-1}, attributable to its lactone group. In the irin spectrum the first and strongest of these bands appears to be shifted to 1280 cm^{-1} (some esters are known to absorb as high as this), and the remaining bands (at 1170–1042) coincide in location, but not in intensity, with those of dodecalactone; the weak band at 968 cm^{-1} is present in some lactones.

The absorption due to isolated double bonds is usually weak in non-conjugated compounds (Bellamy, 1958, chapter 3), and occurs most frequently within the range 1680–1620 cm^{-1}, although in some unsaturated steroids it can be found as low as 1590 cm^{-1}; *cis* isomers absorb 20 cm^{-1} lower than *trans*. The weakness of the double-bond absorption is clearly seen in the spectrum of the unsaturated (*cis*) ricinoleic acid, where it appears to be lost in the falling phase of the 1710 cm^{-1} band. The same applies to the irin spectrum in which the double-bond absorption could be either of the small humps at 1660 or at 1600 cm^{-1}. The weakness of these absorptions would appear to exclude the presence of a conjugated system of double bonds in the irin molecule.

The large 1410 cm^{-1} band of ricinoleic acid is due to the seven CH_2 groups which adjoin the terminal carbonyl group. In dodecalactone there would be only three such methylene groups (inside the lactone ring); the 1410 cm^{-1} band appears to have shifted as in some steroids (Bellamy, 1958, p. 23) and is seen as the hump marked by an asterisk at 1440 cm^{-1}. Neither the 1410 cm^{-1} band nor the 1440 cm^{-1} hump is discernible in the irin spectrum, which suggests fewer methylene groups adjoining carbonyl than even in dodecalactone. This may be a hint that the double bond in irin is endocyclic, which would leave only a solitary CH_2 group inside the lactone ring.

The absorption at 1340 cm^{-1} in the dodecalactone and ricinoleic spectra is probably due to their —CH group. In irin this band is either lost in the rising phase of the 1280 cm^{-1} band or is absent; this could perhaps be due to the effect of a nearly double bond.

We are indebted to Dr L. J. Bellamy, Ministry of Supply, to Mr J. F. Grove and Dr L. A. Duncanson, I.C.I. Akers Research Laboratory, Welwyn, for much useful advice on the interpretation of these spectra, and to Miss Paula Wood and Mrs S. M. Beech for preparing some of the irin specimens.

REFERENCES

AMBACHE, N. (1957*a*). Properties of irin, a physiological constituent of the rabbit's iris. *J. Physiol.* **135**, 114–132.

AMBACHE, N. (1957*b*). A rapid purification of irin and the use of the hamster colon for its assay. *J. Physiol.* **138**, 6–7*P*.

AMBACHE, N. (1957*c*). The unsaturated nature of irin and its interaction with lactones. *J. Physiol.* **140**, 24–25*P*.

AMBACHE, N. & REYNOLDS, M. (1959). Irin-like activity of some further unsaturated hydroxy-fatty acids. *J. Physiol.* (in the Press).

BAILEY, W. A. & ROY, A. (1946). Danger of explosion in handling ether. *J. Soc. Chem. Ind.* **65**, 421–422.

BELLAMY, L. J. (1958). *The Infra-red Spectra of Complex Molecules*, 2nd ed. London: Methuen.

BERGSTRÖM, S. (1949). Prostaglandinets Kemi. *Nord. med.* **42**, 1465–1466.

BROCKLEHURST, W. E. (1956). A slow reacting substance in anaphylaxis—'SRS–A'. *Ciba Foundation Symposium on Histamine*, pp. 175–179. London: Churchill.

CALDWELL, B. P. & PIONTKOWSKI, F. A. (1934). The addition of iodine and thiocyanogen to unsaturated compounds. *J. Amer. chem. Soc.* **56**, 2086–2089.

CAVALLITO, C. J. & HASKELL, T. H. (1945). The mechanism of action of antibiotics. The reaction of unsaturated lactones with cysteine and related compounds. *J. Amer. chem. Soc.* **67**, 1991–1994.

CAVALLITO, C. J. & HASKELL, T. H. (1946). α-Methylene butyrolactone from *Erythronium americanum*. *J. Amer. chem. Soc.* **68**, 2332–2334.

