REFERENCES

- Aitken, E. H. & Preedy, J. R. K. (1953). *J. Endocrin.* **9**, 251.
- Axelrod, L. R. (1953). *J. Amer. chem. Soc.* **75**, 6301.
- Bates, R. W. (1954). *Recent Progr. Hormone Res.* **9**, 95.
- Bates, R. W. & Cohen, H. (1950a). *Endocrinology*, **47**, 166.
- Bates, R. W. & Cohen, H. (1950b). *Endocrinology*, **47**, 182.
- Bernstein, S. & Lenhard, R. H. (1953). *J. org. Chem.* **18**, 1146.
- Bierry, H. & Gouzon, B. (1936). *C.R. Soc. Biol., Paris*, **122**, 147.
- Bompiani, A. (1951). *Quad. Clin. Ostet. Ginecol.* **6**, 539.
- Boscott, R. J. (1948). *Nature, Lond.*, **162**, 577.
- Braunsberg, H. (1952). *J. Endocrin.* **8**, 11.
- Bush, I. E. (1952). *Biochem. J.* **50**, 370.
- Clark, I. (1955). *Nature, Lond.*, **175**, 123.
- Diczfalusy, E. (1953). *Acta endocr., Copenhagen*, **12**, suppl. 12.
- Dirscherl, W. & Breuer, H. (1954). *Z. Vitam.- Horm.- u. Fermentforsch.* **6**, 287.
- Finkelstein, M. (1952). *Nature, Lond.*, **169**, 929.
- Finkelstein, M., Hestrin, S. & Koch, W. (1947). *Proc. Soc. exp. Biol., N.Y.*, **64**, 64.
- Garst, J. B., Nye, J. F., Maron, D. M. & Friedgood, H. B. (1950). *J. biol. Chem.* **186**, 119.
- Gold, V. & Tye, F. L. (1952). *J. chem. Soc.* p. 2172.
- Goldzieher, J. W., Bodenchuk, J. M. & Nolan, D. (1952). *J. biol. Chem.* **199**, 621.
- Goldzieher, J. W., Bodenchuk, J. M. & Nolan, D. (1954). *Analyt. Chem.* **26**, 853.
- Gornall, A. G. & Kalant, H. (1955). *Analyt. Chem.* **27**, 474.
- Graff, M. M. (1952). *J. biol. Chem.* **197**, 741.
- Kalant, H. (1954). *Rev. canad. Biol.* **13**, 476.
- Levine, V. E. & Tao, A. H. (1953). *Fed. Proc.* **12**, 238.
- Linford, J. H. (1952). *Canad. J. med. Sci.* **30**, 199.
- Linford, J. H. (1957). *Canad. J. Biochem. Physiol.* **35**, 299.
- Linford, J. H. & Fleming, O. J. (1953). *Canad. J. med. Sci.* **31**, 182.
- Linford, J. H. & Paulson, O. B. (1952). *Canad. J. med. Sci.* **30**, 213.
- McAleer, W. J. & Kozlowski, M. (1956). *Arch. Biochem. Biophys.* **62**, 196.
- Marrian, G. F. (1930). *Biochem. J.* **24**, 435.
- Neher, R. & Wettstein, A. (1951). *Helv. chim. acta*, **34**, 2278.
- Newman, M. S. & Deno, N. C. (1951). *J. Amer. chem. Soc.* **73**, 3651.
- Nowaczynski, W. J. & Steyermark, P. R. (1955). *Arch. Biochem. Biophys.* **58**, 453.
- Pontius, D., Beckmann, I. & Voigt, K.-D. (1955). *Acta endocrin., Copenhagen*, **20**, 19.
- Reichstein, T. & Shoppee, C. W. (1943). *Vitam. & Horm.* **1**, 345.
- Schwenk, E. & Hildebrandt, F. (1932). *Naturwissenschaften*, **20**, 658.
- Simpson, S. A., Tait, J. F., Wettstein, A., Neher, R., Euw, J. von, Schindler, O. & Wettstein, T. (1954). *Helv. chim. acta*, **37**, 1163.
- Steyermark, P. R. & Nowaczynski, W. J. (1955). *Arch. Biochem. Biophys.* **59**, 1.
- Sweat, M. L. (1954). *Analyt. Chem.* **26**, 773.
- Tauber, H. (1952). *Analyt. Chem.* **24**, 1494.
- Umberger, E. J. & Curtis, J. M. (1949). *J. biol. Chem.* **178**, 275.
- Wieland, H., Straub, W. & Dorfmueller, T. (1929). *Hoppe-Seyl. Z.* **186**, 97.
- Wintersteiner, O. & Pfiffner, J. J. (1936). *J. biol. Chem.* **116**, 291.
- Zaffaroni, A. (1950). *J. Amer. chem. Soc.* **72**, 3828.
- Zaffaroni, A. (1953). *Recent Progr. Hormone Res.* **8**, 51.
- Zaffaroni, A., Burton, R. B. & Keutmann, E. H. (1950). *Science*, **111**, 6.

