Biochem. J. (1967) **105**, 1085
Printed in Great Britain

Partition of Porphyrins between Cyclohexanone and Aqueous Sodium Acetate as a Function of pH

DETERMINATION OF UROPORPHYRIN AND OF HYDROPHILIC PORPHYRIN CONJUGATES

BY C. RIMINGTON AND A. BENSON
Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(*Received* 22 *May* 1967)

1. The partition of uroporphyrins I and III, coproporphyrins I and III, haematoporphyrin IX, porphyrin *c* and a hydrophilic porphyrin–peptide fraction from variegate-porphyria faeces has been studied in systems of equal volumes of cyclohexanone and sodium acetate buffers of varying pH and concentration. 2. The concentration of acetate in the aqueous phase has little effect on the partition of porphyrin *c*, but markedly influences that of uroporphyrin. At 50% acetate saturation and pH 4·5, only 5% enters the cyclohexanone phase whereas 60% of porphyrin *c* is extracted under similar conditions. 3. This circumstance forms the basis of a method for the determination of hydrophilic porphyrin–peptides in variegate-porphyria urine. Its reliability has been checked in model experiments. 4. At pH 1·5 and an aqueous phase half-saturated with sodium acetate, an equal volume of cyclohexanone removes 95–97% of uroporphyrin and about 55% of porphyrin *c*. Uroporphyrin may therefore be determined as a second step in the method. 5. For the routine determination of uroporphyrin in systems free from other hydrophilic porphyrins, cyclohexanone extraction may be performed at any pH in the range 1·0–3·0.

The determination of uroporphyrin in biological materials is rendered difficult by its insolubility in the usual organic solvents. Dresel & Tooth (1954) found, however, that uroporphyrins I and III could both be extracted by ethyl acetate from solutions within the narrow pH range 3·0–3·2. Kennedy (1953) noted the extractability of uroporphyrin from acid solutions by cyclohexanone and this observation led to the quantitative technique of Dresel, Rimington & Tooth (1956), which has since become the standard procedure for determination of uroporphyrin in urine (Rimington, 1961). In their description they direct that the pH of the solution containing the uroporphyrin be adjusted to 1·5 before extraction with cyclohexanone. This pH or the narrow range 1·5–1·8 (Dressel & Falk, 1956) has been followed generally by other workers.

During work on the porphyrins excreted in porphyria variegata, the presence was observed in urine of hydrophilic porphyrin that was fairly readily extracted by cyclohexanone at pH 2·9–3·0 but hardly at all at pH 1·5. This material was later identified as porphyrin–peptide conjugate, similar in many respects to porphyrin *c* (C. Rimington, W. H. Lockwood & R. V. Belcher, unpublished work). The possibility of separating it from uroporphyrin by a partition procedure demanded much more detailed information on the behaviour of each material in the cyclohexanone–sodium acetate buffer system and has prompted the investigation reported below. The opportunity was taken of examining also the behaviour of coproporphyrins I and III and of haematoporphyrin IX, which arises rather readily as a result of degradation of the porphyrin–peptide complexes.

MATERIALS

Chemicals. Cyclohexanone (technical grade; Hopkin and Williams Ltd., Chadwell Heath, Essex) was distilled under reduced pressure, collecting the fraction with constant boiling point. Ether (peroxide-free; May and Baker Ltd., Dagenham, Essex) was used without further treatment. Hydrochloric acid (A.R. grade; British Drug Houses Ltd., Poole, Dorset) was standardized and found to be 11·6N (42·3%, w/v). All dilutions were prepared on this basis and are recorded as weight of acid per 100ml.

Porphyrins. Uroporphyrin I octamethyl ester, m.p. 293°, was isolated from a case of congenital porphyria (Rimington & Miles, 1951). Uroporphyrin III octamethyl ester, m.p. 253–260°, was prepared from turacin. Coproporphyrin I

tetramethyl ester, m.p. 246–248°, was isolated from the same source as uroporphyrin I octamethyl ester. Coproporphyrin III tetramethyl ester, m.p. 155–170°, was obtained from *Corynebacterium diphtheriae* by the method of Gray & Holt (1948). Haematoporphyrin dihydrochloride was a gift from Dr G. Y. Kennedy. Porphyrin *c* was a gift from Dr S. Sano. This sample contained some impurities and was purified by chromatography on Celite (Tuppy & Paléus, 1955), yielding two fractions, samples of which were examined by lutidine paper chromatography (Eriksen, 1958). The first band to leave the column contained the impurities and gave spots in the positions for tricarboxylic (strong) and dicarboxylic porphyrins (faint). The main band consisted of porphyrin *c*, giving spots equivalent in position to coproporphyrins I and III, together with a relatively very small admixture of the tricarboxylic material of fraction 1. Both fractions were used in the experiments described below.

