Br. J. Pharmac. Chemother. (1968), 33, 162–170.

A SIMPLE CHEMICAL PROCEDURE FOR DISTINGUISHING E FROM F PROSTAGLANDINS, WITH APPLICATION TO TISSUE EXTRACTS

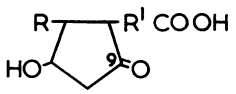
BY

N. AMBACHE AND HILARY C. BRUMMER

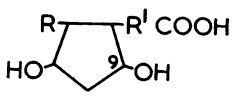
From the Medical Research Council, Royal College of Surgeons of England, London W.C.2

(*Received December* 6, 1967)

This paper describes an easy and relatively rapid procedure for distinguishing the ketonic prostaglandins E (PGEs) from the non-ketonic prostaglandins F (PGFs) and other hydroxy-acid lipid spasmogens.



PGE



PGF

The method depends on the ability of certain hydrazine derivatives to combine specifically with keto groups. The condensation reaction leads to the formation of hydrazones according to the equation:

$$RR'CO + H_2N.NHR'' \rightleftharpoons RR'C=N.NHR'' + H_2O$$

To obtain this ketonic condensation we have used Girard's reagent T (trimethylammonium-acetohydrazide chloride, $H_2N.NH.CO.CH_2.N(CH_3)_3Cl$), which is one of a series of reagents developed by Girard & Sandulesco (1936); these reagents are soluble in water and insoluble in non-hydroxylated organic solvents. The hydrazones formed from ketones and reagent T are also more water and less ether-soluble than the original ketones. In preliminary trials with known compounds it was found that treatment of ketonic PGEs in aqueous solution with reagent T leads, as expected, to an apparent retention of the product in the water phase during subsequent partitions with diethyl ether at pH 3–4; this reduced extractability of PGEs will be referred to as an apparent inactivation. On the other hand, the non-ketonic PGFs were recovered fully from the ether phase, indicating absence of reaction with the reagent, as expected from the difference in its structure at C9, which is shown above.

Reagent T is itself insoluble in ether and did not interfere with the assays of prostaglandins.

The reduction in ether-extractability of PGEs by reagent T was applied for the detection of prostaglandin-like spasmogenic keto-hydroxy-acids previously found in this laboratory in extracts of the iris and cerebral hemispheres (Ambache, 1957, 1959; Ambache & Reynolds, 1960; Ambache, Reynolds & Whiting, 1963).

A preliminary account of these experiments has appeared elsewhere (Ambache, Brummer, Rose & Whiting, 1966).

METHODS

Girard's reagent T is highly hygroscopic and was stored in a desiccator. After some weeks, because of repeated opening of the bottle, the reagent deteriorates and develops a stronger fishy odour; when this occurred, a fresh batch of reagent (laboratory reagent grade) was obtained from British Drug Houses Ltd.

Condensation reaction. Weighed amounts of the solid reagent were added to dilute solutions of various prostaglandins (in 0.9% NaCl) to give a final concentration of 100 mg of reagent T/ml. Simultaneously controls were prepared of each prostaglandin without the reagent and of the reagent without prostaglandins. All these samples were stood for 1 hr either at room temperature (21°–25° C) or at 0° C and were then partitioned with ether after acidification; in a few experiments the partition was carried out after 0.5 hr. Because Girard & Sandulesco (1936) mention that the condensation product formed is more stable at low temperatures, the treated and control samples in some experiments were kept at room temperature for 1 hr and then stored overnight in a deep freeze (−15° C) and thawed next morning immediately before partition.

For partition all samples were acidified to pH 3–4.3 with 0.1 N or 0.5 N HCl, the exact amount of acid having been predetermined by titration of an aliquot. Treated and control samples were then immediately extracted twice with 1 volume of freshly distilled ether; delay was avoided after acidification, because Girard & Sandulesco (1936) state that the condensation reaction is rapidly reversed by "strong mineral acids." The separated ether phases were evaporated to dryness in a stream of nitrogen; these will be referred to as ether-purified residues. For bio-assay the residues were taken up in a suitable volume of de Jalon's colon solution of the same composition as used previously (Ambache, 1959).

