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The Relationship of Opsopyrroledicarboxylic Acid to the Biosynthesis of Porphyrin

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Porphobilinogen is known to be a specific precursor of porphyrins (Bogorad & Granick, 1953; Falk, Dresel & Rimington, 1953) and, by use of radioactive porphobilinogen, it was shown by Dresel & Falk (1956*b*) that porphobilinogen alone is a sufficient substrate for the formation of haem in haemolysates prepared from fowl red cells.

The steps involved in the conversion of porphobilinogen into porphyrins of series III are not known; since the only derivatives of porphyrins to be used biologically are of series III and include chlorophyll, haemoglobin and other haem enzymes, the problem is of general importance. The particular difficulty in formulating a mechanism by which porphobilinogen might be converted into a porphyrin III arises as follows. The condensation of four molecules of porphobilinogen in the manner shown would, on cyclization, give rise to a series-I structure, which may be described briefly as (AP·AP·AP·AP). The series III structure is (AP·AP·AP·PA), the side chains being those of uroporphyrin. The state of oxidation of the tetramer need not be considered at present; only the relative positions of the β-side chains are relevant. Mechanisms proposed have included (1) condensation of porphobilinogen to give a radial trimer which might be split in two ways to give either (AP·AP) or (AP·PA) (Bogorad & Granick, 1953; Shemin, Russell & Abramsky, 1955), (2) migration of the α-side chain of porphobilinogen (Robinson, 1955; Bullock, Johnson, Markham & Shaw, 1958; D. Mauzerall, personal

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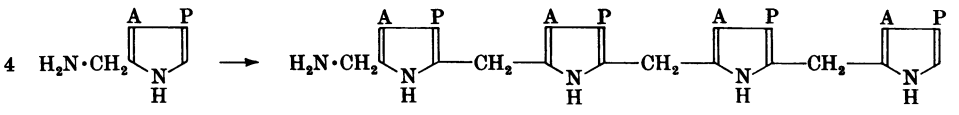
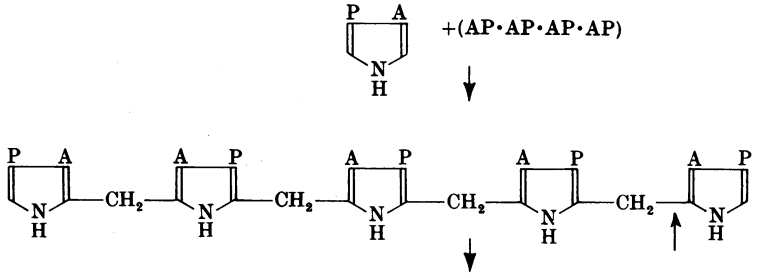


(A = $-CH_2 \cdot CO_2H$; P = $-CH_2 \cdot CH_2 \cdot CO_2H$)



communication), and (3) involvement of a coenzyme considered by Jackson & MacDonald (1957) and by ourselves, and investigated in the work to be described.

If the compound (AP·AP·AP·AP) were to condense with a molecule of opsopyrroledicarboxylic acid [3(4)-carboxymethylpyrrole-4(3)-β-propionic acid] before cyclization, the following pentamer could result, provided that addition of opsopyrroledicarboxylic acid were controlled enzymically to give an A·A linkage. On rupture of the bond indicated and cyclization, a series III compound would be formed and opsopyrroledicarboxylic acid would be regenerated. It would therefore need to be present only in catalytic amounts; furthermore, its formation *ab initio* from porphobilinogen is quite conceivable.

Studies of the conversion of porphobilinogen into uroporphyrin III suggest that at least two enzymes may be involved in plant tissues (Bogorad & Granick, 1953; Bogorad, 1955, 1957). The first enzyme, 'porphobilinogen deaminase', is believed to convert porphobilinogen into 'uroporphyrinogen I', a compound which gives uroporphyrin I on oxidation; the enzyme is inhibited competitively by opsopyrroledicarboxylic acid (Bogorad, 1957). The second enzyme, or system of enzymes, 'uroporphyrinogen isomerase', acting in conjunction with the first, converts porphobilinogen into 'uroporphyrinogen III'. In animal tissues, an enzyme system present in red cells converts porphobilinogen into a 'uroporphyrinogen III'; this system can be altered by heating so that a 'uroporphyrinogen I' is the main product (Booij & Rimington, 1957; Lockwood & Rimington, 1957; see also Granick & Mauzerall, 1958). According to the mechanism proposed here, opsopyrroledicarboxylic acid would act as a coenzyme of 'uroporphyrinogen isomerase'.

In the present work, which has been reported briefly (Scott & Smith, 1958), the effect of opsopyrroledicarboxylic acid on the synthesis of haem was studied, by using haemolysates prepared from anaemic fowls (Laver, Neuberger & Udenfriend, 1958). Special problems were posed by the fact that the postulated coenzyme was also known to be an inhibitor of the enzyme involved in the preceding step. Findings are submitted which we believe preclude the action of opsopyrroledicarboxylic acid as a coenzyme in the manner proposed.

