3. There is no correlation between anticholinesterase activity and effectiveness as reactivator. The ratios (potency as reactivator:reactivity with organophosphates), obtained with different oximes, support the interpretation that attachment to the phosphorylated enzyme during reactivation enhances activity. Possible sites of attachment are discussed.

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# The Enzymic Condensation of Porphobilinogen to Porphyrins

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The ability to catalyse the condensation of porphobilinogen to uroporphyrin has been reported for several tissues and extracts. It now appears that the primary product of the enzyme action is uroporphyrinogen, which differs from the porphyrin in having six additional hydrogen atoms. When the reaction is carried out in the presence of oxygen, uroporphyrin is formed by the oxidation of the porphyrinogen.

Although some of the preparations are capable of producing uroporphyrin III from porphobilinogen, all can be so modified that they produce uroporphyrin I only. Bogorad & Granick (1953) obtained *Chlorella* extracts capable of forming uroporphyrin III, together with coproporphyrin III; after heating, the extracts produced uroporphyrin I only. Hoare & Heath (1958) and Heath & Hoare (1959) showed that suspensions of frozen and thawed *Rhodopseudomonas spheroides* as well as the acetone-dried powders from them condense porphobilinogen to uroporphyrin III and coproporphyrin III, but the cell-free supernatants, after centrifuging, and also the heated acetone-dried powders gave uroporphyrin I only. Booij & Rimington (1957) heated vertebrate haemolysates (65° for fowl and 55° for human) which then catalysed the formation of series I porphyrins only. Granick & Mauzerall (1958) found the same for partially purified preparations from rabbit and from fowl erythrocytes. Bogorad (1958*a*, *b*) prepared a preparation from spinach acetone-dried powders which converted porphobilinogen into uroporphyrinogen I. The addition to this of a fraction obtained from wheat-germ extracts, which alone had no action on porphobilinogen, produced a system which formed uroporphyrin III from porphobilinogen.

In this paper, a partially purified preparation from avian erythrocytes, briefly described by Lockwood & Rimington (1957), has been used in a study designed to test proposed mechanisms of the enzymic condensation of porphobilinogen to uroporphyrinogen.

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## MATERIALS AND METHODS

Phosphate buffers were made from $Na_2HPO_4$ and $KH_2PO_4$. Hydrochloric acid was used for making 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer.

Crystalline porphobilinogen hydrate prepared from the urine of patients with acute porphyria was kindly supplied by Professor C. Rimington. [$^{14}$C]Formaldehyde was supplied by The Radiochemical Centre, Amersham, Bucks. Fowl blood was collected as described by Dresel & Falk (1954). Calcium phosphate gel was prepared according to Keilin & Hartree (1951). Uroporphyrin I and uroporphyrin III standards were those described by Falk, Dresel, Benson & Knight (1956).

*Porphobilinogen estimation.* Porphobilinogen was estimated by the method of Rimington, Krol & Tooth (1956). It is not clear from their paper whether the extinction coefficients given refer to the free base, the stable hydrate or to the hydrochloride. Their extinction coefficient ($E^{1\,cm.}_{555\,m\mu}$ 0·114 for a mixture of equal parts of their reagent and a solution containing 1 $\mu$g. of porphobilinogen/ml.) agrees with our findings with solutions of known concentration when the porphobilinogen concentration was expressed as the hydrate. This corresponds to molar extinction of 55 500 for the complex.

*Porphyrin estimation.* Uroporphyrin was estimated in 5% (w/v) HCl solution by measuring the light absorption at 430, 406 and 380 m$\mu$. A molar value of $9{\cdot}50 \times 10^5$ was used for the expression $2E^{1\,cm.}_{406} - E^{1\,cm.}_{430} - E^{1\,cm.}_{380}$ corresponding to a specific value for the expression, 1·143, for a solution containing 1 $\mu$g./ml.

*Estimation of enzyme activity.* To estimate enzyme activity a suitable amount of the solution to be tested was incubated for 1–3 hr. at 37° with 25 $\mu$g. of porphobilinogen. The total volume was 0·5 ml. and the solution was 0·05 M with respect to phosphate and at pH 7·3–7·5. The reaction was stopped by adding 0·7 ml. of water and 0·4 ml. of 20% trichloroacetic acid. The proteins were centrifuged off and 1 ml. of the supernatant was diluted to a final volume of 6·4 ml., having 5% (w/v) HCl concentration. Porphobilinogen was estimated in a 2 ml. sample. Under these conditions the colour produced with the aldehyde was stable for at least 5 min. after mixing. The amount of enzyme preparation and the time were chosen so that about half the porphobilinogen disappeared during the incubation. Combined uroporphyrin and uroporphyrinogen were estimated by spectrophotometric measurement of the remainder of the 6·4 ml. dilution after it had been kept in the dark for 25 hr. to allow maximum oxidation of uroporphyrinogen to uroporphyrin. Bogorad (1958*a*) has since shown that higher yields of uroporphyrin are obtained when the oxidation is carried out with iodine than when it is allowed to occur by keeping in air. A small amount of porphyrin also remains adsorbed to the protein. No attempt has been made to correct for either of these losses. Enzyme activities were expressed either as $\mu$mole of porphobilinogen consumed/hr. or as $\mu$mole of uroporphyrin produced/hr. The latter has been used where possible since porphobilinogen-consumption rates were much more variable with substrate concentration than porphyrin-formation rates.

Isomer type of the uroporphyrins was determined by paper chromatography of the octamethyl ester by the method of Falk & Benson (1953). The uroporphyrin III spot was normally accompanied by the spot ascribed by Falk *et al.* (1956) to *pseudo*uroporphyrin, particularly in the porphyrins from fresh enzyme preparations.