Aqueous extracts of rabbit and cat irides and blood-free cerebral hemispheres were prepared and purified by partition with ether at pH *ca.* 3, as described previously (Ambache, 1959; Ambache, Reynolds & Whiting, 1963). The ether phases were evaporated in a stream of nitrogen and the residues obtained in this way were dissolved in 0.9% NaCl solution and subdivided into samples, some of which were treated with the reagent (100 mg/ml.) for 1 hr; a single crude aqueous extract of cat iris was also treated with the reagent.

Bio-assays. These were carried out on ascending colon preparations from various gerbilline species, namely the Libyan or Mongolian jirds (*Meriones shawi* or *Meriones unguiculatus*) and the gerbil proper (*Gerbillus pyramidum tarabuli*), using animals weighing 50–100 g. The muscles were suspended at 30° C in oxygenated de Jalon's colon solution containing atropine sulphate (usually 10^{-7} g/ml.) and, in most of the experiments, methysergide bimaleate 4×10^{-7} g/ml. The 1.8 ml. organ bath was of the jointed type described by Ambache, Kavanagh & Whiting (1965, Fig. 1). After allowing 0.5–1 hr for relaxation, contractions were recorded with Kavanagh's (1962) lever at a load of 0.4–0.6 g and a ×12 magnification. A small constant flow of de Jalon's solution was maintained through the organ bath throughout the experiments, except during the 30 or 60 sec contact periods with the test samples; the rapid relaxation after contractions of these gerbilline muscles permitted dose-intervals of 3–5 min, and sometimes of 2.5 min. Unless otherwise stated, drug and extract doses refer to the total amounts injected into the organ bath.

RESULTS

Prostaglandins of the F series are unaffected by Girard's reagent T

The amounts of $PGF_{1\alpha}$ and of $PGF_{2\alpha}$ recovered from the evaporated ether phases after partitions at pH 3–4 were the same from samples exposed to Girard's reagent T for 1 hr as from parallel untreated samples; in both cases the recovery of activity—that is, the extractability from the aqueous phase—was usually more than 90%. Control samples

of evaporated ether phases from partitions of the reagent alone (in 0.9% NaCl solution) were inactive and did not depress responses to prostaglandin standards, presumably because of the exclusion of the reagent from the ether phase.

Figure 1 shows an assay on a *Meriones shawi* ascending colon preparation in the presence of atropine. With $PGF_{2\alpha}$ the responses to treated and control samples were