EXPERIMENTAL

Materials

[α-^{14}C]Glycine, diethyl [α-^{14}C]malonate and [1:4-$^{14}C_2$]-succinic acid were supplied by the Radiochemical Centre, Amersham. Celite 545 (Johns-Manville and Co.) was washed with acid to remove iron oxide and traces of other metals. It was covered with conc. HCl for 2 days and then filtered off and washed thoroughly with water to remove the acid. After drying at 300° for 24 hr., it was transferred while hot to jars having corks fitted with $CaCl_2$ tubes. All contact with metals was avoided during this treatment.

Methods

[α-^{14}C]*Malonic acid.* Diethyl [α-^{14}C]malonate (100 μc; 7·4 mg.) was diluted with unlabelled malonate (525·5 mg.) and saponified overnight with 3 ml. of cold 20% (w/v) NaOH (Ropp, 1950). The solution was then stirred for 3 hr. with Zeo-Karb 225 (8 g., Permutit Co. Ltd.) in the H^+ form. The slurry was filtered and the resin washed thoroughly with water. The filtrate and washings were similarly treated with a further 8 g. of resin; the final solution (about 80 ml.) was concentrated by distillation under reduced pressure from a bath at 40–50° and the residue was dried in a vacuum desiccator, giving a quantitative yield of malonic acid (350 mg.; 33 μc/m-mole).

Synthesis of opsopyrroledicarboxylic acid labelled with ^{14}C. In view of the low overall yield, based on malonic acid, in the conversion of 2-ethoxycarbonyl-3-ethoxycarbonylmethyl-5-methylpyrrole-4-aldehyde (I) into opsopyrroledicarboxylic acid (OPD) by the published methods [(I) into ethyl 2-ethoxycarbonyl-3-ethoxycarbonylmethyl-5-methylpyrrole-4-β-propionate (MacDonald, 1952) and the latter into OPD (MacDonald & MacDonald, 1955)], variations of the procedure were tried and certain modifications were made. The yield of the pyrrole-4-β-acrylic acid, obtained from the condensation of the aldehyde (I) with malonic acid, was not increased by using absolute instead of aqueous ethanol as solvent, or by substituting piperidine or dimethylaniline for aniline; the use of pyridine as

catalyst resulted in a product of low m.p. which could not be hydrogenated satisfactorily. However, a reduction of 25% in the amount of malonic acid used did not lower the yield of the pyrrole-4-β-acrylic acid. With this modification, the published procedure was carried through, starting with 350 mg. of [α-^{14}C]malonic acid and 860 mg. of the aldehyde (I), up to the final stage except that OPD was isolated from the products of heating in a sealed tube in the following way. The contents of the tube were diluted to 3 ml. with water and adjusted to pH 3 with 2N-HCl. The solution was then extracted continuously with ether for 1 hr. The ethereal extract was dried ($MgSO_4$) and concentrated, and *n*-hexane was added dropwise until OPD started to crystallize. After leaving overnight at $-18°$, OPD was separated by centrifuging; after one recrystallization it had m.p. 137–139° (1·8 mg.; 0·3% based on malonic acid). The specific activity was 30·3 μc/m-mole. Non-radioactive OPD, prepared similarly, had the same m.p., which was not depressed after mixing with a sample (m.p. 139°) kindly supplied by Dr S. F. MacDonald. All melting points were determined with a micro melting-point apparatus (A. Gallenkamp and Co. Ltd.) and are uncorrected.

Determination of opsopyrroledicarboxylic acid. OPD was determined by measurement of the colour obtained with Ehrlich's aldehyde reagent. The aqueous solution (0·1 ml.) containing OPD was diluted with ethanol (2 ml.) and 0·1 ml. of a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde in ethanolic 2N-HCl was added. A magenta colour developed slowly and its maximum extinction at 548 mμ was measured, against a reagent blank, 30 min. after addition of the reagent, with a Unicam SP. 500 spectrophotometer and 0·5 cm. silica cells. E_{548} (ϵ 61 900) was found to be constant between 25 and 35 min. after addition of the reagent, and during this time its value was about 5 times that obtained 5 min. after addition of the reagent.

Solutions of OPD in ether–chloroform (1:4, v/v) were determined similarly, but the maximum extinction was then at 550 mμ, and readings were taken at this wavelength (ϵ 74 600). The determination was modified for urine samples; the sample (0·5 ml.) was diluted with water (2·5 ml.) and 0·2 ml. of a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde in 50% (v/v) conc. HCl (sp.gr. 1·18) was added. The maximum extinction, at 574 mμ (ϵ 78 600), was read 30 min. after addition of the reagent.

In each of the three methods, $E_{max.}$ increased linearly with concentration of OPD over the range used (0·5–10 μg./ml.).

Preparations from fowl red cells. Hens were made anaemic and the blood was collected and haemolysed by the method of Laver *et al.* (1958). $MgCl_2$ was added to the haemolysates to give a final concentration of 0·002M.

For the whole-cell preparations, the red cells were washed twice by centrifuging in cold 0·9% NaCl and the packed cells were resuspended in sufficient cold 0·9% NaCl to restore the original blood volume. Such preparations were haemolysed, immediately after incubation, by freezing in acetone–solid CO_2 and thawing, before isolation of the protoporphyrin. Incubation of both whole cells and haemolysates was always for 2 hr. at 37° under the conditions of Laver *et al.* (1958), the preparations being kept at 0° until incubated.

Isolation of protoporphyrin. Protoporphyrin methyl ester was prepared from haemoglobin by the method of Grinstein (1947) and plated for counting as the crystalline copper complex (Dresel & Falk, 1954).