Fluorimetric Measurement of Adrenocortical Steroids in Concentrated Acid Solution

By H. KALANT

Department of Pathological Chemistry, University of Toronto, Toronto, Canada

(Received 29 July 1957)

A number of steroids of various types, when dissolved in concentrated sulphuric, phosphoric or other acids, exhibit visible fluorescence under suitable ultraviolet or visible illumination. This phenomenon has been adapted quantitatively for fluorophotometric measurement of various oestrogenic steroids, and numerous modifications of technique have been devised (see review by Bates, 1954). Very much less attention seems to have been directed to the quantitative possibilities of the fluorescence shown by adrenocortical steroids in acid solution.

Interest in this phenomenon was revived by the finding of Neher & Wettstein (1951) that all the physiologically active corticosteroids, when isolated on paper chromatograms, fluoresce with distinctive colours under ultraviolet illumination, after the chromatogram has been heated with phosphoric or sulphuric acid or acetic anhydride. Finkelstein (1952) developed a quantitative fluorophotometric assay for certain corticosteroids dissolved in 85 % phosphoric acid with the aid of heat. Sweat (1954) reported a more sensitive method, in which the steroids were dissolved in concentrated

sulphuric acid at room temperature. Both authors, however, found the method to be applicable only to corticosterone and 17-hydroxycorticosterone, and at a considerably lower level of sensitivity to 11-deoxycorticosterone. The colour of fluorescence observed in all cases was green.

In a previous paper (Kalant, 1958) it was shown that a considerably wider range of steroids can fluoresce when dissolved in concentrated acid, as numerous investigators had found previously, and that these steroids do in fact fluoresce with different colours as reported by Neher & Wettstein (1951). These colours were found to be composite, i.e. each reaction mixture emitted two or more fluorescence bands, the relative intensities of which varied according to the temperature, acid concentration, primary illumination and other reaction and measurement conditions. These findings suggested that the high specificity, or conversely the narrow range of applicability, of the methods of Finkelstein (1952) and of Sweat (1954) might be readily altered by simple changes in experimental conditions. The present paper describes some of the changes which permit the extension of the acid-fluorescence method to the measurement of a number of steroids other than corticosterone and hydrocortisone.

EXPERIMENTAL

Apparatus and materials

Fluorophotometric measurements were made in a Coleman Model 12 Electronic Photofluorometer. The original ultraviolet source in the instrument was replaced by a General Electric H85C3 mercury-vapour lamp. The jacket of this lamp is made of a glass with a high degree of transparency to ultraviolet radiation. The transmission properties of the various primary and secondary filters employed may be found in the catalogue of the Corning Glass Co., and are summarized briefly at relevant points in the text below. A specially made adapter permitted the use of 10 mm. $\times$ 150 mm. Pyrex cuvettes, so that 4 ml. samples could be used instead of the 8 ml. ordinarily required in the Coleman instrument. The reaction vessels were 25 ml. Pyrex test tubes fitted with ground-glass stoppers of standard taper.