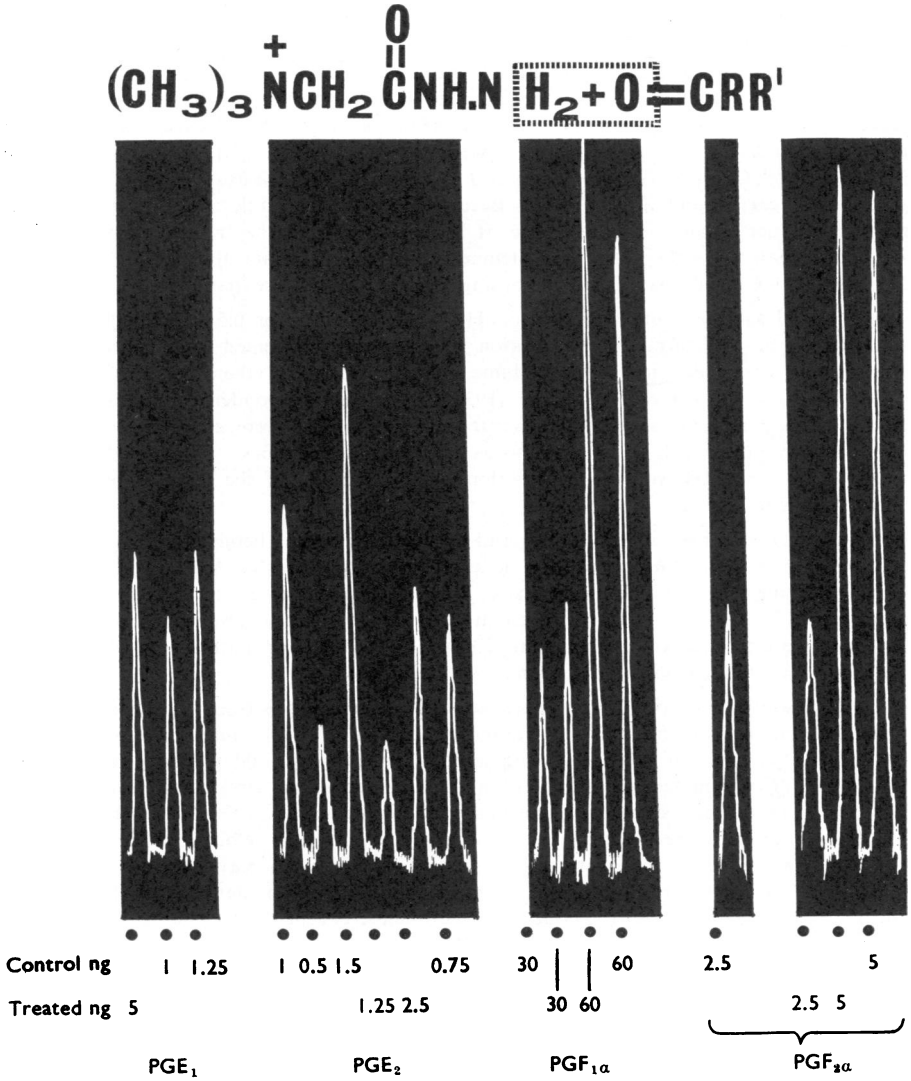


Fig. 1. Apparent inactivation of E prostaglandins by treatment for 1 hr at room temperature with Girard's reagent T, caused by the reaction with the ketonic group, as shown above the tracing, and retention in the aqueous phase on subsequent partition with ether. The non-ketonic F prostaglandins are unaffected. Assay of treated, and untreated control, ether-partitioned samples of PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ on ascending colon preparation of *Meriones shawi* in 1.8 ml. bath ; atropine sulphate 10^{-6} g/ml. Contacts 30 sec.

virtually the same at two dose levels, 2.5 and 5 ng. With $PGF_{1\alpha}$, to which this preparation was 12 times less sensitive than to $PGF_{2\alpha}$, the response to the treated sample was slightly greater than that obtained with the control sample at the 30 ng and the 60 ng levels.

These results suggested that the reagent fails to react with non-ketonic prostaglandins, as expected.

Effect of Girard's reagent T on prostaglandins of the E series, leading to apparent inactivation

Whereas the F prostaglandins were unaffected, there was in the same experiments a marked reduction in the ether-extractable activity recovered from the evaporated ether phases of the parallel treated samples of PGE_1 and PGE_2. As shown also in Fig. 1, the dose of the treated PGE_1 sample had to be raised to " 5 ng " in order to obtain a match with 1.25 ng of its control (the parallel untreated sample of PGE_1), thus indicating an apparent inactivation of 75%. With PGE_2 the response to " 2.5 ng " of the treated sample was considerably less than that to 1 ng of the control and only just greater than the response to 0.75 ng of the control ; thus, the apparent inactivation was 70%.

The results of these experiments are summarized in Table 1, which shows that the reagent affected the ether-extractability of PGE_2 and PGE_1 equally.

TABLE 1

APPARENT INACTIVATION OF KETONIC PROSTAGLANDINS E BY TREATMENT FOR 1 HR WITH GIRARD'S REAGENT T, EITHER AT 0° C OR AT ROOM TEMPERATURE, WITH OR WITHOUT SUBSEQUENT FREEZING

	Total number of experiments	Reduction in recovery in ether phase (%)	
		Range	Mean
PGE_1	6	55–97·5	72·9
PGE_2	10	50–98	75·5
$PGF_{1\alpha}$	4	0	0
$PGF_{2\alpha}$	5	0	0

In four other experiments in which the treatment with the reagent at room temperature lasted only 0.5 hr, the average inactivation of PGE_1 or PGE_2 was 65.5%, and in a fifth at 0° C it was 75%.