Degradation of the copper complex of protoporphyrin methyl ester. The procedure was essentially the same as that described by Muir & Neuberger (1949) for the oxidation of mesoporphyrin dimethyl ester. The amount of chromic anhydride necessary was determined iodometrically as by Muir & Neuberger; the amount of CO_2 evolved and recovered as $BaCO_3$ represented 90–98% (average of 5: 95%) of ten molecular equivalents when 30 mg. or larger quantities of the copper complex were oxidized. The overall process may be represented by the following equation, assuming that each of the two vinyl side chains is oxidized to give CO_2 and a carboxylic acid which is then decarboxylated spontaneously:

$$C_{36}H_{36}O_4N_4Cu + 22CrO_3 + 34H_2SO_4 \rightarrow 10CO_2 + 2C_8H_9O_4N + 2C_5H_5O_2N + 11Cr_2(SO_4)_3 + CuSO_4 + 38H_2O. \quad (1)$$

In the degradation of radioactive samples, only 10–20 mg. quantities of the copper complex were available; 25 equiv. of CrO_3 was used and recoveries of CO_2 on this scale were 75–90% (average of 10, 84%) of the theoretical amount.

Assay of radioactivity. Porphyrin samples, weighing not less than 12 mg., were counted on 1 cm.^{2} polythene disks with an end-window counter; the counting rates of samples weighing less than 20 mg./cm.^{2} were corrected to give the expected rate at infinite thickness (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). Samples of $BaCO_3$ were counted on 2 cm.^{2} disks at infinite thickness. All counting rates were relative to a 1 cm.^{2} Perspex standard (Radiochemical Centre) having 1 μmc/mg. and giving about 700 counts/min. under the conditions of assay; the background rate was about 12 counts/min. The standard error of all rates stated was less than 3%. Radioactive OPD was counted in a 0·3 cm.^{2} Perspex disk. Radioactivity in eluates of the Celite columns was detected as described below.

Ultrafiltration by centrifuging. Small volumes of incubated haemolysate were ultrafiltered to remove the protein which interfered with the estimation and isolation of OPD. A conical centrifuge tube (15 ml.) was one-third filled with glass beads (diameter 2–3 mm.) and a small pad of non-adsorbent cotton wool was placed over them. A cylindrical collar was made from a rectangle (6 cm. × 7 cm.) of stainless-steel gauze (80 mesh) and a length of cellophan paper was wrapped round the vertical edges of the gauze to cover any sharp ends. The collar was inserted to extend from the cotton wool to the top of the tube and a length of polythene tubing (0·5 mm. bore; Allen and Hanbury Ltd.) was inserted, leading from the beads to the top of the tube, to permit air to escape. Dialysis tubing (Visking; $\frac{5}{32}$ in.), tied to form a sac the same length as the collar and containing about 2 ml. of haemolysate, was placed on the cotton wool with the lower knotted end pulled to one side. The tube was then centrifuged (1750 *g*) at 0°. In 4 hr. about 0·5–0·6 ml. of ultrafiltrate had collected round the beads.

Ethereal extracts of ultrafiltrate (0·3–0·5 ml., adjusted to pH 3 with HCl) were prepared by continuous extraction for 1 hr. in a small-scale liquid–liquid extractor with 5 ml. of ether; the height of the aqueous phase was about 20 cm./ml. Of the known precursors of porphyrins, succinic acid, but not glycine, δ-aminolaevulic acid (ALA) or PBG, was extracted under these conditions.

Free porphyrins. Changes in the approximate concentration of total porphyrins and porphyrinogens in haemolysates containing OPD were measured relative to their concentration in a control sample, to which no OPD had been added. After incubation in the presence of ALA, haemolysate samples were ultrafiltered for 5 hr. Ultrafiltered samples (0·1 ml.) were acidified with conc. HCl (sp.gr. 1·18; 0·2 ml.) and diluted to 5 ml. with water. The resulting solutions were left for one week in the dark in tubes plugged with cotton wool and the maximum absorption was then at 404 mμ. Extinctions were read at this wavelength on a Unicam SP. 500 spectrophotometer. The extinctions did not change on standing for a further 24 hr.; porphyrinogens originally present were thus assumed to be totally oxidized to porphyrins. If measurements were made within 8 hr. of incubation, values approximately 20–30% lower were obtained and steady readings could not be taken.

Determination of δ-aminolaevulic acid. This was carried out by the method of Shuster (1956) as modified by Laver *et al.* (1958).

Preparation and running of Celite columns for the separation of opsopyrroledicarboxylic acid and succinic acid. Acid-washed Celite 545 (2 g.) was stirred with 0·8 ml. of an aqueous buffer solution and suspended in ether which had been equilibrated with the buffer. The buffer was made by adding 2·5 ml. of 0·1 N-HCl to 7·5 ml. of 0·1 M-glycine, giving a pH of 2·85. The Celite slurry was packed in small portions into a tube of 9 mm. internal diameter, and the column so formed was washed with ether–chloroform (1:4, v/v), previously equilibrated with the buffer. Fractions of 1·1 ml. were collected (every 2 min. approx.) and OPD was determined colorimetrically in each. A 0·1 ml. portion of each fraction was applied to lens tissue 105 (J. Barcham Green Ltd., Maidstone) on a 2 cm.² polythene disk; after drying, radioactivity was measured with an end-window counter. OPD was eluted between fractions 12 and 28 and succinic acid between fractions 36 and 55. Recovery of 1 mg. of OPD from such a column was 75–80%; with 0·1 mg. only 20% was recovered.