*Preparation of enzymes.* Fowl erythrocytes were washed twice with 0·9% NaCl and haemolysed with 3 vol. of water. To each 10 vol. of haemolysate, 1 vol. of $CHCl_3$ was added and the mixture was shaken gently on a mechanical shaker for 3–5 hr. The $CHCl_3$ and precipitated haemoglobin were removed by centrifuging, and the solution, which still contained some haemoglobin, was dialysed overnight against running tap water. Small amounts of protein which precipitated during dialysis were removed by centrifuging. The solution was run through a column consisting of cellulose powder and calcium phosphate gel (10 g. of cellulose powder plus 15–20 ml. of gravity-packed gel for each 250 ml. of diffusate; cf. Walker, 1957). Some haemoglobin was retained on the column. The column was then washed with water (about 500 ml.) followed by an equal volume of 0·5% NaCl. This removed much of the haemoglobin and apparently considerable quantities of nucleic acids, together with a small fraction of the enzyme activity. The column was then eluted with phosphate buffer (0·01 M with respect to phosphate in 0·5% NaCl; $Na_2HPO_4$–$KH_2PO_4$, 4:1). The enzyme activity was eluted with a narrow coloured band running at the phosphate buffer front. It contained appreciable quantities of haemoglobin or a pigment spectroscopically resembling haemoglobin. From 250 ml. of fowl blood the yield was about 25 ml. of enzyme preparation with an activity of 0·2–0·4 $\mu$mole of porphyrin/ml./hr. with a protein content of about 20 mg./ml. For some purposes this solution was concentrated by pressure-filtration through Viscose tubing as demonstrated by Grant, Rowe & Stanworth (1958). This gave a thick syrupy liquid without significant loss of activity.

Human erythrocytes contain the same enzyme system but it is less stable; particularly it easily loses its ability to produce uroporphyrin III; this is exemplified by sensitivity to heat. After heating at 50° for 15 min. the enzyme system from human erythrocytes is capable of producing uroporphyrin I only, whereas that from fowl erythrocytes appears not to be affected by this treatment. Acetone precipitation either of the erythrocytes or of the haemolysate gave an active dried powder (although occasionally a preparation in which uroporphyrin I and not III was produced), but although there was little, if any, loss of activity in preparing the acetone-dried powder, the precipitation achieved no purification. Little purification was achieved by $(NH_4)_2SO_4$ precipitation because the enzyme activity precipitated in the same range as the haemoglobin, e.g. during $(NH_4)_2SO_4$ precipitation of an acetone-dried powder extract of human erythrocytes 15% of the total activity came down at 40% saturation, 35% at 50% saturation and 5% at 55% saturation; the remaining activity was lost. Some separation could be achieved but the enzyme became increasingly sensitive to dialysis as the purification proceeded. Ethanol–$CHCl_3$ precipitation of the haemoglobin was very destructive of enzyme activity.

The preparation described produces from porphobilinogen uroporphyrin only. It appears equally active in the presence or absence of oxygen. In the absence of oxygen no uroporphyrin is formed but a precursor which gives uroporphyrin on oxidation in air. This precursor is believed to be uroporphyrinogen; on exposure to air there is first an increase of absorption at 500 m$\mu$ followed by a decrease as

the porphyrin spectrum develops (Bogorad, 1958*a*, *b*, and Granick & Mauzerall, 1958). All experiments described have been carried out in air. The ability to form coproporphyrin (or coproporphyrinogen) is completely lost after treatment of the haemolysate with $CHCl_3$. Short treatment of chicken erythrocytes with benzene produces a haemolysate which can no longer synthesize protoporphyrin, but which still retains the ability to synthesize coproporphyrin (coproporphyrinogen). The preparation was stable for several weeks. There seemed little difference in the stability at room temperature, at 2° and at −10°.

## RESULTS

*Effects of heat.* The results of heat-treatment of the enzyme preparations were quantitatively similar to the findings of Booij & Rimington (1957). The human enzyme was the more sensitive; after heating at 55° for 15 min. and at pH 7·4, it yielded with porphobilinogen uroporphyrin I only. The fowl enzyme was more resistant; after heating at 55° for 15 min. at pH 7·4, it still produced uroporphyrin III but no detectable amounts of uroporphyrin I. When heated at 60–62° the enzyme appeared to be completely altered after 10 min., giving no detectable amounts of uroporphyrin III. After heating for 2 min. or 5 min. there was partial alteration of the enzyme system. The sensitivity of the system to heating was influenced by pH; heating a fowl enzyme system for 15 min. at 55° at pH 8·5 completely altered it to a uroporphyrin I-producing system. To produce an altered (uroporphyrin I-producing) system for further studies the intact system was heated at 60–62° for 15 min. at pH 7·4.

Heating the enzyme preparation at 65° or less had little effect on the over-all rates of porphyrin production and porphobilinogen consumption. At higher temperatures there was significant loss of enzyme activity. Table 1 gives the results of heating at 55° and at 60°. The slight increase in activity was often, but not invariably, found. It agrees with the findings of Booij & Rimington (1957), who observed an increase in the rate of total porphyrin formation in fowl erythrocytes after heating at 65°.

