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The Effects of Oxygen Concentration on Porphyrin Biosynthesis in Chicken-Erythrocyte Preparations

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In the biosynthesis of haem pigments, the concentration of oxygen appears to play a regulatory role at the sites of specific reactions in the pathway. The release of iron from storage in liver ferritin (Green & Mazur, 1956) and the catalysis, by soluble liver ferrochelatase preparations, of the incorporation of Fe^{2+} ions into protoporphyrin to form haem (Porra & Jones, 1963) are both favoured by anoxic conditions.

There is considerable evidence that oxygen concentration controls the rate of synthesis of the porphyrin nucleus itself. In avian erythrocytes, oxygen is necessary for the conversion of acetate and glycine into δ-aminolaevulic acid (Gibson, Laver & Neuberger, 1958) and of coproporphyrinogen III into protoporphyrin IX (Falk, Dresel & Rimington, 1953; Granick & Mauzerall, 1958; Sano & Granick, 1961; Porra & Falk, 1961). These requirements for oxygen explain the rise, towards an optimum oxygen concentration of about 7% (v/v), found by Falk, Porra, Brown, Moss & Larminie (1959) for the rate of formation of protoporphyrin from glycine; the decreases found at oxygen concentrations higher than 7% have now been studied further.

By the use of preparations from washed chicken erythrocytes that had been haemolysed, the inhibition by higher oxygen concentrations has now been traced to a reversible inhibition by oxygen of the rate of conversion of porphobilinogen into uroporphyrinogen III. These findings are discussed in relation to the known effects of oxygen concentration on haemopoiesis in higher animals and on the cytochrome concentrations in certain micro-organisms.

EXPERIMENTAL

Materials

δ-Aminolaevulic acid. This was supplied by the Monadnock Research Institute, Antrim, N.H., U.S.A.

Ehrlich's reagent. A 2% (w/v) solution of *p*-dimethylaminobenzaldehyde (analytical reagent) in 5% (w/v) HCl was used.

Porphobilinogen. A crystalline sample of porphobilinogen hydrochloride was a gift from Professor C. Rimington, University College Hospital Medical School, London.

Haemolysates. These were prepared from washed chicken erythrocytes by the method of Dresel & Falk (1956*b*).

Haemolysate supernatants. These were prepared by centrifuging the haemolysates for 60 min. at 30 000 rev./min. at 4° in the no. 30 rotor of a Spinco model L centrifuge. The clear red supernatant was carefully removed with a pipette. Both haemolysates and haemolysate supernatants could be stored at −15° for several weeks without loss of enzymic activity.

Methods

Incubations. All incubations were carried out in 100 ml. conical flasks fitted with Bunsen valves, and gas mixtures of the compositions indicated in the text were passed through the flasks. Except where otherwise stated, the haemolysate (10·5 ml.) or haemolysate supernatant (10·5 ml.) was incubated at 37° with $MgCl_2$ (0·05 M), KCl (0·1 mM) and δ-aminolaevulic acid or porphobilinogen at concentrations shown in the text. The pH of the preparation (7·2) was maintained by the buffering capacity of the erythrocyte proteins. Flasks were shaken at 120 oscillations/min. with an amplitude of 6 cm.

Gas mixtures. All gas mixtures contained 5% (v/v) of CO_2. The concentrations of O_2 and N_2 are expressed throughout as % (v/v), and were regulated by the use of the constant-flow apparatus of Bailey (1954). The gas mixtures were passed over the surface of each incubation mixture at the rate of 10 ml./min.; the pH of the haemolysate preparations (7·2) was lowered by only 0·16 unit even after incubation for 6 hr.

Extraction, isolation and determination of uroporphyrins, coproporphyrins and protoporphyrins. It is now well established that uro- and copro-porphyrinogens are intermediates in the biosynthesis of protoporphyrin IX. That a reduced form of protoporphyrin IX is an intermediate that has been shown by Porra & Falk (1964). In the present paper the porphyrinogens and the porphyrins formed from them by oxidation were determined together as the corresponding porphyrins, and the results are, therefore, expressed in terms of porphyrins.