Paper chromatography. Chromatograms were run overnight on Whatman no. 1 paper, with butanol–acetic acid–water (63:10:27, by vol.; upper phase) as solvent. Pyrroles were detected by spraying with 2% (w/v) *p*-dimethylaminobenzaldehyde in 50% (v/v) conc. HCl (sp.gr. 1·18). OPD gave a purple spot having R_F 0·7–0·8 (porphobilinogen, $R_F \sim 0{\cdot}2$; succinic acid, $R_F \sim 0{\cdot}8$, detected radioautographically).

Intraperitoneal injection of opsopyrroledicarboxylic acid into a rat. An aqueous solution (pH 7) (2 ml.) of OPD (10 mg.) was injected intraperitoneally into a female albino rat (wt. 251 g.) in a metabolism cage. Urine samples and cage washings (total vol. 5 ml.) were collected hourly for 8 hr. and after 24 hr. Ether extracts were prepared in the same way as from ultrafiltrates.

RESULTS

With a system synthesizing protoporphyrin from labelled glycine or labelled succinate, addition of an excess of OPD should, as a result of the mechanism proposed, cause a reduction in the specific activity of the porphyrin equivalent to loss of one of the four labelled pyrrolic residues. The radioactivity of the bridge carbon atoms of the porphyrin would be unaffected. The α-carbon atom of glycine supplies all 4 bridge and 4 of the 16 ring carbon atoms (Muir & Neuberger, 1950; Radin, Rittenberg & Shemin, 1950; Wittenberg & Shemin, 1950), while the carboxyl groups of succinic acid supply 8 ring and 2 side-chain carbon atoms of protoporphyrin (Shemin & Kumin, 1952). It would thus be expected that, in the presence of an excess of OPD, the radioactivity of the porphyrin derived from [α-^{14}C]glycine would be reduced by one-eighth, and that derived from [1:4-$^{14}C_2$]succinate by three-tenths, assuming no interference with biosynthesis by the relatively large amounts of OPD added. The radioactivity of porphyrin synthesized in the presence of added OPD should therefore decrease more rapidly with increase in concentration of OPD when succinate rather than glycine provides the radioactivity. This would still be true if the OPD added were also partially inhibiting synthesis; but were the OPD to act as an inhibitor only, then the decrease in specific activity would be independent of the nature of the labelled substrate.

Fig. 1 shows the effect on the radioactivity of the haem of adding increasing concentrations of OPD to haemolysate samples incubated in the presence of labelled precursors. It may be seen that there was no difference in radioactivity of the haem, whether derived from labelled glycine or from

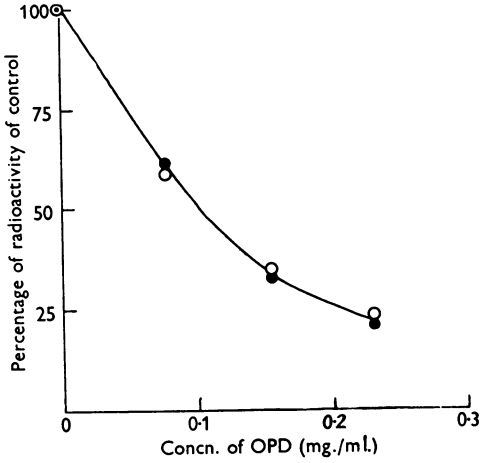


Fig. 1. Effect of opsopyrroledicarboxylic acid (OPD) on the incorporation of glycine and succinate into haem. Haemolysates (20 ml.), prepared from the pooled blood of three anaemic hens, were incubated with 0·019 M-glycine, 0·012 M-potassium succinate, KH_2PO_4–K_2HPO_4 (pH 7·3, 0·01 M with respect to P) and OPD in a total volume of 21·9 ml. ●, [α-^{14}C]Glycine (14 μc/m-mole); ○, [1:4-$^{14}C_2$]succinate (90 μc/m-mole). The radioactivities of the control samples to which no OPD was added were respectively 270 and 654 counts/min./cm.² of protoporphyrin methyl ester copper complex.

Table 1. *Distribution of radioactivity in haem synthesized in the presence of opsopyrroledicarboxylic acid*

Conditions of incubation are described in Fig. 1; for the methods of isolation and degradation of protoporphyrin dimethyl ester copper complex, see text. The source of radioactivity was [α-^{14}C]glycine (14 μc/m-mole) except for the sample described in the first row, for which [1:4-$^{14}C_2$]succinic acid (90 μc/m-mole) was used. The recovery of CO_2 as $BaCO_3$ was calculated, assuming the production of 10 CO_2/mol. [see equation (1)]. Rows which are bracketed represent an experiment with a single haemolysate preparation. Figures in parentheses indicate standard deviations calculated from eight assays.