METHODS

All porphyrins were used in the free state; esters were hydrolysed in 25% (w/v) HCl for 42 hr. at room temperature and evaporated to dryness *in vacuo* over solid NaOH, and the residue was washed with a little water and again dried. The porphyrin was dissolved in 0·1 M-$NaHCO_3$ and its concentration determined spectrophotometrically, after appropriate dilution with 5% (w/v) HCl, with a Unicam SP. 500 spectrophotometer, by using accepted extinction coefficients (Rimington, 1956) and the correction formula of Rimington & Sveinsson (1950). For porphyrin *c* the same extinction data were used as for uroporphyrin. The stock solutions were adjusted to a final concentration of 20–40 μg./ml. of 0·1 M-$NaHCO_3$.

Conduct of partition experiments. Partition experiments were carried out over the pH range 0·5–6·0, with slight variations within this range. The experimental technique was as follows: to each of ten 250 ml. beakers was added in the following order 25 ml. of 10% (w/v) HCl, a quantity of stock solution to contain 20–25 μg. of porphyrin and then 75 ml. of saturated sodium acetate (372 g./l.). The mixture was rapidly brought to the required pH by addition of conc. HCl or saturated sodium acetate, the volume was adjusted to 140 ml. with glass-distilled water and each solution was shaken with 140 ml. of cyclohexanone for 30 sec. The solutions were allowed to stand for 20 min. to ensure good separation of the phases. The aqueous layer was then checked for fluorescence under ultraviolet light and removed. To the organic layer was added 280 ml. of ether (2 vol.) and, for porphyrin *c*, in addition about 50 ml. of light petroleum (b.p. 40–60°) to facilitate extraction of the porphyrin by acid. The organic layer was then extracted to completion by repeated shakings with 5% (w/v) HCl and the porphyrin content of the extract determined spectrophotometrically. The results are conveniently expressed as percentages passing into the cyclohexanone.

Separation of a mixture of uroporphyrin III and porphyrin c. A model separation experiment was performed with a mixture of the two hydrophilic porphyrins uroporphyrin III and purified porphyrin *c* (tetracarboxylic fraction; see the Materials section) to assess the validity of the findings in actual practice. An added reason for this experiment was the similar physical properties of porphyrin *c* and the hydrophilic porphyrin–peptide conjugates excreted by variegate-porphyria patients. From the stock solutions, uroporphyrin III (18·72 μg.) and porphyrin *c* (20 μg.) were added to 25 ml. of 10% (w/v) HCl and 75 ml. of saturated sodium acetate and the mixture was brought to pH 4·5. The volume was adjusted to 120 ml. Two successive shakings were performed with an equal volume of cyclohexanone and the total porphyrin extracted was determined by transfer to acid and spectrophotometry. The aqueous phase, the pH of which was 5·0, was then brought to pH 2·0 and twice extracted with an equal volume of cyclohexanone. Porphyrin in the cyclohexanone extracts was determined by acid extractions and spectrophotometry as already described.

Application of the method to a faecal extract. The material was a crude 'X porphyrin' fraction (C. Rimington, W. H. Lockwood & R. V. Belcher, unpublished work) obtained from faeces of a variegate-porphyria patient. Partition between half-saturated sodium acetate and an equal volume of cyclohexanone was determined on samples adjusted to pH 1·5, 2·3, 3·0, 4·0 and 5·0.

Effect of concentration of acetate in the aqueous phase. In all the experiments so far described the aqueous phase was approximately half-saturated in sodium acetate. The effect of varying acetate concentration on the partition between cyclohexanone and the aqueous phase at pH 4·5 was studied as follows.

From a normal urine, coproporphyrin was removed by ether extraction and to 25 ml. portions was added sufficient saturated sodium acetate to achieve percentage saturations of 50, 25, 10, 5 and 2·5 respectively. Uroporphyrin III (0·67 ml., 23·5 μg.) was added to each and the pH adjusted to 4·5 with either conc. HCl or 2 N-NaOH and the mixture was shaken with an equal volume of cyclohexanone. The porphyrin extracted was determined as described above. This experiment was repeated with porphyrin *c* (16·55 μg.) added to portions in which the percentage saturation was 50, 25, 10 and 2·5 respectively. Finally, the partition (at pH 4·5) was examined of uroporphyrin between cyclohexanone and porphyrin-free urine from a case of variegate porphyria. To prepare this, the urine was twice extracted with ether–acetic acid, adjusted to pH 4 and shaken with talc, which was removed by centrifugation. The conduct of the experiment then followed that described above with coproporphyrin-free normal urine.