CHARGAFF, E. & DAVIDSON, J. N. (1955). *The Nucleic Acids*, Vol. I. New York: Academic Press Inc.

COLOWICK, S. P. & KAPLAN, N. O. (1957). *Methods in Enzymology*, Vol. 3, p. 244. New York: Academic Press Inc.

DASLER, W. & BAUER, C. D. (1946). Removal of peroxides from organic solvents. *Industr. Engng Chem.* (*Anal.*) **18**, 52–54.

DE JALÓN, M. G. & DE JALÓN, P. D. (1946). Aportacion a la farmacologia de los detergentes sintéticos. *Farmacoterapia Actual*, p. 833.

DEUEL, H. J., Jr. (1951). *The Lipids*, Vol. I. *Chemistry*. New York: Interscience Publications Inc.

ELIASSON, R. (1957). A comparative study of prostaglandin from human seminal fluid and from prostate gland of sheep. *Acta physiol. scand.* **39**, 141–146.

ELIASSON, R. (1958). The spasmolytic effect of patulin. *Experientia*, **14**, 460–463.

FEIGL, F. (1956). *Spot Tests in Organic Analysis*, 5th ed. London: Elsevier Publishing Co.

FELDBERG, W., HOLDEN, H. F. & KELLAWAY, C. H. (1938). The formation of lysocithin and of a muscle-stimulating substance by snake venoms. *J. Physiol.* **94**, 232–248.

FORD, M. A., PRICE, W. C., SEEDS, W. E. & WILKINSON, G. R. (1958). Double-beam infrared microspectrometer. *J. Optical Soc. Amer.* **48**, 249–254.

GABR, Y. (1954). *The Chemical Nature of a Substance in Human Plasma which Gives a Slow Constrictor Response in Guinea-pig Gut* in vitro. Ph.D. Thesis, University of London.

GABR, Y. (1956). Observations on a substance in human plasma which gives a slow contraction of guinea-pig gut *in vitro*. *Brit. J. Pharmacol.* **11**, 93–98.

GADDUM, J. H., PEART, W. S. & VOGT, M. (1949). The estimation of adrenaline and allied substances in blood. *J. Physiol.* **108**, 467–481.

GUNSTONE, F. D. (1952). Fatty acids. Part I. 9-hydroxyoctadec-12-enoic acid, a new hydroxy-acid occurring in *Strophanthus sarmentosus* seed oil. *J. chem. Soc.* pp. 1274–1278.

GUNSTONE, F. D. (1954). Fatty acids. Part II. The nature of the oxygenated acid present in *Vernonia anthelmintica* (Willd) seed oil. *J. chem. Soc.* pp. 1611–1616.

HACK, M. H. (1953). Analysis of lipids by spot tests on filter-paper disk chromatograms. *Biochem. J.* **54**, 602–605.

HILDITCH, T. P. (1956). *The Chemical Constitution of Natural Fats*, 3rd ed. London: Chapman and Hall, Ltd.

HOFMAN, T. (1953). The biochemistry of the nitrifying organisms. 3. Composition of *Nitrosomonas*. *Biochem. J.* **54**, 293–295.

HOUGH, L., JONES, J. K. N. & WADMAN, W. H. (1950). Quantitative analysis of mixtures of sugars by the method of partition chromatography. Part V. Improved methods for the separation and detection of the sugars and their methylated derivatives on the paper chromatogram. *J. chem. Soc.* Part II, 1702–1706.

JAMES, A. T. & WHEATLEY, V. R. (1956). The determination of the component fatty acids of human forearm sebum by gas-liquid chromatography. *Biochem. J.* **63**, 269–273.

JAMES, A. T. & WHEATLEY, V. R. (1957). Studies of sebum. 7. The composition of the sebum of some common rodents. *Biochem. J.* **65**, 36–42.

JOELS, N. & MACNAUGHTON, J. I. (1957). A micro-pH electrode system suitable for routine laboratory use. *J. Physiol.* **135**, 1–2*P*.

KARRER, P. (1946). *Organic Chemistry*, 3rd ed. London: Elsevier Publishing Co.

KING, E. J. (1946). *Micro-analysis in Medical Biochemistry*. London: Churchill.