OPD added (μg./ml. of lysate)	Copper complex: Amount oxidized (mg.)	Copper complex: Total ^{14}C (10^{-3} × mg. × counts/min./cm.^{2})	Barium carbonate: Amount recovered (mg.)	Barium carbonate: Recovery (%)	Barium carbonate: Corrected $BaCO_3$-^{14}C (10^{-3} × calc. mg. × counts/min./cm.^{2})	Percentage of ^{14}C in methene-C	Specific activity of the Cu complex (% of value when no OPD added)
0	13·90	9·94 (0·16)	33·73	80·3	0·680 (0·093)	6·85 (1·05)	100
0	12·66	5·46 (0·04)	31·81	83·3	2·80 (0·12)	51·2 (2·6)	100
0	14·30	6·30 (0·04)	37·38	86·4	3·09 (0·13)	49·0 (2·3)	100
50	17·62	6·08 (0·14)	47·18	88·8	2·81 (0·14)	46·3 (3·4)	78·1
100	16·60	5·33 (0·03)	37·82	75·5	2·42 (0·08)	45·4 (1·8)	72·6
200	16·35	4·63 (0·07)	41·85	84·8	2·15 (0·08)	46·5 (2·4)	64·1

labelled succinate, indicating that OPD was probably not incorporated in biosynthesis but that it acted as an inhibitor.

Results obtained with different samples of haemolysate could not be compared directly, because of the considerable variation in activity from one preparation to another. The less active the preparation, the more marked was the inhibition.

In view of the variation in activity of different preparations, it was considered desirable to study the effect of OPD by a method which is independent of the degree of inhibition. When [α-^{14}C]-glycine is used as the source of radioactivity, replacement of one pyrrole nucleus of the porphyrin by an unlabelled OPD residue should cause dilution of the specific activity of the pyrrolic but not of the methene-bridge carbon atoms. Considering total rather than specific radioactivities, the ratio (bridge ^{14}C)/(porphyrin ^{14}C) would therefore increase from 0·50 (i.e. 4/8) towards 0·57 (i.e. 4/7) with increasing concentration of OPD. Table 1 shows that, while the specific activity of the whole porphyrin decreased, the ratio (bridge ^{14}C)/(porphyrin ^{14}C), although not quite constant, did not increase. This result supports the conclusions of the experiments shown in Fig. 1.

As a final test radioactive OPD was used, the ^{14}C being in a position where it would be retained irrespective of the particular pyrrolic residue replaced. 3(4)-Carboxymethylpyrrole-4(3)-β-[α-^{14}C]propionic acid (30·3 μc/m-mole) was accordingly added to give a concentration of 0·045 mM in the haemolysate, which was then incubated with unlabelled glycine and succinic acid. No activity was detected in the copper complex subsequently isolated.

Effect of opsopyrroledicarboxylic acid on successive stages of porphyrin synthesis. OPD was found

Table 2. *Effect of opsopyrroledicarboxylic acid on the utilization of δ-aminolaevulic acid in a haemolysate*

Haemolysate was incubated with 0·018 M-glycine, 0·010 M-potassium succinate and the concentration of OPD shown. Unincubated samples contained 23 μM-ALA.

Concn. of OPD (μg./ml.)	Concn. of ALA (μM)
0	36
97	28
195	43
390	31

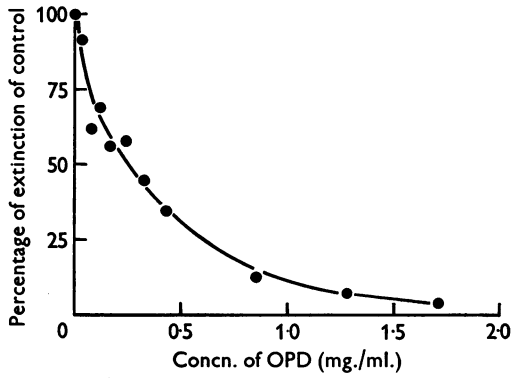


Fig. 2. Effect of opsopyrroledicarboxylic acid (OPD) on the formation of free porphyrin from δ-aminolaevulic acid (ALA). Samples (2 ml.) of a single preparation of haemolysate were incubated with 0·05 M-ALA (added as a solution of the hydrochloride adjusted to pH 7) and OPD as shown, in a total volume of 2·5 ml., isotonic with respect to KCl. Ultrafiltrates of the incubated haemolysate were treated as described in the text to obtain a relative measure of the total porphyrin concentration. The method of assay does not distinguish between porphyrins and porphyrinogens. Optical density was measured at 404 mμ and was 0·710 in the control, to which no OPD had been added.

to have no effect, up to a concentration of 1·5 mM, on the rate of formation of ALA in the 'washed particle' system described by Laver *et al.* (1958). If, on the other hand, OPD were to inhibit the conversion of ALA into PBG by ALA dehydrase (Gibson, Neuberger & Scott, 1955), it would cause accumulation of ALA in systems synthesizing porphyrin. Table 2 shows that the increase in concentration of ALA on incubation of haemolysates with glycine and succinate is not altered significantly by the presence of OPD. However, the conversion of ALA into porphyrinogens and porphyrins is markedly inhibited by OPD, as shown in Fig. 2. Inhibition must therefore have taken place at a stage subsequent to formation of PBG.

Failure of opsopyrroledicarboxylic acid to enter intact cells. OPD did not inhibit the formation of haem in whole cells (Table 3); as inhibition is marked in haemolysates (Fig. 1), it is likely that OPD does not penetrate into intact red cells. Dresel & Falk (1956*a*) reported similar findings for PBG.