Application to determination of uroporphyrin and hydrophilic porphyrin–peptide in urine from a variegate-porphyria patient and in control normal urine. Two experiments were carried out with the porphyric urine. First, from two 25 ml. portions coproporphyrin was removed by shaking with ether, the ether being washed twice by 3% (w/v) sodium acetate and the washings being returned to the aqueous phases. Solid sodium acetate was added to one aqueous phase to 50% saturation and to the other to 25% saturation. Each was adjusted to pH 4·5 and shaken twice with equal volumes of cyclohexanone. The aqueous phases were adjusted to pH 1·5 and again shaken twice with cyclohexanone. Extracted porphyrin was determined as described above.

In the second experiment 500 ml. of urine passed by this patient was rendered coproporphyrin-free (the ether extract not being washed with acetate) and then half-saturated with sodium acetate added as solid. The pH was adjusted to 4·5 and the mixture shaken once only with an equal volume of cyclohexanone. The resulting aqueous phase was again extracted once at pH 1·5. Normal urine (500 ml.) was treated in a similar manner.

Electrophoresis. Electrophoresis of extracted porphyrins was performed in a Shandon Universal electrophoresis tank with Whatman no. 1 chromatography paper and 0·05M-sodium barbital buffer, pH 8·6. The voltage was 10v/cm. and current 4mA.

Paper chromatography. The lutidine–water method of Eriksen (1958) was used. The acid extracts were evaporated to dryness *in vacuo*, and the residues were esterified with methanol containing HCl (20%, w/v), transferred to ether, washed and evaporated to dryness. The purified esters were then hydrolysed and the free porphyrin from each sample was run together with uroporphyrin I, coproporphyrins I and III, deuteroporphyrin IX and purified porphyrin *c* as markers.

For rapid identification it was possible to use Whatman no. 1 chromatography-paper strips (12·5cm. × 2cm.) standing in stoppered test tubes (15cm. × 2·5cm.) containing 2ml. of the lutidine–water mixture (Eriksen, 1958). An ammonia atmosphere was provided by a small strip of filter paper soaked in aq. ammonia (sp.gr. 0·88) and secured by the stopper at the top of the tube. A run normally took about 2hr. and resolution of uroporphyrin, coproporphyrin and dicarboxylic porphyrins was generally good. A strip with markers was run at the same time in a separate tube. For this rapid test, purification by esterification followed by hydrolysis may usually be omitted.

RESULTS

Uroporphyrins I and III. The results depicted in Figs. 1(*a*) and 1(*b*) reveal that there is a fairly wide range of pH over which 90% or more of either porphyrin is extracted by an equal volume of cyclohexanone. Below pH 1·0 and above pH 3·5 extractability falls off sharply and at pH 4·5 not more than about 5% enters the cyclohexanone phase. It is clear that the pH range 1·5–1·8 used by Dresel & Falk (1956) is unnecessarily narrow.

Coproporphyrins I and III. Coproporphyrin I passes practically quantitatively into the cyclohexanone phase at all pH values between 0·5 and 5·0; at pH 6·0 partition is 50% (Fig. 1*c*). Coproporphyrin III is not quite so readily extracted (Fig. 1*d*) and a falling off of that in the cyclohexanone phase is noticeable at pH 0·5.

Haematoporphyrin IX. Haematoporphyrin IX behaves very similarly to coproporphyrin III, but even at pH 6·0 over 85% enters the cyclohexanone phase (Fig. 2*a*).

Porphyrin c. The behaviour of the purified tetracarboxylic material is shown in Fig. 2(*b*). The partition is much less favourable to cyclohexanone at all pH values, which is understandable in view of the hydrophilic nature of this porphyrin. A clearly defined range of maximum extractability (70–74%) does occur, however, between pH 2·3 and 3·5. It is also evident that at pH 4·5 as much as 60% may be removed by a single shaking with an equal volume of cyclohexanone, whereas at this pH only about 5% of uroporphyrin is extracted.