A.C.S. reagent-grade concentrated H_2SO_4 (Canadian Industries Ltd.) was used without further purification.

Phosphoric acid was AnalA.R.-grade concentrated orthophosphoric acid, supplemented with AnalA.R.- P_2O_5 , to give almost 100% H_3PO_4 (sp.gr. 1.86), as described elsewhere (Kalant, 1958). For convenience, this is referred to below as 100% H_3PO_4 .

Absolute methanol and ethanol were distilled over 2:4-dinitrophenylhydrazine and then redistilled before use, and were stored in dark bottles in the cold.

Outline of procedures investigated

Development of fluorescent products from steroids dissolved in concentrated H_2SO_4 was carried out by the method of Sweat (1954). The procedure, with 100% H_3PO_4 , was essentially the same as that used by Finkelstein (1952) with 85% H_3PO_4 . A detailed study of this latter procedure

as applied to oestrogens was carried out by Braunsberg (1952), and in the present work many of her observations were found to be valid for the measurement of corticosteroids. Essentially the method consists of adding a quantity of acid to dry steroid, and heating in a water bath at a selected temperature in the dark, for a measured time. As shown by Braunsberg (1952), exposure to light during the heating period decreases the fluorescence yield, the loss being greater the longer the period of heating.

As described below, optimum results in the fluorophotometry of hydrocortisone were found with a shorter period of heating than that used by Finkelstein (1952) and by Braunsberg (1952). It was found, however, that with short periods of heating, solution of dry steroid samples in the very concentrated H_3PO_4 was incomplete and variable. This difficulty was easily overcome by adoption of the following procedure. The solution of steroid in methanol or ethanol was measured into the reaction tube and evaporated to dryness in an oven at 100°. When cool, the dry residue was redissolved in exactly 0.05 ml. of absolute ethanol. The concentrated acid was then added, and mixed thoroughly before heating. This acid is very viscous at room temperature, but is much more readily pipetted if warmed first.

Immediately on completion of the measured period of heating, the tubes were placed in a cold-water bath. When the contents were approximately at room temperature, they were transferred to cuvettes for fluorimetry. As shown by Bates & Cohen (1950) and by Diczfalusy (1953), the intensity of fluorescence is inversely related to the temperature of the sample; this is a general characteristic of fluorescent solutions (Pringsheim, 1949). However, it was not found practicable to cool the samples below room temperature, because of the risk of condensation on the cuvettes.

RESULTS

Influence of initial acid concentration

Samples of 100% H_3PO_4 were diluted with sufficient water to give a series of acids which were 90, 77, 56 and 43% (v/v) H_3PO_4 . With each of these acids, the fluorescence procedure was carried out as described above in 'Outline of procedures investigated', with duplicate samples of 0.35 μg . of hydrocortisone 21-acetate and a corresponding reagent blank. The reaction mixtures were heated in a boiling-water bath for 5 min. Fluorescence was measured with the Coleman B-2 primary filter (principal transmission 436 $m\mu$ mercury line) and the PC-2 secondary filter (sharp cut-off below 525 $m\mu$), as recommended by Finkelstein (1952). The results are given in Table 1.

Table 1. *Effect of initial concentration of phosphoric acid on fluorescence of hydrocortisone 21-acetate*

Concn. of acid (%, v/v)	Specific gravity	Fluorescence (scale reading)
100	1.86	94
90	1.74	82
77	1.60	51
56	1.39	3
43	1.27	1

These results are in full agreement with those found by Braunsberg (1952) when applying the reaction to oestrogens. Ethanol was found to exert the same effect when it was used as the diluent in place of water, which indicates the importance of using a measured and minimal amount of ethanol for redissolving the dried steroid residue before the addition of acid.