In view of this finding with whole red cells, the effect of OPD on bacterial growth was briefly investigated. OPD produced no detectable inhibition of growth when 0·1 ml. portions of neutral solutions (containing 1–10 mg. of OPD/ml.) were placed in cups cut from agar plates flooded with the following micro-organisms: *Proteus vulgaris, Escherichia coli* (V_2), *Pseudomonas pyocyanea, Staphylococcus aureus* or *Corynebacterium diphtheriae*, which are known to synthesize porphyrins and haem from simple precursors. These findings, which are discussed below, are believed to result similarly from inability of the pyrrole to enter the bacterial cells.

Reisolation of opsopyrroledicarboxylic acid from incubated haemolysate. The results so far described show that OPD is not incorporated into porphyrins during biosynthesis, but it was considered possible that it might be formed enzymically from PBG. Attempts were therefore made to detect radioactivity in OPD reisolated after its addition to haemolysates synthesizing porphyrin from radioactive glycine or succinate. The concentration of OPD used would have caused not more than 60% inhibition of synthesis.

After incubation of haemolysate with glycine, [1:4-$^{14}C_2$]succinate and OPD, an ethereal extract of the ultrafiltrate was prepared as described and applied to a Celite column. The OPD recovered from the column represented 20% of that added to the haemolysate. There were two peaks of radioactivity in the column eluates, the earlier corresponding with that of the OPD measured colorimetrically, and the later peak corresponding with succinic acid. However, similar peaks of radioactivity, but no OPD, were detected in the eluate corresponding with a haemolysate to which no OPD had been added (Table 4). Recovery experiments indicated that quantities of OPD too small to be detected colorimetrically (less than about 1 μg./ml. of ultrafiltrate) would probably be lost on the column, and it was presumed that the radioactivity was due mainly to a metabolite of succinate which was eluted from the column at the same rate as OPD. However, when the experiment was repeated, but with [α-^{14}C]glycine (140 μc/m-mole) in place of labelled succinate, radioactivity, although very much less intense, was again obtained in the fractions containing OPD (Table 4). On this occasion there was virtually no radioactivity in the corresponding fractions obtained from the haemolysate incubated without OPD. Fractions 10–28 of experiment 6 (Table 4) were redissolved in 1·3 ml.

Table 3. *Effect of opsopyrroledicarboxylic acid on haem biosynthesis by whole red cells*

Whole-cell preparations (20 ml.) were incubated with [α-^{14}C]glycine (0·017 M; 5·15 μc), 0·01 M-potassium succinate, KH_2PO_4–K_2HPO_4 (pH 7·3, 0·01 M with respect to P) and OPD in a total volume of 21·8 ml.

Concn. of OPD (μg./ml.)	Radioactivity of protoporphyrin methyl ester Cu complex (counts/min./cm.^{2})
0	130
65	126
194	115
324	140

Table 4. *Recovery of opsopyrroledicarboxylic acid from haemolysates incubated with radioactive glycine or succinate*

Preparations of haemolysate (25 ml.) contained 0·02 M-glycine and 0·01 M-potassium succinate with or without OPD. When used as labelled precursors [1:4-$^{14}C_2$]succinate (Expts. 1–3) or [α-^{14}C]glycine (Expts. 4–6) had 90 μc or 140 μc/m-mole respectively. In Expts. 1, 3, 4 and 6 the preparations were incubated; in Expts. 2 and 5 the preparations were maintained at 0° until after ultrafiltration. An ultrafiltrate of the haemolysate was extracted with ether at pH 3 and the extract was applied to a buffered Celite column as described. When estimable colorimetrically OPD was always found entirely between fractions 10 and 28. The radioactivity of fractions 1–10 and 30–35 was always negligible. For further details, see text.

Expt.	Concn. of OPD (mg./ml.)	Radioactivity of fractions 10–28 (counts/min.)
1	0	804
2	0·1	20
3	0·1	1040
4	0	12
5	0·1	13
6	0·1	91

of aq. 2N-ammonia; 0·3 ml. of this solution was extracted continuously with ether (solution *A*). The remaining 1 ml. was dried *in vacuo*, and the residue was redissolved in ethanol (solution *B*).

The whole of the material in solutions *A* and *B* was then applied to a paper chromatogram in such a way as to give a single compact spot from each. After development and spraying with Ehrlich's reagent only one spot was seen (R_F 0·70); this corresponded with the OPD in solution *B*. A radioautograph was made from the sprayed and dried chromatogram, and after one month's exposure radioactivity was detected in the positions of OPD (R_F 0·70) both from solution *A* and solution *B*, the intensity of *B* being greater than that of *A*. The material from solution *B* also produced a second radioactive spot (R_F 0·44; Ehrlich-negative) of intensity similar to that of the OPD from *B*. This second spot was not identified; it could not have been glycine, ALA or PBG.

Excretion of opsopyrroledicarboxylic acid after intraperitoneal injection. About 53 % of the OPD administered intraperitoneally to a rat appeared in the urine within 4 hr.; 65 % was excreted in 24 hr. The nature of the Ehrlich-positive material in the urine and in an ethereal extract was examined by chromatography on paper. Unchanged OPD was the only detectable pyrrole present.