Table 1. *Effect of heat on activity of enzyme preparation*

Enzymic activity was estimated in 0·11 M-tris buffer, pH 8·3, with porphobilinogen 0·13 mM at 37°. The limits given for the accuracy of the values express the errors of the spectrophotometric readings and do not include losses and other systematic errors in the method of estimation.

| Treatment | | Subsequent activity (% of activity of unheated enzyme preparation) | |
|---|---|---|---|
| Time (min.) | Temp. | Porphobilinogen consumption | Porphyrin formation |
| 5 | 55° | 100±3 | 100±1 |
| 10 | 55 | 100±3 | 106±1 |
| 30 | 55 | 102±3 | 99±1 |
| 5 | 60 | 106±3 | 115±1 |
| 10 | 60 | 102±3 | 114±1 |
| 30 | 60 | 92±3 | 91±1 |

*Incorporation of formaldehyde.* Shemin, Abramsky & Russell (1954) isolated [$^{14}$C]formaldehyde, formed from porphobilinogen, labelled with $^{14}$C in the amino methyl group, during enzymic condensation of the porphobilinogen to porphyrin. We were interested to investigate whether [$^{14}$C]formaldehyde would be incorporated into porphyrin during the enzymic condensation of non-radioactive porphobilinogen. [$^{14}$C]Formaldehyde (1·4 μg. with an activity of 0·05 μc) was incubated at 37° for 10 hr. with porphobilinogen hydrate (25 μg.) and excess of enzyme in tris buffer, pH 8·5 (total volume 0·55 ml.). A tube containing the same quantities of porphobilinogen and formaldehyde in 0·1 N-HCl but without enzyme was heated for 15 min. in a boiling-water bath. The times were sufficient to give maximum yield of porphyrin in both tubes. As a control, porphobilinogen and formaldehyde were incubated at 37° in tris buffer, but without enzyme. At the end of the 10 hr. a very faint fluorescence showed negligible porphyrin production in the control tube. Formaldehyde inhibits the enzyme but at the concentration used the inhibition cannot be detected. The uroporphyrin from the two tubes (enzymically-formed porphyrin and chemically-formed porphyrin) was isolated, esterified and crystallized. Carrier formaldehyde (1 mg.) was added to the washings obtained during the isolation of the enzymically-formed porphyrin and the dimedone compound was isolated and counted at infinite thickness. A count of 32 000 counts/min. showed that formaldehyde was still available in the enzyme solution at the end of the experiment. The porphyrin was prepared for counting by allowing approx. 4 μg. in chloroform solution to dry on a lens-tissue circle on the planchet; this approximates to an infinitely thin layer. The background count was 13 counts/min., giving a correction for background, for the counts done on 4 μg. samples, of −3 counts/min./μg. The activity of the enzymically-prepared porphyrin was not significantly different from the background (1 count/min./μg. after correction). The chemically-prepared porphyrin gave a corrected count of 385 counts/min./μg. In another experiment carried out at pH 7·4 the count of the enzymically-prepared porphyrin (31 counts/min./μg.) was not negligible, although less than a tenth of that of the chemically-prepared porphyrin (405 counts/min./μg.). Spontaneous porphyrin formation increases with decrease of pH. In a radioautograph of a chromatogram of the chemically-prepared porphyrin most of the activity coincided exactly with

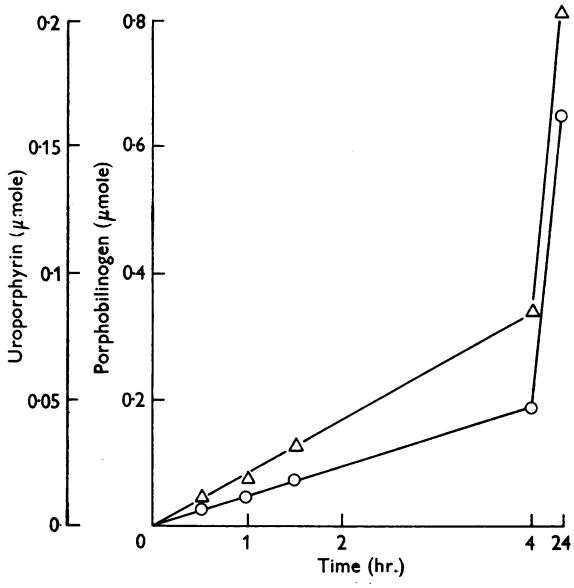



Fig. 1 (*a*). Porphobilinogen consumption and uroporphyrin formation by intact enzyme. △, Porphobilinogen consumed; ○, porphyrin formed. System contained: 0·4 ml. of intact enzyme, 0·88 μmole of porphobilinogen, phosphate buffer, pH 7·4 (final concn. 0·05 M), to 4 ml. Temperature 37°. Samples (0·5 ml.) were taken for analysis.

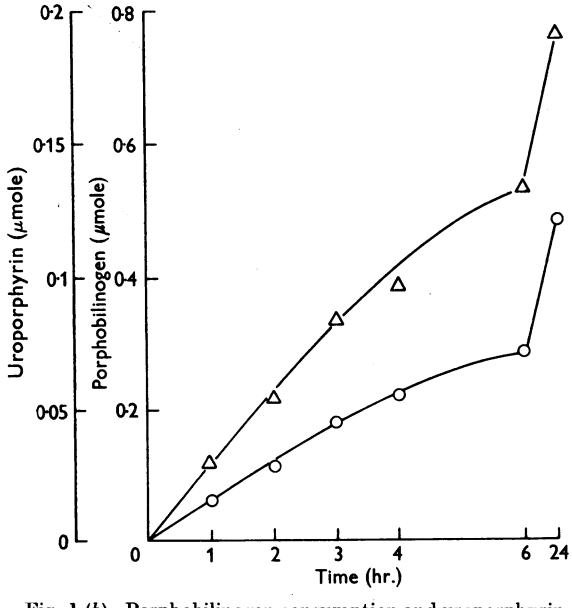



Fig. 1 (*b*). Porphobilinogen consumption and uroporphyrin formation by heated enzyme. △, Porphobilinogen consumed; ○, porphyrin formed. System contained: 0·2 ml. of enzyme which had been heated for 30 min. at 60°, 0·78 μmole of porphobilinogen, tris buffer, pH 8·3 (final concn. 0·04 M), to 5 ml. Temperature 37°. Samples (0·5 ml.) were taken for analysis.

the uroporphyrin III methyl ester spot, although some activity remained at the point of application. Enzymically-prepared porphyrins, both isomer III from intact enzyme and isomer I from heat-altered enzyme, showed on the radioautographs only faint traces of activity. The traces coincided with the respective porphyrin spots.