As with the PGFs, the recovery of activity from the ether phases in the partitions of the parallel untreated control samples of PGE_1 or PGE_2 was usually more than 90%.

As shown in Fig. 1, the *M. shawi* preparation is highly sensitive to PGE_2 (threshold on average 0.56 ng/ml., but occasionally even as low as 0.1 ng/ml.) and less sensitive to PGE_1. The sensitivity to PGE_2 was also on average greater than that to $PGF_{2\alpha}$, the activity ratio $E_2/F_{2\alpha}$ varying between 1 and 1.66 (mean 1.1).

Effect of temperature

As shown in Table 2, the apparent inactivation was not significantly different whether the initial treatment with reagent was carried out at room temperature (21°–25° C) or at 0° C. In those experiments in which the tubes were left overnight at −15° C between the 1 hr treatment at room temperature and the partitions, however, a statistically significant ($P<0.05$) increase in the apparent inactivation of PGEs was noted, while the PGFs

TABLE 2

EFFECT OF TEMPERATURE ON THE REACTION BETWEEN GIRARD'S REAGENT T AND PROSTAGLANDINS

Values indicate "apparent inactivations"—that is, % reductions in recovery from ether phases on acid partition. Number of experiments in brackets.

	1 hr treatment at room temperature (21°–25° C)	1 hr treatment at 0° C	1 hr treatment at room temperature then frozen overnight
PGE_1 or PGE_2	50–80 (6) Mean 66·2	54–90 (4) Mean 67·3	66–98 (6) Mean 86·3
$PGF_{1\alpha}$ or $PGF_{2\alpha}$	0 (3)	0 (2)	0 (4)

remained unaffected. The recovery from untreated controls was unaffected by freezing and thawing.

Inactivation of mixtures of prostaglandins

Apparent inactivation also occurred when known mixtures of one E and one F prostaglandin were treated with reagent T.

Experiments with active lipids extracted from tissues

Iris extracts

Aqueous extracts of rabbit or cat irides contain atropine-resistant spasmogenic unsaturated hydroxy-acids which, after partition with peroxide-free ether at pH *ca.* 3, are recovered from the ether phase (Ambache, 1957, 1959). The residues of the evaporated ether phases will be referred to as ether-purified irins, and their doses will be given in ≡mg of original tissue. Column chromatography has previously revealed the presence of at least two components in ether-purified rabbit irin prepared in this laboratory (Vogt, 1960; Ambache & Reynolds, 1961, graph B). Some of these chromatographically purified fractions possessed vasodilator activity (Ambache, 1961).

In the present experiments ether-purified cat and rabbit irin residues were redissolved in 0.9% NaCl, subdivided, and treated with reagent T before partition with ether at pH *ca.* 3 in parallel with untreated samples of the same irins. Evidence of inactivation by the reagent was obtained in a total of thirteen out of sixteen trials, assayed on *M. shawi* (seven out of eight on cat and six out of eight on rabbit samples). For these thirteen trials the apparent inactivation averages were: cat samples 59.8%, and rabbit samples 43%; in repeats of the three negative trials on parallel samples of the same irins apparent inactivations of 40% or more were recorded. One of the eight rabbit irin samples was treated with reagent T at room temperature for only 0.5 hr, with an apparent inactivation of 43%. All the other rabbit and cat irin samples and the one crude cat iris extract were treated with the reagent for 1 hr either at room temperature or at 0° C. One rabbit and one cat irin sample were stored overnight at −15° C after treatment for 1 hr at room temperature; their inactivations were 50 and 95%, respectively.