The fraction 1 that was obtained during column

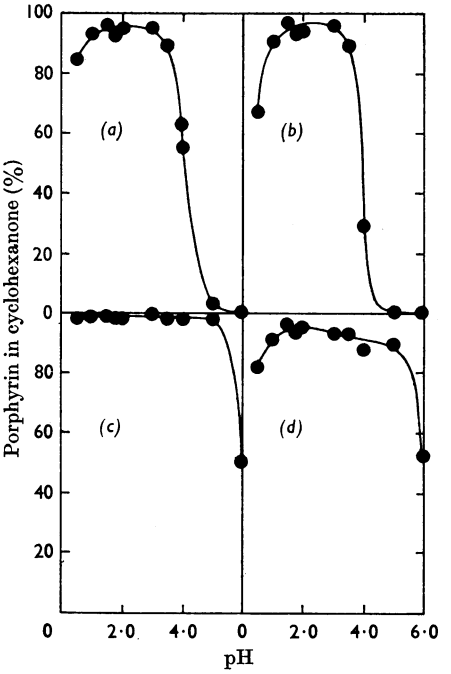

Fig. 1. Partition of porphyrins between cyclohexanone and an equal volume of aqueous half-saturated sodium acetate (186g./l.) adjusted to differing pH values. (*a*) Uroporphyrin I; (*b*) uroporphyrin III; (*c*) coproporphyrin I; (*d*) coproporphyrin III.

chromatographic purification of porphyrin *c* and that consisted mainly of tricarboxylic porphyrin together with some dicarboxylic porphyrin, presumably haematoporphyrin, gave somewhat irregular results in the partition experiment (Fig. 2*c*). It was, in general, more efficiently extracted than porphyrin *c*, about 90% passing into cyclohexanone at the optimum pH 3·0. Extractability fell away at higher pH values, as does that of porphyrin *c*, and also diminished between pH 3·0 and 1·0, but at pH 0·5 it appeared to rise again. This complex behaviour is possibly due to the presence of haematoporphyrin etc. in the mixture.

Separation of a mixture of uroporphyrin III and porphyrin c. Results with pure materials suggested that at every shaking at pH 4·5 with an equal volume of cyclohexanone, 60% of the porphyrin *c* and 5% of the uroporphyrin would be expected to pass from the aqueous to the organic phase. When, eventually, the aqueous phase was adjusted to pH 2·0 almost all (95%) of the uroporphyrin and 67% of the porphyrin *c* remaining should enter the cyclohexanone at each shaking.

The quantity of porphyrin removed by cyclohexanone at pH 4·5 was 14·5μg.; that expected by

calculation was 18·6 μg. The quantity subsequently removed at pH 2·0 was 19·3 μg. whereas the expected value was 19·7 μg. The agreement with the calculated partitions is good except that rather less porphyrin *c* than expected was removed at pH 4·5. The fault may have been failure to readjust the aqueous phase to pH 4·5 before the second shaking with cyclohexanone; it had risen to pH 5·0 after this was completed.

Confirmation that a good separation had been achieved was obtained by paper chromatography (Eriksen, 1958) of the materials extracted at pH 4·5 and subsequently at pH 2·0. A trace of dicarboxylic porphyrin (probably haematoporphyrin) appeared in the pH 4·5 extract, but this was undoubtedly due to some decomposition of the very labile porphyrin *c* having occurred during the manipulations necessary for recovery and chromatography.

Application of the method to a faecal extract. The partition of porphyrin contained in this material between half-saturated sodium acetate and cyclohexanone is recorded in Fig. 2(*d*). Its behaviour is similar to that of porphyrin *c* (fraction 1), indicating that at pH 4·5 a good separation should be possible from any uroporphyrin present in such extracts; other porphyrins such as coproporphyrin and haematoporphyrin would not be eliminated by this procedure, but could be extracted by ether.

Effect of concentration of acetate in the aqueous phase. The results of these experiments shown in Fig. 3 indicate that the concentration of acetate has little effect on the partition of porphyrin *c*, but greatly affects that of uroporphyrin. By working at about half-saturation, optimum conditions are achieved for a separation of these substances at pH 4·5.

Uroporphyrin and hydrophilic porphyrin in urine from a variegate-porphyria patient. The results of the first experiment in which two different concentrations of sodium acetate were used are presented in Table 1. They show good all round agreement and indicate that 85–90% of the total ether-insoluble porphyrin was extractable at pH 4·5, like porphyrin–peptide. Their identity was confirmed by labelling with 1-fluoro-2,4-dinitro-[U-^{14}C]benzene and radioautography (C. Rimington, W. H. Lockwood & R. V. Belcher, unpublished work).