DISCUSSION

Tests of the incorporation of opsopyrroledicarboxylic acid into haem

Before the results of the experiment using radioactive OPD can be accepted, the sensitivity of the method must be examined. The radioactive OPD had twice the molar specific activity of the [α-^{14}C]-glycine added on other occasions; however, the OPD would supply only one ^{14}C/mol. of porphyrin whereas glycine is known to supply eight. At the concentration of OPD used, inhibition would have been negligible, so that if OPD were incorporated in the manner proposed the specific activity of the haem isolated should be one-quarter of that obtained when [α-^{14}C]glycine was used instead. Unfortunately a control sample which had been incubated with [α-^{14}C]glycine was lost, and the experiment could not be repeated owing to shortage of radioactive OPD. A direct comparison is therefore not available because of the difference of the enzymic activity in different preparations of haemolysates. From other samples of haemolysate similarly prepared the specific activities of the copper complex isolated after incubation with [α-^{14}C]glycine ranged from 270 to 604 counts/min./cm.^{2} (average of 6 samples, 448 counts/min./cm.^{2}), so it is reasonably certain that incorporation of radioactive OPD would have been detected had it taken place. Dilution of the radioactive OPD by porphyrin synthesis would cause only a slight reduction in specific activity since the amount of OPD added (2·46 μmoles) must have been 10–100 times the amount of porphyrin synthesized (Dresel & Falk, 1956*a*; Laver *et al.* 1958), depending on the activity of the particular preparation used. It is considered, therefore, that the experiments reported preclude the possibility that OPD plays any role in the synthesis of haem which involves actual incorporation. The formation of porphyrins of series III may nevertheless involve the action, as a coenzyme, of some other pyrrole related to PBG, such as '*iso*porphobilinogen' (Prasad & Raper, 1955); attempts to synthesize this and related compounds are in progress.

Specificity of the oxidative degradation of protoporphyrin

It was required to measure only the porphyrin ^{14}C and the methene-bridge ^{14}C present in very small quantities of protoporphyrin ester copper complex (10–15 mg.); prior conversion into mesoporphyrin would have caused serious losses on this scale. Oxidation of mesoporphyrin methyl ester on a larger scale but otherwise under similar conditions was found by Muir & Neuberger (1949) to yield 6CO_2/mol.; these were shown to come almost entirely from the four bridge and the two ester carbon atoms, and both of the substituted maleimides expected were isolated. In addition, with derivatives of protoporphyrin methyl ester, the two vinyl groups would be expected to give on oxidation 2 mol. of CO_2 and the corresponding carboxylic acids, by analogy with the behaviour of β-vinylpyrroles on treatment with CrO_3 under conditions in which the pyrrolic α-carbon atoms are not attacked (Fischer & Zeile, 1928). When, however, oxidation of the vinyl groups is accompanied by oxidation of the pyrrolic rings the resulting imide carboxylic acids would be expected to decarboxylate spontaneously and, in fact, no β-carboxymaleimides have ever been isolated. An equation such as (1) would accordingly be expected, but its validity has been established here only with respect to the amounts of CrO_3 consumed and of CO_2 evolved; imides were not isolated. The precise course of the reaction has not, therefore, been established in the present case, but it is considered that the $^{14}CO_2$ produced did nevertheless represent mainly the methene-bridge ^{14}C. This is borne out by the finding that the observed ratios ($^{14}CO_2$)/(porphyrin ^{14}C) had the values expected for (bridge ^{14}C)/(porphyrin ^{14}C). The values were close to 0·5 (Table 1) when [α-^{14}C]glycine was the source of labelling. After use of [1:4-$^{14}C_2$]succinic acid, which should label neither the four bridge nor the four vinyl carbon atoms, the value of 0·07 then

obtained is not greater than that to be expected from the finding of Muir & Neuberger (1949) that up to 10 % of the CO_2 evolved during oxidation (of mesoporphyrin ester) may arise from unknown sources as a result of side reactions.

Formation of opsopyrroledicarboxylic acid in haemolysates

It has been shown that a compound closely resembling OPD is formed in haemolysates synthesizing haem. Formation of this compound requires incubation (Table 4) but it has not been proved that the process is enzymic. Identification of the substance with OPD rests at present solely on the similarity of solubility properties in ether/water (pH 3), on the Celite column and on the paper chromatogram. Although suggestive, these findings cannot be considered as sufficient evidence to establish OPD as a metabolite. In particular it has not been excluded that the compound was an intermediate (formed from PBG) which was accumulating as a result of inhibition by OPD.

Inhibition of haem synthesis by opsopyrroledicarboxylic acid

In view of the possibility that OPD is formed by red cells its inhibition of porphyrin synthesis, observed by Bogorad (1957) and here confirmed, is of interest in relation to factors controlling biosynthesis *in vivo*. The haemolysate used in obtaining the data in Fig. 1 was in fact the least active used, as judged by the specific activity of the haem synthesized from [α-^{14}C]glycine under the standard conditions of incubation without OPD. Even with this preparation it may be seen that a decrease of only 5 % in the amount of haem synthesized would have required a concentration of about 10 μg. of OPD/ml., a value ten times the limit of detection of OPD. In fact OPD was never found in the extracts of ultrafiltrates from untreated haemolysates. It is therefore considered unlikely that endogenous OPD, if present in the cells studied, had any significant effect on the rate of synthesis of haem.