As described below, it was found advantageous in the measurement of certain steroids to dilute the reaction mixtures, after heating, with sufficient water to bring the final acid concentration down to 12.5% (v/v). In these cases, dilution of the acid before the heating period was found to have the same deleterious effect on the production of fluorescence as that noted above.

Influence of temperature and duration of heating

A series of equal quantities of hydrocortisone acetate and a number of reagent blanks were treated with 100% H_3PO_4 as described above, in a boiling-water bath at 100°. At intervals of 1, 2, 3, 4, 5, 10, 20 and 30 min. after immersion in the bath, a reagent blank and three reaction mixtures were removed and cooled for fluorophotometric measurement. Similar experiments were carried out at 90°, 80° and 70°, in a thermoregulated bath. Fluorometric measurements were made with the Coleman B-2 and PC-2 filters. The results of the first three experiments are shown graphically in Fig. 1. The results at 70° were too erratic to permit valid graphic representation.

The findings were considered indicative of two

processes: one involved in the production of the fluorescent material and the other in its destruction. The assumed destructive process would appear to have a lower rate and a lower thermal coefficient than the productive process, since highest results were obtained with a heating period of 5 min. at 100°.

Influence of oxidizing and reducing agents

Oxidizing agents are known to be capable of destroying the fluorescent products formed from oestrogens in concentrated acid (McAnally & Hausman, 1954). Because of the possibility that the destructive process postulated above might be oxidative in nature, the effects of several oxidizing and reducing agents on the fluorescence of hydrocortisone in 100% H_3PO_4 and in concentrated H_2SO_4 were tested. Saturated KNO_3 and KClO_3 solutions, and BaO_2 in trace amounts, were all found capable of preventing or destroying the fluorescence. Of the reducing agents tried, NaHSO_3 and 5% Metol (*p*-methylaminophenol sulphate) reagent completely prevented the development of fluorescence. Small amounts of FeSO_4 , however, produced a considerable increase in the intensity of fluorescence in H_3PO_4 . An increase of over 60% in fluorescence intensity was obtained by addition of 2 mg. of FeSO_4 to each tube; larger or smaller amounts were less effective.

Comparison of phosphoric acid from different sources

The findings described in the two preceding sections were compatible with the assumption that some contaminant was present in the H_3PO_4 , possibly an oxidizing agent. Therefore the fluorogenic efficacies of H_3PO_4 from several sources were compared. These included AnalaR (British Drug Houses Ltd.), A.C.S. grade (Fisher Scientific Co.) and Erco A.C.S. (Electroreduction Co.) grade acids, supplemented as described above with British Drug Houses Ltd. Certified, Fisher A.C.S. or Baker C.P. grades of P_2O_5 . Considerable differences were found. The best results were obtained with AnalaR H_3PO_4 supplemented with Fisher A.C.S. grade P_2O_5 . With this combination, FeSO_4 produced no increase in fluorescence, which suggests that its previous beneficial effect had indeed been due to some protective action against an impurity in the acid used.

Influence of dilution after heating

The green fluorescence of corticosterone and hydrocortisone in concentrated H_3PO_4 or H_2SO_4 has been shown to be due to a stable product which continues to fluoresce even after extreme dilution of the acid solutions (Kalant, 1958). With such dilution, however, a primary illumination of

Fig. 1. Effect of temperature and duration of heating on intensity of fluorescence of hydrocortisone 21-acetate in 100% H_3PO_4 . ●, 100°; □, 90°; ▲, 80°. Encircled points indicate identical triplicate values.

436 m μ wavelength or longer becomes progressively less effective in producing fluorescence, whereas 365 m μ or shorter radiation becomes more effective.