KOECHLIN, B. A. (1955). On the chemical composition of the axoplasm of squid giant nerve fibers with particular reference to its ion pattern. *J. biophys. biochem. Cytol.* **1**, 511–529.

KORPÁCZY, S. (1934). Bestimmung der Ranzigkeit von Fetten mittels der Reaction nach Stamm. *Z. Untersuch. Lebensmitt.* **67**, 75–79.

KUMLER, W. D. & EILER, J. J. (1943). The acid strength of mono and diesters of phosphoric acids. The *n*-alkyl esters from methyl to butyl, the esters of biological importance, and the natural guanidine phosphoric acids. *J. Amer. chem. Soc.* **65**, 2355–2361.

LASER, H. (1950). The isolation of a haemolytic substance from animal tissues and its biological properties. *J. Physiol.* **110**, 338–355.

LIGTHELM, S. P. (1954). A new hydroxy acid from the oil of *Ximenia Caffra* Sond. *Chem. & Ind. (Rev.)* 249–250.

LIPMANN, F. (1941). Metabolic generation and utilisation of phosphate bond energy. *Advanc. Enzymol.* **1**, 99–162.

LUCK, J. M. (1949). The combination of fatty acid anions with proteins. *Disc. Faraday Soc.* **6**, 44–52.

MARKLEY, K. S. (1947). *Fatty Acids.* New York: Interscience Publications Inc.

PARTRIDGE, S. M. (1949). Aniline hydrogen phthalate as a spraying agent for chromatography of sugars. *Nature, Lond.*, **164**, 443.

RALSTON, A. W. (1948). *Fatty Acids and Their Derivatives.* London: Chapman and Hall Ltd.

REINDEL, F. & HOPPE, W. (1953). Über eine neue Färbemethode zum Nachweis von Aminosaüren, Peptiden und Eiweisskörpern auf Papierchromatogrammen und -elektropherogrammen. *Naturwissenschaften*, **40**, 221.

ROTHMAN, E. S., WALL, M. E. & EDDY, C. R. (1954). Steroidal sapogenins. XI. Steroidal C-ring lactones. *J. Amer. chem. Soc.* **76**, 527–532.

RYDON, H. N. & SMITH, P. W. G. (1952). A new method for the detection of peptides and similar compounds on paper chromatograms. *Nature, Lond.*, **169**, 922–923.

SAVARY, P. (1954). Contribution à l'étude de la chromatographie sur papier des acides gras. *Bull. soc. chim. biol.* **36**, 927–932.

SEIDELL, A. (1941). *Solubilities of Organic Compounds* (3rd ed.), **2**, 194. New York: D. van Nostrand Co. Ltd.

STERN, J. R., DEL CAMPILLO, A. & RAW, I. (1956). Enzymes of fatty acid metabolism, I and II. *J. biol. chem.* **218**, 971–1002.

TOH, C. C. & MOHIUDDIN, A. (1958). Vasoactive substances in the nasal mucosa. *Brit. J. Pharmacol.* **13**, 113–117.

VOGT, W. (1955). Eigenschaften und Gewinnung von Darmstoff-präparaten. *Arch. exp. Path. Pharmak.* **227**, 224–233.

VOGT, W. (1957*a*). Pharmacologically active lipid-soluble acids of natural occurrence. *Nature, Lond.*, **179**, 300–304.

VOGT, W. (1957*b*). Pharmacologically active substances formed in egg yolk by cobra venom. *J. Physiol.* **136**, 131–147.

VOGT, W. (1957*c*). The chemical nature of Darmstoff. *J. Physiol.* **137**, 154–167.

VOGT, W. (1958). Naturally occurring lipid-soluble acids of pharmacological interest. *Pharmacol. Rev.* **10**, 407–435.

VON EULER, U. S. (1936). On the specific vasodilating and plain muscle stimulating substances from accessory genital glands in man and certain animals (Prostaglandin and Vesiglandin). *J. Physiol.* **88**, 213–234.

WALEY, S. G. (1957). The structure of ophthalmic acid. *Proc. chem. Soc.* p. 25.

ZETSCHE, F., LÜSCHER, E. & MEYER, H. E. (1938). Die Kennzeichung von Carbon-saüren als Ureīde mit Hilfe der Carbodi-imide (I. Mitteil). *Ber. dtsch. chem. Ges.* **71**, 1088–1093.