*Rate of porphyrin formation.* Figs. 1 (*a*) and 1 (*b*) show porphobilinogen consumption and porphyrin production for both intact and heat-altered enzyme preparations. Both heated and unheated enzyme preparations show initially linear porphyrin production. There is no evidence of any lag. Porphobilinogen consumption is more irregular.

*Dependence of* pH. Fig. 2 (*a*) shows porphyrin formation by both the intact and the heat-altered enzyme systems as a function of pH. Fig. 2 (*b*) shows the same for porphobilinogen consumption. At lower pH values there is appreciable porphobilinogen consumption without porphyrin production but elsewhere the shape of the four curves is identical within the limits of experimental error. The maximum of pH 8·0–8·5 reported by Lockwood & Rimington (1957) applies to the human enzyme system. The maxima found and the shape of the curve for the fowl enzyme system agree with those reported by Granick & Mauzerall (1958).

*Michaelis constants.* Michaelis constants were calculated on porphobilinogen consumption. For the intact preparation values of 8, 9, 15, 17, 19 and 19 μM were obtained and for the heat-altered preparation values of 6, 6, 8, 11 and 19 μM. The value decreased on storage of the enzyme preparation. When both estimations were carried out on the same preparation, heat treatment caused a decrease in the Michaelis constant; thus pairs of values before and after heat treatment were 19 and 8 μM respectively and, for another preparation, 17 and 11 μM. If this increase in affinity for the enzyme is not accompanied by a change in the reaction rate of the enzyme–substrate complex (namely, if $V_{max.}$ remains constant) it could explain the slight increase in the activity of the heat-treated enzyme shown in Table 1. The activities quoted in Table 1 were measured at a substrate concentration of 0·2 mM. A decrease of $K_m$ from one-tenth of the substrate concentration to one-twentieth should change the rate from 0·9 $V_{max.}$ to 0·95 $V_{max.}$. Apparent values of the Michaelis constant based on porphyrin formation were higher than those based on porphobilinogen consumption. Figs. 3*a* and 3*b* show graphs used to calculate the Michaelis constants. Porphyrin-production values are also plotted for comparison.

*Porphyrin yield.* Yields of porphyrin based on porphobilinogen consumption varied from 50 to 90% of the theoretical required for the formation

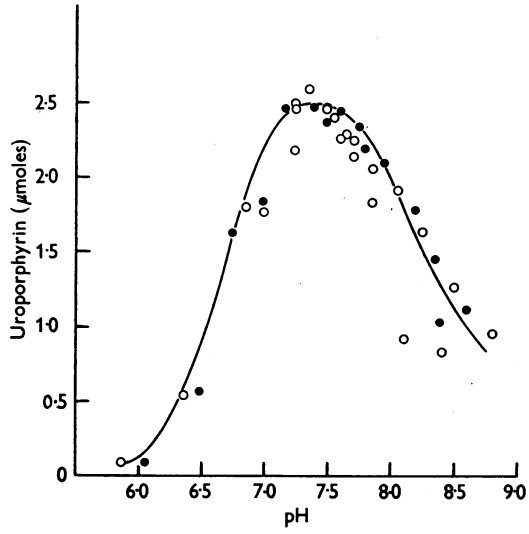



Fig. 2 (*a*). Effect of pH on porphyrin formation. System contained: enzyme, 0·2 ml. and porphobilinogen, 0·4 μmole. Incubation was at 37° for 2 hr. Volume, 10 ml. Phosphate and tris buffers of final concentrations 0·04 and 0·03 M respectively were used. ○, Intact enzyme; ●, heated enzyme; the intact and heated enzymes did not come from the same batch. The estimate is in terms of maximum activity, excluding some apparently aberrant estimations. Half the volume was used for pH measurement. The other half was incubated. A portion (5 ml.) was used for analysis as given in the Materials and Methods section except that 2 ml. of trichloroacetic acid and 1 ml. of 11 N-HCl were added without further dilution.

