Studies on Ferrochelatase

THE EFFECTS OF THIOLS AND OTHER FACTORS ON THE DETERMINATION OF ACTIVITY

BY R. J. PORRA, KARIN S. VITOLS, ROBERT F. LABBE* AND NORMA A. NEWTON
Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, A.C.T., Australia

(*Received* 28 *October* 1966)

1. Haems are unstable under aerobic conditions in the presence of thiols, which are used to activate the ferrochelatase enzyme; catalase inhibits this degradation of haem. In addition, thiols interfere with the determination of protohaem as its pyridine haemochromogen derivative. 2. Three ferrochelatase assays are described that minimize interference by these two reactions. Two of these assays involve measurement of porphyrin utilization, one spectrophotometrically and the second spectrofluorimetrically. The third assay measures haem formation by a pyridine haemochromogen technique. Results obtained with these three methods were in close agreement at a GSH concentration of 4mM. 3. The stimulatory effect of GSH on ferrochelatase has been confirmed. The spectrum of the haem formed is dependent on GSH concentration; at high GSH concentrations (20mM) the haem is in the reduced state, but at low concentration (4mM) the spectrum of the product resembles that of an oxidized haemoprotein such as ferrihaemoglobin. 4. The inhibitory effect of oxygen on ferrochelatase activity has been confirmed by spectrophotometric assay of porphyrin disappearance.

The enzyme protohaem ferro-lyase (EC 4.99.1.1), also known as ferrochelatase (Rimington, 1958), haem synthase (Porra & Ross, 1965) or haem synthetase (Riethmüller & Tuppy, 1964), catalyses the insertion of Fe^{2+} ions into various porphyrins, with the release of two protons, to form the corresponding haems. Krueger, Melnick & Klein (1956) and Goldberg, Ashenbrucker, Cartwright & Wintrobe (1956) established the enzymic nature of the reaction by measuring the incorporation of $^{59}Fe^{2+}$. Other assays, depending on the measurement of newly formed haem, include the methods of Oyama, Sugita, Yoneyama & Yoshikaya (1961) and Porra & Jones (1963*a*,*b*,*c*). Ferrochelatase has also been assayed by measuring the disappearance of porphyrin substrate either after deproteinizing the reaction mixture (Labbe, 1959) or by continuously following the disappearance of the Soret band (Labbe, Hubbard & Caughey, 1963) or of band IV (Johnson & Jones, 1964; P. A. Trudinger & A. Johnson, personal communication). Assays that measure the disappearance of Fe^{2+} ion have been reported by Oyama *et al.* (1961) and Yoneyama, Tamai, Yasuda & Yoshikawa (1965).

In the present paper we show that haems are unstable under aerobic conditions in the presence of thiols or ascorbic acid, which are necessary activators of ferrochelatase. Further, in the presence of thiols there is some additional alteration of protohaem, but not of mesohaem. This interferes with the determination of ferrochelatase activity by the haemochromogen method when protoporphyrin IX is the substrate. Some properties of these two interfering reactions are described and a modified pyridine haemochromogen assay of ferrochelatase is presented that minimizes the effects of these reactions. In addition, we describe two new assays based on the disappearance of porphyrin.

EXPERIMENTAL

Materials

Emasol 4130 (polyoxyethylene sorbitan mono-oleate) was a gift from the Kao Soap Co., Tokyo, Japan. Tween 20 (polyoxyethylene sorbitan monolaurate) was supplied by L. Light and Co. Ltd., Colnbrook, Bucks. Mesoporphyrin IX was obtained from Fluka A.-G., Basle, Switzerland. Mesohaem IX was prepared from mesoporphrin IX by the ferrous sulphate method described by Falk (1964, p. 133). Protohaem IX was prepared from chicken blood by the method of Labbe & Nishida (1957). Crystalline catalase was supplied by General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. The most satisfactory sample of GSH was supplied

* Present address: Department of Pediatrics, University of Washington, Seattle, Wash., U.S.A.

by Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; some other samples gave rise to turbidity in assay mixtures after several minutes' incubation at 38°. Antifoam AF Emulsion, a silicone type, was supplied by Anax (Pty.) Ltd., Sydney, N.S.W., Australia.

Rat-liver ferrochelatase preparation

Adult Wistar rats were anaesthetized with ether and their livers were removed and chilled on ice. All subsequent procedures were performed at 4°. The livers were homogenized with 9vol. of 0·25M-sucrose in a Waring Blendor, the homogenate was centrifuged for 10min. at 1000*g* and the residue was washed once with a small volume of 0·25M-sucrose. The combined supernatants were centrifuged for 10min. at 20000*g* and the residue of mitochondria was washed twice with 0·02M-tris–HCl buffer, pH8·2, to remove smaller particles and soluble protein. The washed mitochondria were suspended in 0·1M-tris buffer, pH10·4 (1·5ml./g. of liver). While the suspension was being stirred vigorously, 0·1vol. of aq. 10% (w/v) Tween 20 solution followed by 0·1vol. of ethanol were slowly added. After gentle stirring for 2·5hr., the mixture was centrifuged for 10min. at 20000*g*. The supernatant, after dialysis overnight against 30vol. of 0·02M-tris–HCl buffer, pH8·2, was centrifuged for 60min. at 80000*g* and the yellow supernatant was removed with a pipette. A narrow turbid layer at the top of the supernatant had no effect on the ferrochelatase activity; it was not, therefore, separated from the rest of the supernatant. Protein concentrations, determined by the method of Lowry, Rosebrough, Farr & Randall (1951), varied from 5 to 10mg./ml. and the final pH was 8·3. This ferrochelatase preparation was stored at −15° as 10ml. samples and was stable for several months.