At the end of incubations, 10 vol. of ethyl acetate–acetic acid (3:1, v/v) was added to the incubation mixtures with rapid stirring and the mixtures were exposed to light and air for 1 hr. to convert any porphyrinogens into porphyrins (cf. Sano & Granick, 1961). The porphyrins were then isolated and determined as described by Dresel & Falk (1956*a*), the uroporphyrin being isolated by the cyclohexanone method.

Determination of porphobilinogen. The porphobilinogen content of incubation mixtures was determined, after precipitation of the proteins by trichloroacetic acid, as described by Dresel & Falk (1956*c*).

RESULTS

Effect of oxygen concentration on the conversion of δ-aminolaevulic acid into porphyrins by haemolysates. When haemolysates of chicken erythrocytes were incubated with non-limiting amounts of δ-aminolaevulic acid (see Fig. 3 of Dresel & Falk, 1956*b*) under a range of oxygen concentrations, the maximum rate of protoporphyrin biosynthesis was obtained when the oxygen concentration in the gas phase was 7% (Fig. 1). The curve for protoporphyrin formation is reminiscent of that obtained for the conversion of glycine into protoporphyrin in chicken blood and in intact washed chicken erythrocytes (Falk *et al.* 1959). In the latter experiments coproporphyrin formation increased steeply from nil in nitrogen to a maximum rate in an atmosphere containing between 0·1 and 1·0% of oxygen. This reflects the known oxygen requirement of the reactions leading to the formation of δ-aminolaevulic acid from glycine. There is no such requirement when δ-aminolaevulic acid is the substrate (cf. Falk *et al.* 1953), and the results shown in Fig. 1 confirm this.

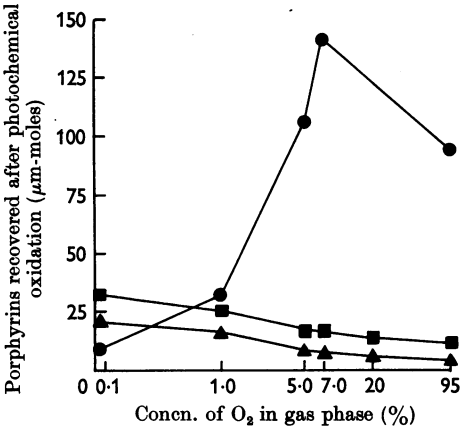

Fig. 1. Effect of oxygen concentration on the conversion of δ-aminolaevulic acid into haem intermediates by chicken haemolysates. Porphyrinogens were determined as porphyrins after photochemical oxidation as described in the Methods section. Haemolysate (10·5 ml.) was incubated as described in the text for 2 hr. at 37° with 6·7 μ-moles of δ-aminolaevulic acid in a final volume of 12·0 ml. ■, Uroporphyrin; ▲, coproporphyrin; ●, protoporphyrin. The O_2 concentrations are plotted on a logarithmic scale with an arbitrary zero.

Table 1. *Effect of oxygen concentration on the conversion of δ-aminolaevulic acid into intermediates in haem biosynthesis*

Haemolysate supernatant (10·5 ml.) was incubated as described in the text for 2 hr. at 37° with 7·6μmoles of δ-aminolaevulic acid (950μm-moles of 'porphyrin equivalent') in a final volume of 12·0 ml. [Eight molecules of δ-aminolaevulic acid are required to form one porphyrin molecule, and are thus one 'porphyrin equivalent' as are four molecules of porphobilinogen (cf. Dresel & Falk, 1956*b*).] Porphyrinogens were determined as porphyrins after photochemical oxidation as described in the Methods section.

Concn. of O_2 in the gas phase (%)	Porphyrins recovered after oxidation (μm-moles)		
	Uroporphyrin	Copro-porphyrin	Proto-porphyrin
1	82	14	2
5	66	15	2
7	50	14	2
14	28	14	0
20	28	13	0
95	21	11	0

Effect of oxygen concentration on the conversion of δ-aminolaevulic acid into porphyrins by haemolysate supernatants. In these preparations the particle-bound enzymes, coproporphyrinogen oxidative decarboxylase (coprogenase) and protoporphyrinogen oxidase (cf. Porra & Falk, 1964), responsible for the conversion of coproporphyrinogen III into protoporphyrin IX, have been removed (Dresel, 1955; Porra & Falk, 1961). The decrease in uroporphyrinogen formation observed when these preparations were incubated with δ-aminolaevulic acid under increasing concentrations of oxygen (Table 1) indicates that high concentrations inhibit the conversion of δ-aminolaevulic acid into uroporphyrinogen. That this inhibition was in fact operating after the conversion of δ-aminolaevulic acid into porphobilinogen was demonstrated in the following experiments, where porphobilinogen itself was used as substrate.