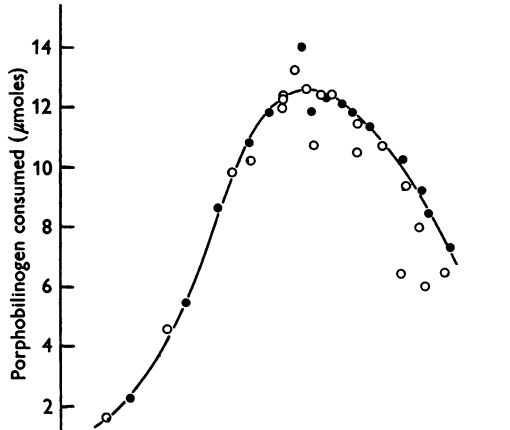
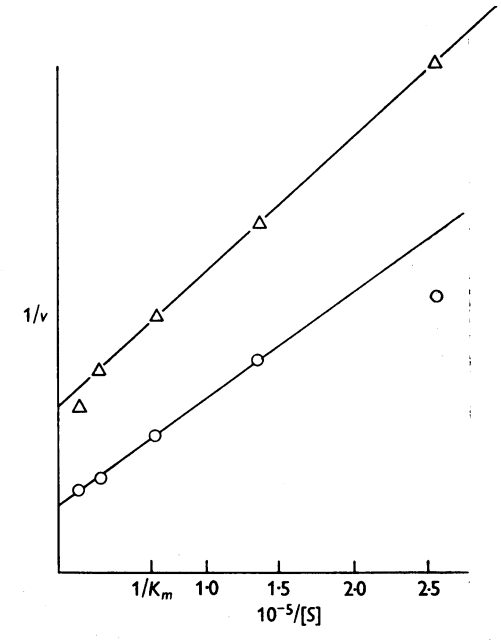
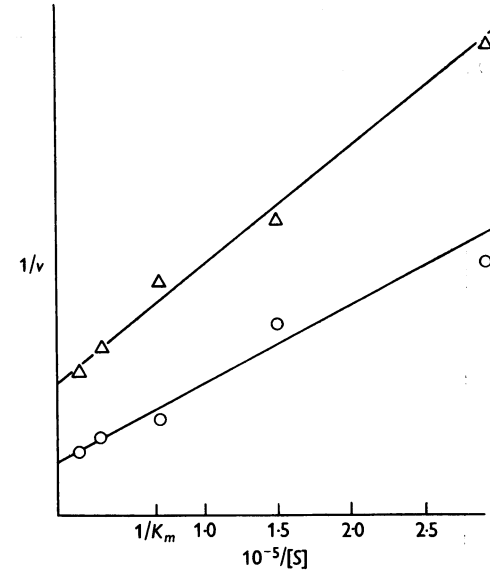



Fig. 2 (*b*). Effect of pH on porphobilinogen consumption. Details were as given in Fig. 2 (*a*).




(*a*)




(*b*)

Figs. 3 (*a*) and 3 (*b*). Curves used for determination of Michaelis constant. (*a*) Intact enzyme. (*b*) Enzyme which had been heated at 60° for 30 min. Tris buffer, pH 8·3 (final concn. 0·03 M). Volume of each system, 5 ml.; enzyme 0·05–0·2 ml. with increasing substrate concentration; incubation was at 37° for 2 hr. ○, Porphobilinogen-consumption rates; △, porphyrin-formation rates. Ordinate in arbitrary units.

of 1 molecule of porphyrin from 4 molecules of porphobilinogen. Yields above 80%, that is less than 5 molecules of porphobilinogen/molecule of porphyrin for both intact and heat-altered enzyme were obtained in experiments in which the porphyrin formation was rapid.

*Inhibitors.* In Table 2 are shown the effects of possible inhibitors. The inhibition by metal ions such as copper, cobalt, etc., occurs at relatively high concentrations and probably has little significance. Table 3 shows that $Hg^{2+}$ ions inhibit and the inhibition is reversed by sulphide. The system is also inhibited by *p*-chloromercuribenzoate and the inhibition is reversed by 1:2-dimercaptopropan-3-ol. The reversible inhibition by mercury compounds is evidence that the system depends on SH groups for its activity. Systems partially inhibited by $Co^{2+}$ ions and systems in which *p*-chloromercuribenzoate inhibition has been reversed by dimercaptopropanol produce isomer III. It can be seen from the Table that there is no inhibition by $CN^-$ or $N_3^-$ ions. However, in the presence of either cyanide or azide (mM) the enzyme system produces isomer I only. The system was slightly inhibited by relatively high concentrations of formaldehyde and of dimedone.

*Paper electrophoresis.* Attempts were made further to purify the enzyme system by paper

Table 2. *Action of some common inhibitors*

Each tube contained inhibitor, 0·1 ml. of intact enzyme preparation, tris buffer, pH 8·3 (0·03 M final concentration), 0·1 μmole of porphobilinogen. The porphobilinogen was added 5 min. after the addition of the inhibitor. Total volume, 0·5 ml. Incubation was at 37° for 3 hr. Estimations of porphobilinogen and porphyrins were as described in the Materials and Methods section.

| Inhibitor | Concn. (mM) | Rate (% of control without inhibitor) Porphobilinogen consumption | Porphyrin formation |
|---|---|---|---|
| $Co^{2+}$ | 0·03 | 100* | 83* |
| | 0·2 | 87 | 57 |
| $Zn^{2+}$ | 0·03 | 100* | 104* |
| | 0·2 | 35 | 15 |
| $Ni^{2+}$ | 0·03 | 100* | 102* |
| | 0·2 | 82 | 87 |
| $Cu^{2+}$ | 0·03 | 100* | 91* |
| $Pb^{2+}$ | 0·03 | 100* | 102* |
| $Hg^{2+}$ | 0·002 | 109 | 99 |
| | 0·02 | 5 | 0 |
| | 0·02 | — | 4 |
| | 0·2 | — | 3 |
| | 0·2 | 0 | 1 |
| | 0·1 | 0 | 0 |
| Chloromercuribenzoate | 0·1 | 22 | 1 |
| | 1 | 7 | 1 |
| $S^{2-}$ | 0·01 | 96 | 95 |
| | 0·1 | 99 | 96 |
| | 0·1 | — | 97 |
| | 1 | 89 | 78 |
| | 1 | — | 77 |
| | 1 | 100* | 81* |
| Dimercaptopropanol | 0·1 | 98 | 100 |
| | 1 | 92 | 108 |
| | 2 | 102 | 118 |
| Iodoacetate | 1 | 98 | 84 |
| $CN^-$ | 1 | 109 | 109 |
| | 1 | 100* | 94* |
| $N_3^-$ | 1 | 98 | 91 |
| | 1 | 100* | 94* |
| $F^-$ | 1 | 100 | 89 |
| | 1 | 100* | 96* |
| $AsO_2^-$ | 1 | 104 | 97 |
| | 1 | 100* | 92* |

* The substrate in these experiments was completely exhausted in the control without inhibitor.