The second experiment, employing a larger quantity of urine from this patient, gave a similar result, 90% of the ether-insoluble porphyrin behaving as porphyrin–peptide. Evidence of the efficiency of separation of uroporphyrin and

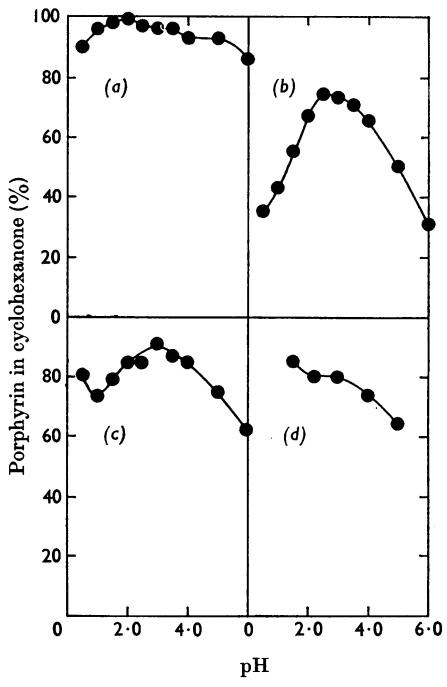

Fig. 2. Partition of porphyrins between cyclohexanone and an equal volume of aqueous half-saturated sodium acetate (186 g./l.) adjusted to differing pH values. (*a*) Haematoporphyrin IX; (*b*) porphyrin *c*; (*c*) tricarboxylic fraction accompanying porphyrin *c*; (*d*) hydrophilic porphyrin–peptide fraction (crude 'X porphyrin') from faeces of a case of variegate porphyria.

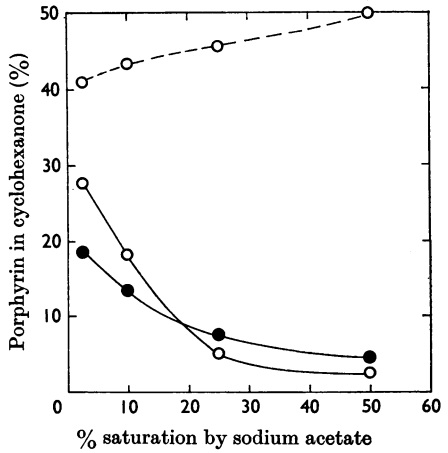

Fig. 3. Effect of concentration of sodium acetate on partition of uroporphyrin III (——) and of porphyrin *c* (– – – –) between cyclohexanone and porphyrin-free urine of a normal subject (○) and a case of variegate porphyria (●) at pH 4·5.

Table 1. *Determination of uroporphyrin and porphyrin–peptide in urine (25ml.) from a case of variegate porphyria by partition at* pH 4·5 *between cyclohexanone and aqueous phase* 50% *or* 25% *saturated with sodium acetate*

Prior ether extraction had removed coproporphyrin (52·8 and 51·1 μg.) from the two samples. Calculation of the porphyrins expected in each extract was based on Figs. 1(*b*) and 2(*b*), showing extraction of 60% of porphyrin–peptide and 5% of uroporphyrin by each shaking with cyclohexanone. For further details see the Methods section.

	Porphyrin (μg.)					
	Extracted			Expected by calculation		Porphyrin extracted at pH 4·5
% saturation with sodium acetate	At pH 4·5	At pH 1·5	Total	At pH 4·5	At pH 1·5	(% of total)
50	5·99	1·17	7·16	6·02	0·7	84
25	6·48	0·63	7·11	5·97	0·7	91

porphyrin–peptide in this experiment was obtained as follows. The two fractions extracted into cyclohexanone at pH 4·5 and 1·5 respectively were made slightly alkaline by addition, with cooling, of 40% (w/v) sodium hydroxide, and then calcium hydroxide was added to adsorb the porphyrin. The mixture was centrifuged and the adsorbent was washed repeatedly with 50% (v/v) ethanol, which removed much impurity. Sufficient 3N-hydrochloric acid was added to the chilled tubes to redissolve the precipitates and the pH then adjusted to 3·5–4·0 with sodium acetate. Two extractions with butan-1-ol removed the porphyrins, which were recovered in the dry state by evaporation of the solvent after washing twice with water. They were then dissolved in 2N-ammonia, applied to Whatman no. 1 paper and subjected to electrophoresis in 0·05M-barbital buffer, pH 8·6, the markers being coproporphyrin and uroporphyrin. The result showed the pH 4·5 fraction to contain slow-moving porphyrin–peptide (confirmed by lutidine chromatography), but no uroporphyrin, whereas the pH 1·5 fraction contained uroporphyrin together with porphyrin–peptide, as expected from the partition behaviour of these materials. Normal urine (500ml.) treated in the same way contained no measurable amount of porphyrin extractable at pH 4·5.