Reversible inhibition by oxygen of the conversion of porphobilinogen into uroporphyrinogen. The results in Table 2 show that, as the oxygen concentration was increased, less porphobilinogen was used and less uroporphyrinogen was formed. The incomplete recoveries, due to the loss of uroporphyrinogen on the protein precipitated during this procedure, render the values for uroporphyrinogen formation inaccurate. This loss, however, is a constant proportion of the total uroporphyrin (Dresel & Falk, 1956*a*) and hence there is little doubt that there is a real decrease in synthesis as the oxygen concentration increases. The porphobilinogen assay is not subject to such inaccuracies, and the decreased utilization of porphobilinogen as the oxygen concentration increases confirms this inhibition. Uroporphyrinogen decarboxylase is a soluble enzyme, and the formation of coproporphyrin in this experiment shows that this enzyme is present in the supernatant. Coprogenase, however, is bound to particulate matter in avian erythrocytes; the formation of very small amounts of protopor-

Table 2. *Inhibition of the conversion of porphobilinogen into intermediates in haem biosynthesis by high oxygen concentrations*

Haemolysate supernatant (10·5 ml.) was incubated as described in the text for 2 hr. at 37° with 1·2 μmoles of porphobilinogen (300 μm-moles of 'porphyrin equivalent') in a final volume of 12·0 ml. (The 'porphyrin equivalent' is defined in Table 1.)

Concn. of O_2 in the gas phase (%)	Porphobilinogen remaining (μm-moles of 'porphyrin equivalent')	Porphyrins recovered after oxidation (μm-moles)			Total recovery (μm-moles of 'porphyrin equivalent')
		Uroporphyrin	Copro-porphyrin	Proto-porphyrin	
0	36	169	43	8	256
1	53	170	35	6	264
5	58	135	56	6	255
7	67	123	39	7	236
20	—	65	49	6	—
95	88	74	50	5	217

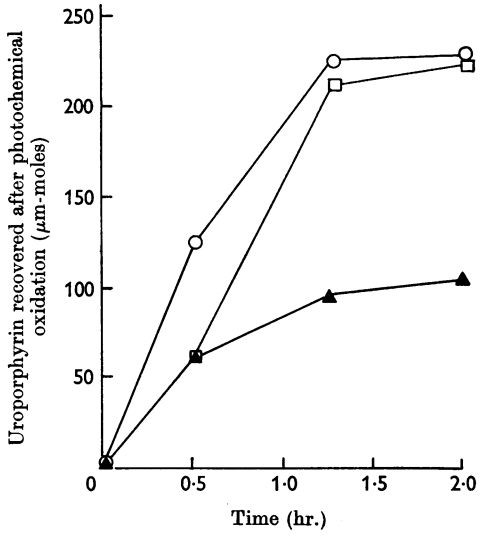

Fig. 2. Inhibition of the conversion of porphobilinogen into uroporphyrinogen (determined as uroporphyrin). Haemolysate supernatant (10·5 ml.) was incubated as described in the text with 1·2 μmoles of porphobilinogen at 37° in a final volume of 12·0 ml. ○, In N_2; ▲, in 20% O_2; □, in 20% O_2 for 0·5 hr. followed by N_2 for 1 hr.

phyrin suggests that a small proportion of this enzyme is solubilized during haemolysis.

The inhibition by oxygen of uroporphyrinogen production is a reversible process; alteration of the atmosphere from 20% oxygen to nitrogen increased the rate of uroporphyrinogen formation to that found when incubation was under nitrogen throughout (Fig. 2). In both cases the same final amount of uroporphyrinogen was formed, demonstrating that porphobilinogen, uroporphyrinogen, and intermediates in the conversion are not irreversibly changed by oxygen.