Table 3. *Reversal of inhibition*

Conditions were as given for Table 2. The sulphide and the dimercaptopropanol were added 5 min. after the addition of the inhibitor.

| Substance | Concn. (mM) | Rate (% of control without test substance) | |
|---|---|---|---|
| | | Porphobilinogen consumption | Porphyrin formation |
| $S^{2-}$ | 1 | — | 77 |
| $Hg^{2+}$ | 0·2 | — | 3 |
| $Hg^{2+} + S^{2-}$ | As above | — | 8 |
| $S^{2-}$ | 0·1 | — | 97 |
| $Hg^{2+}$ | 0·02 | — | 4 |
| $Hg^{2+} + S^{2-}$ | As above | — | 89 |
| Dimercaptopropanol | 2 | 102 | 118 |
| Chloromercuribenzoate | 1 | 7 | 1 |
| Dimercaptopropanol + chloromercuribenzoate | As above | 89 | 90 |
| Chloromercuribenzoate | 0·1 | 22 | 1 |
| Dimercaptopropanol + chloromercuribenzoate | As above | 96 | 101 |

electrophoresis. It was thought that if there were an independent (but in the intact system, inhibited) enzyme which forms uroporphyrin I, it might be possible after electrophoresis to obtain an active enzyme from the intact system which no longer gave a uroporphyrin I-forming enzyme after heating. The possibility of obtaining a separation of a condensing (uroporphyrin I-forming) enzyme and an isomerizing enzyme similar to those described by Bogorad (1958*a*, *b*) was also considered. The enzyme was run on Whatman 3MM paper on a water-cooled apparatus at potential pressures varying from 15 to 50v/cm. Buffers used were phosphate, pH 6·8 and 7·4, concentration 0·01–0·1M. Protein staining showed the development of a continuous pattern with haemoglobin-like compounds accumulating at the anodic end of the streak (these components hardly moved at pH 6·8 and moved slightly anodically at pH 7·4). To test for enzyme activity the paper was sprayed with porphobilinogen solution (500 μg./ml.) in 0·2M-phosphate buffer, pH 7·4, and incubated for 3–15 hr. at 37° in a glass jar containing a beaker with a little water to keep the paper moist. Several fluorescent spots developed. Those developing on the electrophoresis papers of heat-altered preparations had moved further cathodically than those of the intact preparations. Porphyrin isolated from the fluorescent spots that developed on the electrophoresis papers of the heat-altered preparations proved, on paper chromatography, to be uroporphyrin I; from the intact system a mixture of uroporphyrins I and III was isolated. Although pure isomer III was not produced no apparent difference could be found in the ratio of the isomers in the porphyrin isolated from different spots. At pH 7·4 an anodic spot of enzyme activity appeared regularly in the electrophoresis paper in a position where there was no detectable activity in the electrophoresis paper of the heat-altered enzyme. Two parallel electrophoresis papers of intact preparation were prepared: in one the spot was identified by spraying with porphobilinogen and from the other the spot was cut out, the circle of paper was suspended in 0·05M-phosphate buffer, pH 7·5, heated for 15 min. at 60° and then incubated with porphobilinogen. The porphyrin was isolated and shown to be uroporphyrin I by paper chromatography.

## DISCUSSION

In the enzyme system described by Bogorad (1958*a*, *b*) the enzyme responsible for the formation of uroporphyrin I is part of the system which forms uroporphyrin III, and Bogorad finds that uroporphyrin I-forming enzyme is an essential part of his reconstituted uroporphyrin III-forming system. This appears to be true also for the system from *Rhodopseudomonas spheroides* as described by Heath & Hoare (1959).

In this paper it has been shown that an avian uroporphyrin III-forming system can be converted into one forming uroporphyrin I with the same pH curve and having very similar affinity for the substrate, and particularly that the overall rate of porphyrin formation remains unchanged after alteration either by heating or by cyanide or azide. We are therefore confident in claiming that in these systems there is no independent uroporphyrin I-forming enzyme, such as an enzyme associated with a heat-labile inhibitor.

From the results with [$^{14}$C]formaldehyde it is possible to exclude the participation of free formaldehyde in the formation of either uroporphyrin I or uroporphyrin III. The evidence does not exclude the transfer of bound formaldehyde

from porphobilinogen to an enzyme and thence to the free α-position of another molecule of porphobilinogen or to that of an intermediate, but does require that such an enzyme should be incapable of reacting with free formaldehyde.

Bogorad (1958*a*, *b*), using good methods of estimation of combined uroporphyrinogen and uroporphyrin, has shown that less than 4·5 molecules of porphobilinogen are required for the synthesis of 1 molecule of uroporphyrin I or III. Granick & Mauzerall (1958) have shown the same for uroporphyrin III formation by avian enzyme. With methods of porphyrin estimation involving greater losses, we have nevertheless shown that under favourable conditions less than 5 molecules of porphobilinogen are necessary for the formation of either porphyrin isomer.