In addition, two further treated samples (one cat and one rabbit irin) were assayed on a *Meriones unguiculatus* ascending colon preparation which was 4–5 times more sensitive to PGE_2 than to $PGF_{2\alpha}$ (Fig. 2). These samples had also been stored overnight at −15° C after treatment for 1 hr at room temperature, and their inactivations were high: for the rabbit sample, 75%; for the cat sample, 85%.

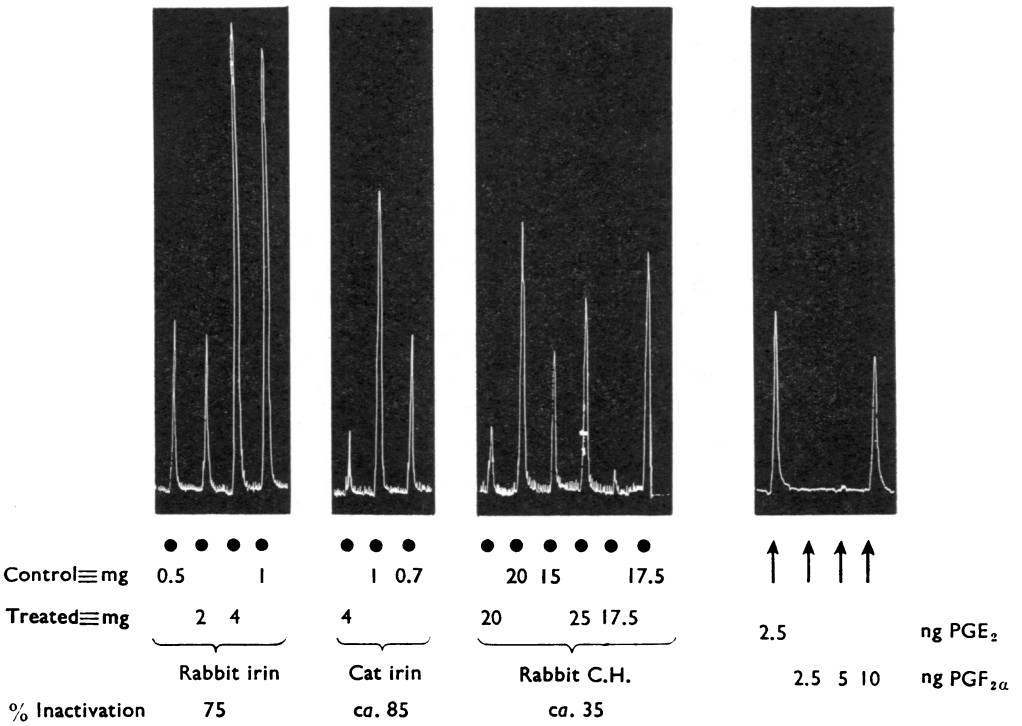


Fig. 2. Apparent inactivation by Girard's reagent T of ketonic components in purified rabbit and cat irins, and in ether-purified extracts of rabbit cerebral hemispheres. Assays on ascending colon preparation from *Meriones unguiculatus* in 1.8 ml. bath; atropine sulphate 10^{-6} g/ml. and methysergide bimaleate 4×10^{-7} g/ml. At the dots, doses of treated and control ether-partitioned extracts, as indicated; at the arrows, doses of prostaglandin standards. Contacts 1 min; dose-intervals, 4 min. The threshold for the untreated control cat irin was ≡0.5 mg (not shown).

These experiments show that ketonic hydroxy-acids are present in the iris of the rabbit and of the cat, apparently in slightly higher proportion in the cat. Subsequently, the presence of significant amounts of PGE_2 in the irins of both these species was confirmed by thin-layer chromatography. In these experiments 50–70% of the total activity was destroyed on the plates during chromatography, and could not be located for that reason (Ambache, Brummer, Rose & Whiting, 1966); however, $PGF_{2\alpha}$ was also found in both irins and in cat irin a third unidentified component was demonstrated.