The quantitative importance of this change was determined in the following manner. Equal amounts of hydrocortisone were heated, at 100° for 5 min., with 0.5 ml. of 100 % H₃PO₄. After cooling, half the samples were diluted to 4 ml. with more of the same H₃PO₄, and the others were diluted with water. Each series was read against a corresponding blank, in the Coleman Model 12 fluorophotometer, first with the Coleman B-2 primary filter and then with the Corning 9863 primary filter (broad-band transmission of ultraviolet below 400 m μ). In each case the Coleman PC-2 secondary filter was employed. The results are shown in Table 2.

The influence of dilution on the fluorescence of hydrocortisone in H₂SO₄ was also tested. To each of a series of 0.1 μ g. samples of hydrocortisone was added 0.05 ml. of absolute ethanol, followed by 1 ml. of concentrated H₂SO₄. After thorough mixing of the contents the reaction vessels were left in the dark at room temperature for 1 hr. The mixtures were then diluted to 4 ml. with appropriate amounts of H₂SO₄ and water to give the final acid concentrations shown in Table 3. Each sample was read in the Coleman fluorophotometer with PC-2 secondary filter, compared against an acid-water blank of the corresponding composition.

The anomalous high value in 96 % acid, with the 9863 primary, is probably due to the fact that concentrated H₂SO₄ itself emits a blue fluorescence under this illumination, and the fluorescence serves as an additional excitation source for the steroid fluorogen.

Table 2. *Effect of dilution on fluorescence of hydrocortisone in phosphoric acid*

Final concn. of acid (%, v/v)	Scale readings	
	B-2 Primary	9863 Primary
100	75	15
12.5	4	210

Table 3. *Effect of dilution on fluorescence of hydrocortisone in sulphuric acid*

Final concn. of acid (%, v/v)	Scale readings	
	B-2 Primary	9863 Primary
96	48	28
72	24	4
48	26	12
24	8	26
12*	4	50

* Reaction was carried out with 0.5 ml. of conc. H₂SO₄; final dilution to 4 ml. was made with water.

Routine procedures for measurement of hydrocortisone and corticosterone

In view of the observations described in the preceding sections, the following procedure was adopted for routine work. A sample of solution or extract, known or estimated to contain from 0.05 to 0.35 μ g. of corticosterone or hydrocortisone, is evaporated to dryness in a glass reaction vessel. Absolute ethanol (0.05 ml.) is added, and the tube rotated so that the steroid residue is thoroughly wetted by the alcohol.

For the H₂SO₄ reaction, concentrated H₂SO₄ is added to a total volume of 4 ml., followed by thorough mixing and storage in the dark at room temperature for 1 hr. The mixture is transferred to a cuvette and read against an acid blank in the fluorophotometer, the B-2 and PC-2 filter system being used.

For the H₃PO₄ reaction, 0.5 ml. of the 100 % H₃PO₄ is used; after thorough mixing, the stoppered vessel is heated in a boiling-water bath in a dark room, for 5 min. The reaction mixture is cooled immediately in a running-water bath, water is added to a total volume of 4 ml., and after thorough mixing, the contents are transferred to a cuvette. When at room temperature, and free of bubbles, the solution is read in the fluorophotometer against a corresponding water-acid blank, with the Corning 9863 primary filter and Coleman PC-2 secondary filter. Fig. 2 shows a standard graph for hydrocortisone prepared in this manner.

Fig. 2. Standard graph for fluorophotometric measurement of hydrocortisone by phosphoric acid reaction. Final vol. 4 ml.; final acid concentration 12.5 %. Corning 9863 primary filter, Coleman PC-2 secondary filter. Encircled points indicate identical replicate values.