Bogorad finds that the formation of uroporphyrin I by spinach enzyme and the formation of uroporphyrin III by the reconstituted enzyme are as active in the absence of oxygen as in its presence. The enzyme described in this paper was also found to be active in the absence of oxygen. This fits with findings by several workers, Neve, Labbe & Aldrich (1956), Hoare & Heath (1958), Mauzerall & Granick (1958), that uroporphyrinogen and not uroporphyrin is the substrate for the decarboxylase (uroporphyrinogen decarboxylase), which is the next enzyme in the pathway of protoporphyrin biosynthesis.

It is now possible to make certain postulates for the enzymic condensation of porphobilinogen to porphyrins.

(1) In the formation of both isomers there occurs the same primary enzymic reaction with porphobilinogen.

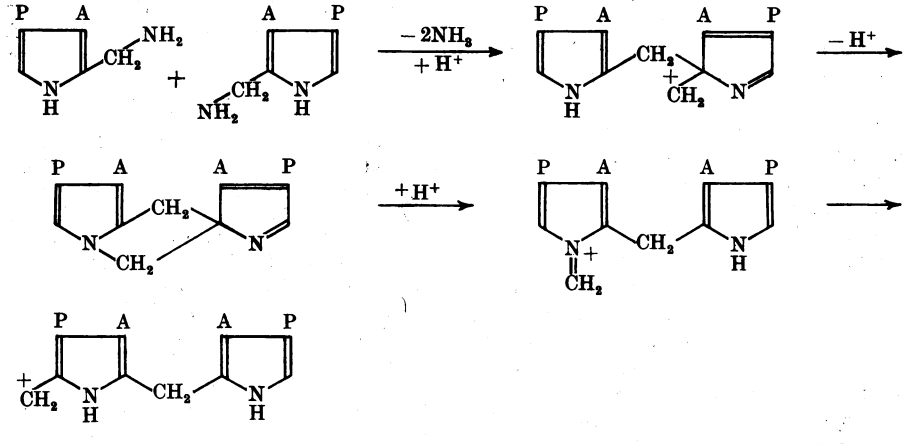
(2) The primary reaction is rate-limiting in both systems.

(3) Free formaldehyde does not participate in the enzymic reaction, although the evidence does not exclude the possibility that an enzyme, incapable of reacting with free formaldehyde, conveys bound formaldehyde from one pyrrole nucleus to another.

(4) Four molecules of porphobilinogen only are required for the formation of either isomer of uroporphyrin.

(5) No oxidation occurs until after cyclization to porphyrinogen and therefore any intermediates formed are in the reduced form, such as dipyrromethanes, not dipyrromethenes.

If these postulates are accepted it is possible immediately to reject certain proposed mechanisms. That proposed by Shemin (1955) requires the loss of one porphobilinogen unit, for example as opsopyrrole dicarboxylic acid [3(4)-carboxymethylpyrrole-4(3)-β-propionic acid]. The findings of Carpenter & Scott (1959), who showed that labelled opsopyrrole dicarboxylic acid is not incorporated into porphyrin during biosynthesis, make unlikely any modification of this scheme whereby opsopyrrole dicarboxylic acid acts as an intermediate or as a cofactor of an isomerizing enzyme. Rimington (1955) has pointed out that the mechanism of the Corwin–Andrews reaction with formation of a tripyrromethane can apply only to the dipyrromethenes, not to the dipyrromethanes, an argument against the mechanisms proposed by Shemin (1955) and by Granick (1955). That proposed by Shlyk (1956) with the participation of a porphobilinogen aldehyde would necessitate either the loss of one pyrrole unit or the formation of a dipyrromethene as an intermediate, and would also




Scheme 1

require an oxidation step for the formation of the aldehyde.

Bullock, Johnson, Markham & Shaw (1958) have proposed the mechanism shown in Scheme 1 to explain the chemical formation of uroporphyrin III rather than I in acid solution.

They propose two repetitions of the same condensation with the formation successively of a tripyrrolic chain ($^{+}CH_2 \cdot PA \cdot PA \cdot AP$) and a tetrapyrrolic chain ($^{+}CH_2 \cdot PA \cdot PA \cdot PA \cdot AP$), where P is $-CH_2 \cdot CH_2 \cdot CO_2H$ and A is $-CH_2 \cdot CO_2H$, and claim that ring closure would then give uroporphyrinogen III. The mechanism could apply equally well to the enzymic formation of uroporphyrinogen. If there is immediate ring closure on the formation of a tetrapyrrolic chain (A, below) then uroporphyrinogen III would be the only isomer formed. If, however, ring closure does not occur immediately on the formation of the tetrapyrrolic chain and a longer polypyrrolic chain is formed, still attached, however, to the enzyme surface, then ring closure could give 1 molecule of uroporphyrinogen III followed by an indefinite number of molecules of uroporphyrinogen I.

(A) $E + 4\ pbg \rightarrow E \cdot CH_2 \cdot PA \cdot PA \cdot PA \cdot AP \rightarrow E + \text{uroporphyrinogen III}$

(B) $E + 5\ pbg \rightarrow E \cdot CH_2 \cdot PA \cdot PA \cdot PA \cdot PA \cdot AP \rightarrow E \cdot CH_2 \cdot PA + \text{uroporphyrinogen III}$

$E \cdot CH_2 \cdot PA + 4\ pbg \rightarrow E \cdot CH_2 \cdot PA \cdot PA \cdot PA \cdot PA \cdot PA \rightarrow E \cdot CH_2 \cdot PA + \text{uroporphyrinogen I}$

(pbg, Porphobilinogen).