Some idea of the high spasmogenic activity extractable from the rabbit iris can be gained from the following comparisons. From assays on *M. shawi* colons the total activity of two different batches of ether-purified rabbit irin could be expressed as 4.68 ng of PGE_2/≡mg of iris tissue (average of ten results; range 1–7.1 ng/≡mg); alternatively, it could be expressed as 8.54 ng of $PGF_{2\alpha}$/≡mg (average of five results on one extract; range 5–12 ng/≡mg). These values constitute upper limits and the true contents of PGE_2 and $PGF_{2\alpha}$ are probably lower in the mixture present in the iris, particularly

since other active components seem to be present. There is, for example, the unidentified substance in cat irin mentioned earlier, and in a chromatogram of sheep irin (Ånggard & Samuelsson, 1964) five peaks of spasmogenic activity were present, two of which were attributed to $PGF_{2\alpha}$ and the others unidentified.

Rabbit cerebral hemisphere extracts

Aqueous extracts of blood-free rabbit cerebral hemispheres contain an irin-like atropine-resistant spasmogenic activity, also accounted for by unsaturated hydroxy-acids extractable into ether at pH *ca.* 3 (Ambache & Reynolds, 1960, 1961; Ambache, Reynolds & Whiting, 1963). Both in the original crude extracts and in their ether-purified residues, however, the activity obtained per mg of brain tissue is usually lower than that of iris extracts from the same species, often considerably so. Moreover, the present experiments suggest that the proportion of the total activity in the hemisphere extracts caused by ketonic hydroxy-acids resembling E prostaglandins is much lower than in the iris.

Subdivided ether-purified residues of a blood-free rabbit cerebral hemisphere extract were redissolved in 0.9% NaCl solution and treated with reagent T for 1 hr at 0° C. In three trials the apparent inactivations, assayed on *M. shawi* colons and measured against parallel untreated samples, were: 0, <9%, and almost 0. In a fourth trial it was 16.7% when assayed on a gerbil colon, on which the activity ratio of PGE_2 to $PGF_{2\alpha}$ has been, on average, 1.5.

In a fifth trial, after treatment for 1 hr at room temperature and storage overnight at −15° C, the apparent inactivation was 35%, as assayed on an ascending colon from *M. unguiculatus* (Fig. 2). The relative insensitivity of this preparation to $PGF_{2\alpha}$ exaggerates the apparent inactivation of PGE_2.

Thin-layer chromatography (Ambache, Brummer, Rose & Whiting, 1966) has confirmed that the ether-extractable material in rabbit cerebral hemispheres contains a preponderance of $PGF_{2\alpha}$, as in ox brain (Samuelsson, 1964), but that a little PGE_2 is also present.

DISCUSSION

The results show the selectivity of Girard's reagent T towards E prostaglandins. The lack of effect of the reagent on PGFs has served as a useful control, showing that the reagent did not interfere with the assays of the evaporated ether-phases, from which it seems to be excluded, as expected. The reduced recovery of activity of PGE_1 and PGE_2 can be presumed to result from the expected combination with the reagent and retention of the product in the aqueous phase on subsequent partition with ether. Although Girard & Sandulesco (1936) mention that refluxing may be necessary for the reaction to occur, this seemed superfluous because satisfactory results were obtained under the milder conditions used by us at room temperature and even at 0° C. Girard & Sandulesco (1936) also mention that the hydrazones are more stable at low temperatures. This might explain the increased inactivation observed when the samples were frozen to −15° C after treatment for 1 hr at room temperature and thawed before partition; possibly the temperature at which these partitions were performed was lower than in our other experiments.

The method has proved useful for detecting qualitatively the presence of E prostaglandins in various tissue extracts. In dealing with mixtures of more than two prostaglandins, however, it is not possible to determine the exact proportions present, because of the widely variable activity ratios on the test muscles of PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$. But if a tissue extract is known to contain one particular PGE with one particular PGF, and parallel samples of a PGE have been virtually 100% inactivated, then it is sometimes possible to deduce the proportion of E to F. The simplest case arises in dealing with a mixture of PGE_2 and $PGF_{2\alpha}$ on a test object with an activity ratio of 1, as found in many *Meriones shawi* preparations: here, the percentage inactivation reflects the exact proportion of PGE_2. But if assayed on *Meriones unguiculatus*, the percentage of inactivation would seem to be higher than the proportion of PGE_2, because the activity ratio $PGE_2/PGF_{2\alpha}$ on this preparation has varied between 2.5 and 5.