Routine procedures for cortisone and aldosterone

Cortisone reacts with concentrated H_2SO_4 or H_3PO_4 to give a product with visible blue fluorescence under near-ultraviolet illumination (Kalant 1958). This product disappears on dilution of the reaction mixture with either water or ethanol. Therefore for the measurement of cortisone, the reactions are carried out in the same manner as described above for hydrocortisone except that the H_3PO_4 reaction mixtures are made to 4 ml. with concentrated acid just as in the H_2SO_4 procedure. For fluorophotometry, the Coleman B-1, PC-1 filter system is used. The B-1 primary filter transmits chiefly the 365 m μ mercury line, whereas the PC-1 secondary filter transmits everything above 420 m μ . Although the Corning 9863 filter would pass more of the 365 m μ line, its transmission range overlaps the lower part of the range of the PC-1 filter and readings are therefore impossible. This fact limits the sensitivity of the method as applied to cortisone, as shown in the accompanying standard graph (Fig. 3).

When solutions of aldosterone in concentrated acid are heated and then diluted with water or ethanol, they exhibit absorption and fluorescence spectra similar to those of undiluted cortisone reaction mixtures. For routine purposes, the sample is most conveniently treated with 2 ml. of concentrated H_2SO_4 in a water bath at 100° for 15 min., cooled to room temperature in running water and diluted to 4 ml. with water or ethanol. Fluorophotometry is carried out with the same filter system as for cortisone, with quantities ranging from 0.1 to 1.0 μg . of steroid. This procedure has not been as well explored as that described for the

other steroids, and it is possible that different conditions of heating and dilution may give still greater sensitivity of measurement.

Deoxycorticosterone and other corticosteroids

For the purposes that prompted the present work, it was not necessary to devise routine methods for the fluorophotometric estimation of deoxycorticosterone, 17-hydroxy-11-deoxycorticosterone or 11-dehydrocorticosterone. However, the methods described above can be extended to the measurement of these compounds and numerous others. For example, deoxycorticosterone in concentrated H_2SO_4 was found to exhibit moderately strong fluorescence bands centred at about 410, 580 and 650 m μ when exposed to near-ultraviolet illumination. When diluted with water to 50 % acid concentration, the solution shows a considerable decrease in the blue fluorescence, but a marked increase in the green band, elicited easily with either the 365 or the 436 m μ mercury line. Fluorescence could therefore be measured either with the B-1 primary filter and a 405 or 410 m μ interference-type secondary filter, in concentrated acid, or with the B-2/PC-2 or 9863/PC-2 filter systems after 50 % dilution.

DISCUSSION

The value of fluorophotometric methods of measurement of adrenocortical, oestrogenic and other steroids lies in their simplicity and marked sensitivity. Claims have also been made for a relatively high degree of specificity towards a restricted number of steroids. It is clear, however, from a number of studies (Boscott, 1948; Neher & Wettstein, 1951; Linford, 1957; Linford & Paulson, 1952; Dirscherl & Breuer, 1954; Pontius, Beckmann & Voigt, 1955; Kalant, 1958) that the chromogenic and fluorogenic properties of steroids treated with concentrated acids result from very complex and relatively unspecific reactions. The course of these reactions can be profoundly influenced by relatively small changes in time and temperature of reaction, dilution, exposure to light, amount of trace contaminants and other reaction conditions. Because of this, a wide variety of reaction procedures can be selected empirically, each of which favours a specific steroid or class of steroids. This implies equally, however, that such specificity is rather precarious, and that minor unseen errors of technique may cause considerable errors of measurement. Strict specificity therefore continues to depend more on the methods of extraction and isolation of steroids from natural sources than on the technique of measurement employed.

The most useful development of fluorophotometric analysis therefore seems to be in the

Fig. 3. Standard graph for fluorophotometric measurement of cortisone by phosphoric acid reaction. Final vol. 4 ml.; acid concentration 100 %. Coleman B-1 primary and PC-1 secondary filters.

direction of extending its applicability as a sensitive micromasurement technique to as many steroids as possible. The present work indicates some of the possibilities for the measurement of adrenocortical steroids other than hydrocortisone and corticosterone. There does appear to be sufficient difference between the optimum conditions of measurement for the individual corticosteroids to make possible the specific measurement of one member of the group in the presence of other members (Kalant, 1958). It is clear, however, that at least a group-specific extraction procedure is necessary.