Stability of porphyrins and intermediates to oxygen. When uroporphyrin was incubated at 37° for 4 hr. with haemolysate, there was no significant difference in the amount recovered whether the gas phase was nitrogen or 20 or 100% oxygen. To test whether copro- and proto-porphyrins, or their reduced precursors, were unstable to oxygen, δ-aminolaevulic acid was incubated with the haemolysate at 37° for 4 hr. in air. One portion of the incubation mixture was analysed at once and three others were exposed at 37° for a further 4 hr. to 50, 70 and 100% oxygen. There was no significant difference in the amount of copro- and proto-porphyrins recovered after any of the four treatments.

DISCUSSION

The present results establish a reversible inhibition by oxygen of the conversion of porphobilinogen into uroporphyrinogen. When the oxygen concentration is raised from about 7 to 20%, there is a marked inhibition of the synthesis of uroporphyrinogen (see Table 1 and Fig. 2), and consequently of copro- and proto-porphyrin (Fig. 1; see also Falk *et al.* 1959). It is thus clear that previous studies *in vitro* of porphyrin and haem biosynthesis by similar preparations in air have been performed under suboptimum conditions. The experiments showing stability to oxygen of the porphyrins and the intermediates demonstrate that the decrease in porphyrin concentrations at higher oxygen concentrations is not due to oxidative degradation of the tetrapyrrole nucleus. It seems, therefore, that this reversible inhibition by oxygen must operate at some enzymic step in the conversion of porphobilinogen into uroporphyrinogen. This inhibition may constitute a biochemical control mechanism for the regulation of haem biosynthesis, additional to the known hormonal mechanisms (cf. Gordon, 1959), and explain in part the phenomenon of increased haemopoiesis at high altitudes. Homeostatic mechanisms would minimize the changes in oxygen concentration in the blood of

animals moving to high altitudes and thus the stimulation of uroporphyrinogen formation would be small. Nevertheless, it is known that increased haemopoiesis on acclimatization to high altitude is a slow process (cf. Grant & Root, 1952), and small changes in the rate of porphyrin synthesis, maintained for several weeks, could well lead to the observed blood haemoglobin concentrations.

Krebs (1959) has pointed out that, though biochemical and hormonal control mechanisms both operate in higher animals, metabolic regulation at the biochemical level assumes great importance in bacteria, which contain no hormones. It is thus conceivable that the regulation by oxygen of haem biosynthesis, described above, may play a part in the changes in haemoprotein concentrations during respiratory adaptation in micro-organisms. Using *Aerobacter aerogenes*, Moss (1956) has shown that the maximum cell content of cytochrome a_2 is obtained when the oxygen concentration in the bacterial culture during growth is 1 μM. Above and below this concentration the cytochrome a_2 content of the cell falls off in a manner similar to that found for protohaem formation *in vitro* (Falk *et al.* 1959).

SUMMARY

1. The effects of oxygen concentration on porphyrin biosynthesis from δ-aminolaevulic acid and porphobilinogen have been studied in whole haemolysates of chicken erythrocytes and in supernatants from these preparations.

2. These studies show that concentrations of oxygen higher than 5–7 % (v/v) inhibit the conversion of porphobilinogen into uroporphyrinogen. This inhibition can be reversed by lowering the concentration of oxygen.

3. The stability of uro-, copro- and protoporphyrins to high concentrations of oxygen has been demonstrated.

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Biochem. J. (1964) **90**, 69

The Enzymic Conversion of Coproporphyrinogen III into Protoporphyrin IX

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Neve, Labbe & Aldrich (1956) were the first to demonstrate that the true tetrapyrrole intermediates in the biosynthesis of protoporphyrin IX were not porphyrins but hexahydroporphyrins (porphyrinogens) (see Fig. 3). The enzyme system converting coproporphyrinogen III into protoporphyrin IX has been obtained in soluble form from liver mitochondria (Sano, 1958; Sano & Granick, 1961; Porra & Falk, 1961; Rimington & Tooth, 1961; Porra, 1962) and has been partly purified by Sano & Granick (1961). For the purposes of the present paper it is presumed that the protoporphyrin formed is isomer IX.

The conversion of coproporphyrinogen III into protoporphyrin IX involves the oxidative decarboxylation of two propionic acid side chains to vinyl groups; the enzyme system catalysing this oxidative decarboxylation has been called coproporphyrinogen oxidative decarboxylase (coprogenase) by Porra & Falk (1961). Although the