SUMMARY

1. Girard's reagent T ($H_2N.NH.CO.\ CH_2N(CH_3)_3.\ Cl$), distinguishes ketonic prostaglandins of the E series from the non-ketonic prostaglandins of the F series.

2. After treatment of E prostaglandins in aqueous solution with the reagent for 1 hr at room temperature or at 0° C, their extraction into ether on partition at pH 3–4 was reduced. Freezing to −15° C after the 1 hr treatment, and thawing before the partition with ether, further reduced the recovery of E prostaglandins.

3. After treatment with the reagent in these different conditions F prostaglandins were recovered fully in all cases.

4. The presence of a PGE-like ketonic component in the hydroxy-acid constituents of rabbit and cat irins was demonstrated; ether-purified extracts of rabbit cerebral hemispheres contain a lower proportion of the ketonic component(s).

This work was done during the tenure of an M.R.C. scholarship by H.C.B. We wish to thank Dr. D. A. van Dorp, Unilever Research Laboratories, Vlaardingen, Holland, and Dr. J. E. Pike, Upjohn Ltd., Kalamazoo, Michigan, for generous gifts of prostaglandins.

REFERENCES

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

AMBACHE, N. (1959). Further studies on the preparation, purification and nature of irin. *J. Physiol., Lond.*, **146**, 255–294.

AMBACHE, N. (1961). Prolonged erythema produced by chromatographically purified irin. *J. Physiol., Lond.*, **160**, 3–4*P*.

AMBACHE, N., BRUMMER, H. C., ROSE, J. G. & WHITING, J. (1966). Thin-layerc hromatography of spasmogenic unsaturated hydroxy-acids from various tissues. *J. Physiol., Lond.*, **185**, 77–78*P*.

AMBACHE, N., KAVANAGH, L. & WHITING, J. (1965). Effect of mechanical stimulation on rabbits' eyes: release of active substances in anterior chamber perfusates. *J. Physiol., Lond.*, **176**, 378–408.

AMBACHE, N. & REYNOLDS, M. (1960). Ether-soluble active lipid in rabbit brain extracts. *J. Physiol., Lond.* **154**, 40*P*.

AMBACHE, N. & REYNOLDS, M. (1961). Further purification of an active lipid acid from rabbit brain. *J. Physiol., Lond.*, **159**, 63–64*P*.

AMBACHE, N., REYNOLDS, M. & WHITING, J. (1963). Investigation of an active lipid in aqueous extracts of rabbit brain, and of some further hydroxy-acids. *J. Physiol., Lond.*, **166**, 251–283.

ÅNGGARD, E. & SAMUELSSON, B. (1964). Smooth muscle stimulating lipids in sheep iris. The identification of prostaglandin $F_{2\alpha}$. Prostaglandins and related factors 21. *Biochem. Pharmac.*, **13**, 281–283.

GIRARD, A. & SANDULESCO, G. (1936). Sur une nouvelle série de réactifs du groupe carbonyle, leur utilisation à l'extraction des substances cétoniques et à la caractérisation microchimiques des aldéhydes et cétones. *Helv. Chim. Acta*, **19**, 1095–1107.

KAVANAGH, L. (1962). A light writing-point pivoted on watch-bearings for frontal levers. *J. Physiol., Lond.*, **163**, 1–2*P*.

SAMUELSSON, B. (1964). Identification of a smooth muscle stimulating factor in bovine brain. *Biochim. Biophys. Acta*, **84**, 218–219.

VOGT, W. (1960). Chromatographie darmerregender lipoidlöslischen Säuren an SiO_2. *Arch. exp. Path. u Pharmak.*, **240**, 196–209.