Effective use of fluorophotometric analysis requires careful attention to the emission properties of the primary light source, the transmission characteristics of the filters and the relative spectral response of the photocell, as pointed out by various workers (Braunsberg & Osborn, 1952; Aitken & Preedy, 1953; Abelson & Bondy, 1955). The significance of these factors will not be reviewed here, but attention is drawn to the fact that the fluorophotometer and filters employed in this work were used because they were available to the author and not because they are the best suited to the needs. The higher sensitivities obtained by Sweat (1954) and by Abelson & Bondy (1955) are explained largely by the fact that they employed Farrand instruments incorporating electron-multiplier tubes rather than the barrier-layer photocell of the Coleman Model 12 fluorophotometer.

The present findings do not indicate any clear preference in the choice between sulphuric and phosphoric acids for the fluorogenic reaction. Concentrated sulphuric acid of adequate purity is obtainable commercially, can be pipetted easily, and gives more intense fluorescence than phosphoric acid does. Suitably concentrated phosphoric acid must be specially prepared and is more difficult to handle, but because of its lower reactivity the reaction products are relatively stable for a long period, permitting greater flexibility of measurement. In addition, the lower reactivity of phosphoric acid may contribute somewhat to greater specificity by lessening the similarities between the groups of fluorescent products derived from the various adrenocortical steroids.

SUMMARY

1. Fluorophotometric methods of measurement of several adrenocortical steroids have been developed, based upon the fluorogenic reaction of these steroids with very concentrated phosphoric or sulphuric acid.

2. The influence of initial acid concentration, time and temperature of heating, contaminants and final dilution with water upon the fluorescence intensity have been studied.

3. Recommended procedures for the routine measurement of hydrocortisone, corticosterone and cortisone are given.

4. Modifications of procedure are also suggested, to permit the fluorophotometric measurement of aldosterone, 11-deoxycorticosterone and other corticosteroids.

This work was carried out during tenure of a Senior Medical Research Fellowship awarded by the National Research Council of Canada.

REFERENCES

- Abelson, D. & Bondy, P. K. (1955). *Arch. Biochem. Biophys.* **57**, 208.
- Aitken, E. H. & Preedy, J. R. K. (1953). *J. Endocrin.* **9**, 251.
- Bates, R. W. (1954). *Recent Progr. Hormone Res.* **9**, 95.
- Bates, R. W. & Cohen, H. (1950). *Endocrinology*, **47**, 166.
- Boscott, R. J. (1948). *Nature, Lond.*, **162**, 577.
- Braunsberg, H. (1952). *J. Endocrin.* **8**, 11.
- Braunsberg, H. & Osborn, S. B. (1952). *Analyt. chim. acta*, **6**, 84.
- Diczfalusy, E. (1953). *Acta endocr., Copenhagen*, **12**, suppl. 12.
- Dirscherl, W. & Breuer, H. (1954). *Z. Vitam.- Horm.- u. Fermentforsch.* **6**, 287.
- Finkelstein, M. (1952). *Nature, Lond.*, **169**, 929.
- Kalant, H. (1958). *Biochem. J.* **69**, 79.
- Linford, J. H. (1957). *Canad. J. Biochem. Physiol.* **35**, 299.
- Linford, J. H. & Paulson, O. B. (1952). *Canad. J. med. Sci.* **30**, 213.
- McAnally, J. S. & Hausman, E. R. (1954). *J. Lab. clin. Med.* **44**, 647.
- Neher, R. & Wettstein, A. (1951). *Helv. chim. acta*, **34**, 2278.
- Pontius, D., Beckmann, I. & Voigt, K.-D. (1955). *Acta endocr., Copenhagen*, **20**, 19.
- Pringsheim, P. (1949). *Fluorescence and Phosphorescence*. New York: Interscience Publishers Inc.
- Sweat, M. L. (1954). *Analyt. Chem.* **26**, 773.