There is no reason why the ring closure suggested in A should not be enzymic and heat- and cyanide-sensitive. Heat would then convert the system from one forming uroporphyrinogen III into one forming uroporphyrinogen I with a small fraction of the III isomer. Such mechanisms would be consistent with the similarity of pH-dependence and Michaelis constants and they could explain the constancy of the reaction rate after alteration of the system by heating and by cyanide. In the terms of this mechanism the enzyme from wheat germ described by Bogorad (1958*b*) as an 'isomerase' would be a cyclizing enzyme.

Bogorad (1958*b*) has shown that the completed system which forms uroporphyrin III consists of at least two enzymes. No evidence has been obtained in the present work of more than one enzyme; in particular electrophoresis showed no evidence of separation into a condensing enzyme with porphobilinogenase activity and an enzyme with 'isomerizing' (or cyclizing) activity. However, the mechanism proposed might be catalysed by two distinct enzymes, such as those described by Bogorad, or it might be catalysed by a single enzyme possessing both forms of activity.


## SUMMARY

1. An enzyme preparation from fowl erythrocytes is described which condenses porphobilinogen to uroporphyrinogen III. After heating the preparation at 60° it forms only uroporphyrinogen I. The reactions with porphobilinogen of the intact and of the heat-altered preparation are compared. Formation of porphyrinogen was measured as the porphyrins formed by their oxidation in air.

2. Neither the intact nor the heat-altered preparation incorporates free formaldehyde into porphyrin.

3. Initial porphyrin formation by both preparations is linear with time.

4. Substrate affinity and pH-dependence of the preparation is not appreciably altered by heat treatment.

5. Mercuric and *p*-chloromercuribenzoate ions inhibit the intact preparation completely. The inhibition is reversed by sulphide ions and by dimercaptopropanol respectively. The isomer formed after reversal of the *p*-chloromercuribenzoate inhibition is uroporphyrin III.

6. Cyanide ions do not inhibit the intact preparation but in the presence of cyanide the intact preparation forms uroporphyrin I.

7. Neither heat treatment nor the presence of cyanide appreciably influences the rate of porphyrin formation.

8. Paper electrophoresis failed to show any separation into fractions with different types of enzyme activity.

9. The findings are discussed in terms of possible mechanisms of porphyrin formation from porphobilinogen.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# Studies on Flavinadenine Dinucleotide-Synthesizing Enzyme in Plants

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There is practically no information on the biosynthesis of flavinadenine dinucleotide in plants. The available evidence relating to this process in animal tissues or micro-organisms is meagre, although the function of flavin nucleotides as components of respiratory enzymes has long been known. Trufanov (1941) suggested that flavinadenine dinucleotide is synthesized in rat-tissue slices by a condensation of riboflavin and adenosine diphosphate and that maintenance of cell structure is essential for the reaction. Schrecker & Kornberg (1950) demonstrated synthesis of flavinadenine dinucleotide from flavin mononucleotide and adenosine triphosphate in yeast. A similar pathway for synthesis of flavinadenine dinucleotide was reported in a riboflavin-secreting mutant yeast (Giri & Krishnaswamy, 1956). Recently Deluca & Kaplan (1958) showed that synthesis of flavinadenine dinucleotide occurs in rat-liver supernatants.

The occurrence of riboflavin in plants has long been known (Bonner, 1942; Bonner & Bonner, 1948; Watson & Nogelle, 1947), but there is no information on the concentration of flavin nucleotide coenzymes and the enzymes synthesizing these.

After the observation on the widespread occurrence of the flavin nucleotides (Giri, Appaji Rao, Cama & Kumar, 1959) and on the biosynthesis of flavin mononucleotide in plants by phosphorylation of riboflavin by adenosine triphosphate (Giri, Krishnaswamy & Appaji Rao, 1958), the mechanism of biosynthesis of flavinadenine dinucleotide was studied, and the results are described and discussed in this paper.

* Deceased.

## MATERIALS AND METHODS

*Substrates and reagents.* Flavin mononucleotide (FMN) was a Hoffman–La Roche and Co. product obtained as a gift from Voltas Ltd., Bombay, and was purified before use. Flavinadenine dinucleotide (FAD; 60% pure) was obtained as a gift from Esai Ltd., Japan, and was purified by preparative circular-paper chromatography before use (Giri, 1954, 1955). Adenosine triphosphate (ATP) used included preparations made according to LePage (1949) of 65–70% purity and another (95% purity) obtained from California Foundation for Biochemical Research. The purity of ATP was determined by the method described by LePage (1949). All other chemicals used were of reagent grade.

*Preparation of alumina Cγ-gel.* Ammonium sulphate (22 g.) was dissolved in 600 ml. of water. Ammonia soln. (100 ml. of 10%; previously standardized by titration) was added to it and the mixture was warmed to 58°. A solution containing 76·7 g. of aluminium ammonium sulphate dodecahydrate in 150 ml. of water was also warmed to 58° and was added all at once to the basic ammonium sulphate solution. This mixture was warmed to 60° and stirred for 15 min. The contents were transferred to a tall jar with 5 l. of water and the precipitate was washed three times with 3 l. of water. To the fourth wash was added 80 ml. of 20% ammonia soln. to decompose the basic sulphate and the gel was washed repeatedly with water. After twenty washings the water remained opalescent and the gel was washed twice more with water. A suspension containing 18 mg. (dry wt.)/ml. was prepared. It was aged at 4° for at least 2 months before use (Willstätter & Kraut, 1923).

*Preparation and purification of the enzyme.* Finely powdered green gram (*Phaseolus radiatus*) was used as the source of the enzyme. An extract with maximum specific activity was obtained by the following procedure.

Freshly powdered green-gram seeds (100 g./300 ml.) were extracted with 0·1 M-sodium bicarbonate soln. with constant stirring at 0–5° for 2 hr. The extract was centrifuged