When it was found that OPD did not apparently enter intact red cells (Table 3), it was thought that, if bacterial cells were permeable, OPD might be of use as a bacteriostatic agent *in vivo*. The failure of OPD to affect bacterial growth under the conditions stated was also probably due to a failure to enter the cells, although the effect of OPD on synthesis by preparations of broken cells from animals or plants has not been shown to extend to bacterial systems.

SUMMARY

1. Radioactive opsopyrroledicarboxylic acid, 3(4)-carboxymethylpyrrole-4(3)-β-[α-^{14}C]propionic acid, has been synthesized.

2. Opsopyrroledicarboxylic acid was shown, by three independent methods, not to be incorporated into haem during synthesis of the latter compound from glycine and succinic acid in lysates of fowl red cells. Opsopyrroledicarboxylic acid cannot, therefore, act as a cofactor in the manner proposed for the biosynthesis of porphyrins of series III.

3. Protoporphyrin methyl ester copper complex was found to give 10 CO_2/mol. on oxidation with chromic anhydride in sulphuric acid. By degradation in this way of protoporphyrin containing ^{14}C, values of the ratio (total $^{14}CO_2$)/(porphyrin ^{14}C) were measured, and were found to be the same, within limits of error, as those expected for the ratio (methene-bridge ^{14}C)/(porphyrin ^{14}C). The effect of opsopyrroledicarboxylic acid on the value of the ratio in haem, synthesized by haemolysates to which had been added [α-^{14}C]glycine and varied concentrations of opsopyrroledicarboxylic acid, was investigated.

4. In haemolysates, opsopyrroledicarboxylic acid inhibits the synthesis of haem at a stage subsequent to the formation of porphobilinogen but preceding the formation of porphyrins.

5. The synthesis of haem by whole red cells and the rates of growth of certain micro-organisms are not affected by the presence of opsopyrroledicarboxylic acid in the medium. It is suggested that these are the results of permeability barriers.

6. A compound closely resembling opsopyrroledicarboxylic acid, in extraction properties and on chromatography in two systems, is formed in haemolysates synthesizing porphyrin from glycine and succinate. There is not yet sufficient evidence to identify this compound with opsopyrroledicarboxylic acid.

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The Isolation, Characterization and Chemical Synthesis of Muramic Acid

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Muramic acid is the trivial name of a hexosamine-like substance which was found in acid hydrolysates of spore peptide (Strange & Powell, 1954) and in the isolated cell walls from a large number of Gram-positive bacteria (Cummins & Harris, 1954, 1956). Recently, Weidel & Primosingh (1957) have found an apparently identical substance in the isolated cell walls of *Escherichia coli*. Park & Strominger (1957) have shown that the acidic hexosamine found by Park (1952) as a constituent of uridine–pyrophosphate–peptide complexes released from penicillin-treated cells of *Staphylococcus aureus* is probably this substance. Muramic acid was isolated in crystalline form from the spore peptide of *Bacillus megaterium* (Strange & Dark, 1956) and provisionally assigned the structure of a 3-*O*-α-carboxyethylhexosamine (Strange, 1956). Kent (1957) synthesized 3-*O*-α-carboxyethyl-D-glucosamine from *N*-acetyl 4:6-*O*-benzylidene-α-methyl-D-glucosaminide (Neuberger, 1941) and showed it to have very similar properties to natural muramic acid.

This paper presents details of the work previously briefly reported (Strange, 1956; Kent, 1957) on the isolation, structure and synthesis of muramic acid.

METHODS AND MATERIALS

Ion-exchange resins. Amberlite IR-4B (OH) and Amberlite IR-120 (H), both A.R. grade, were obtained from British Drug Houses Ltd. IR-4B (OH) was treated several times with 2N-HCl until the supernatant solution was no longer coloured. A column of acid-treated resin was washed with water until the effluent liquid was free from acid and then treated with N-NaOH until the effluent liquid was free from Cl^- ions. The regenerated resin was washed with de-ionized water until free from alkali and partially dried by passage of CO_2-free air. Resin was purified in this manner just before use; on storage it apparently deteriorated and when an acid solution was mixed with resin which had been prepared for some time, soluble pigmented material was obtained. IR-120 (H) was treated several times with 3·6% (w/v) HCl and freed from acid by washing it in a column with water. M-NaH_2PO_4–Na_2HPO_3 buffer, pH 6·05, was run through the column until the effluent fluid was at the pH of the ingoing buffer. Buffer salts were removed from the resin by washing with de-ionized water until the effluent liquid was free from phosphate. Dowex 50 (cross-linkage, ×8; mesh, 200–400) obtained from Dow Chemical Co., Midland, Mich., U.S.A., was washed with 4N-HCl until the supernatant fluid was colourless and the washed resin was stored in 4N-HCl (Gardell, 1953). 0·3N-HCl was passed through a column of the resin until the acid concentration of the effluent liquid was 0·3N.

Source of muramic acid. Spore-exudate peptide from germinating spores of *B. megaterium* was prepared as described by Strange & Powell (1954). Larger yields of less pure but satisfactory material were obtained if the germinated spores were left suspended in water for several weeks.

Isolation of crystalline natural muramic acid. Spore peptide (500 mg.) was hydrolysed with boiling 6N-HCl (20 ml.) under reflux for 2 hr. The hydrolysate was evaporated to dryness at 30–40° under reduced pressure and the residue was freed from most of the residual HCl by repeated evaporation with water under reduced pressure.