DISCUSSION

The separation of mixtures of hydrophilic porphyrins presents considerable difficulty. Partition between cyclohexanone and an aqueous phase half-saturated by sodium acetate is, however, markedly affected by pH and, for uroporphyrin, by concentration of the salt. Porphyrin *c* and the porphyrin–peptide conjugates excreted by variegate-porphyria patients (C. Rimington, W. H. Lockwood & R. V. Belcher, unpublished work) display solubility in the organic phase that falls off fairly rapidly on either side of a maximum at about pH 2·5. Uroporphyrins I and III, on the other hand, are well extracted by shaking the mixture, half-saturated in sodium acetate, once with an equal volume of cyclohexanone over the pH range 1·0–3·5, but only to the extent of about 5% at pH 4·5. These are the only other hydrophilic porphyrins of major importance likely to be present in biological materials.

From these findings, a fairly efficient separation of urinary hydrophilic porphyrin–peptide conjugates from uroporphyrin has been achieved by half-saturating the coproporphyrin-free urine with sodium acetate and extracting with cyclohexanone, first at pH 4·5 and then at pH 1·5–2·0. The method permits reasonably accurate calculation of the amount of each type of porphyrin and should be applicable also to faecal extracts. No detectable amount of porphyrin–peptide was found in a normal urine (pooled specimen). Urines from variegate-porphyria patients contain porphyrin *c*-like conjugates and are likely to contain small amounts of haematoporphyrin arising from partial decomposition of these complexes, but any haematoporphyrin would be removed in the preliminary extraction by ether.

For the routine determination by the cyclohexanone method of uroporphyrin in systems from which other hydrophilic porphyrins are absent, extraction with cyclohexanone may be performed at any pH in the range 1·0–3·0; it is not necessary to adhere to the narrow limits that have customarily been observed hitherto.

There are two ways in which the technique described in this paper could be used. For quantitative determination of porphyrin–peptide (in the absence of large amounts of uroporphyrin) it would appear permissible to give only one extraction at pH 4·5 with an equal volume of cyclohexanone, to

determine the quantity of porphyrin removed and to calculate the total porphyrin–peptide by assuming that this represents 60% of the total quantity actually present in the urine. Subsequent exhaustive extraction at pH 1·5 should remove the remaining 40% of the porphyrin–peptide and all the uroporphyrin. A set of simultaneous equations could, of course, be devised for use when the amount of uroporphyrin in the urine was comparable with that of porphyrin–peptide.

Alternatively, for preparative purposes, extraction with cyclohexanone at pH 4·5 could be continued until nearly all the porphyrin–peptide was removed; two extractions should remove 84%. Less impurity passes into cyclohexanone from urine at pH 4·5 than at pH 1·5.

REFERENCES

Dresel, E. I. B. & Falk, J. E. (1956). *Biochem. J.* **63**, 72.

Dresel, E. I. B., Rimington, C. & Tooth, B. E. (1956). *Scand. J. clin. Lab. Invest.* **8**, 73.

Dresel, E. I. B. & Tooth, B. E. (1954). *Nature, Lond.*, **174**, 271.

Eriksen, L. (1958). *Scand. J. clin. Lab. Invest.* **10**, 319.

Gray, C. H. & Holt, L. B. (1948). *Biochem. J.* **43**, 191.

Kennedy, G. Y. (1953). Ph.D.Thesis: University of Sheffield, p. 167.

Rimington, C. (1956). *Biochem. J.* **75**, 620.

Rimington, C. (1961). *Ass. clin. Path. Broadsheet*, no. 36.

Rimington, C. & Miles, P. A. (1951). *Biochem. J.* **50**, 202.

Rimington, C. & Sveinsson, S. L. (1950). *Scand. J. clin. Lab. Invest.* **2**, 209.

Tuppy, H. & Paléus, S. (1955). *Acta chem. scand.* **9**, 353.