Mesoporphyrin IX solutions

The porphyrin was dissolved in a small volume of aq. 2N-NH_3. The solution was diluted 10–20-fold with water, neutralized to pH8·2 with N-HCl and then made 0·01M with respect to tris–HCl buffer, pH8·2. To this solution was added 0·2vol. of 1% (w/v) Emasol 4130. To standardize this solution, a small sample was diluted 100–200-fold with 0·1N-HCl and the $E^{399}_{1\,cm.}$ value was measured. The concentration of mesoporphyrin was then calculated by using an ϵ^{399}_{mM} value of 445 (Falk, 1964, p. 236).

Determination of ferrochelatase activity

Unless otherwise stated, each ml. of reaction mixture contained 40mμmoles of mesoporphyrin, 0·1ml. of 1% (w/v) Emasol 4130, 0·1ml. of ethanol, 100μmoles of tris–HCl buffer, pH8·2, 4μmoles of GSH, 40mμmoles of $FeSO_4$ and rat-liver ferrochelatase preparation equivalent to 0·1–0·5mg. of protein. Reagents were pipetted in the order indicated. In some experiments specified in the text, 0·26μM-catalase was added to the reaction mixture. Emasol 4130 not only stimulates enzyme activity but also maintains the porphyrin substrate in true solution, thus enabling characteristic spectra of the porphyrin to be obtained (Porra & Jones, 1963*b*). The latter point is important when ferrochelatase activity is measured by the spectrophotometric assay of porphyrin disappearance.

In both of the porphyrin disappearance assays (described below), it is assumed that all the utilized mesoporphyrin is converted into iron mesoporphyrin. This assumption was proved correct under standard assay conditions, in which the GSH concentration was 4mM, by the presence of isosbestic points at 486, 509, 526, 536, 564, 578, 618 and 624mμ (Fig. 5) and by the agreement between results obtained by the porphyrin disappearance methods and the pyridine haemochromogen assay (see the Results section). Under the conditions of these assays, controls performed in the absence of enzyme and in the absence of $FeSO_4$ revealed that there was neither non-enzymic utilization of porphyrin nor enzymic incorporation of other cations such as Zn^{2+} (Mazanowska, Neuberger & Tait, 1966) or Co^{2+} (Labbe & Hubbard, 1961; Porra & Ross, 1965).

(1) *Pyridine haemochromogen method.* Ferrochelatase activity was measured anaerobically in small Thunberg cuvettes or, in some cases, ordinary Thunberg tubes. In each Thunberg tube was placed 2·5ml. of reaction mixture excluding the ferrochelatase preparation, which was placed in the side arm. Use of Thunberg tubes with a small free gas volume minimized degradation of haem. A few drops of antifoam were added to minimize frothing on evacuation. The Thunberg tubes were evacuated and flushed with O_2-free N_2 several times and finally sealed under a slight positive pressure of N_2. The tubes were transferred to a water bath at 38° and 5min. later the incubation was started by tipping enzyme into the reaction mixture. At the end of the incubation period, the evacuation port of the Thunberg tube was opened and 0·5ml. of 0·4M-iodoacetamide was quickly introduced with a hypodermic syringe to minimize the entry of oxygen. The tube was then closed and the contents were gently mixed for several minutes. The cuvette was then opened and pyridine (0·5ml.) and N-NaOH (0·5ml.) were added. The difference spectrum of the reduced and oxidized pyridine haemochromogens was recorded between 500 and 600mμ and the mesohaem formed was calculated by using $\Delta\epsilon_{mM} = \epsilon^{547}_{mM} - \epsilon^{531}_{mM} = 21{\cdot}7$ (Porra & Jones, 1963*a*). If higher sensitivity was required, the haem formed could be determined from the difference spectrum in the Soret region. To avoid interference from $Na_2S_2O_4$ only a minimal amount was added and, to minimize autoxidation, the solution was covered with liquid paraffin. In the Soret region $\Delta\epsilon_{mM}$ for pyridine mesohaemochromogen is $\epsilon^{408}_{mM} - \epsilon^{384}_{mM} = 106$.

(2) *Spectrofluorimetric measurement of porphyrin disappearance.* This method depends on the fact that porphyrins in solution are fluorescent whereas haems are not. Incubations were performed as in method (1). After addition of iodoacetamide, pyridine and alkali, 0·5ml. of the solution was diluted to 5·0ml. with the alkaline pyridine reagent of Paul, Theorell & Åkeson (1953). Such a dilution diminishes the $E^{500}_{1\,cm.}$ value to 0·1 or less; i.e. the porphyrin concentration is diminished to 10μM or less, thus ensuring a satisfactory registration on the spectrofluorimeter used. In addition, this dilution ensures that the fluorescence emission of mesoporphyrin at 618mμ, stimulated by irradiation with light at 500mμ, is proportional to mesoporphyrin concentration; significant departure from linearity due to concentration quenching is not obtained until the porphyrin concentration is approx. 0·1–1mM (S. W. Thorne, personal communication). Fluorescence was measured in a spectrofluorimeter, capable of recording absolute fluorescence emission and excitation spectra, designed in these Labora-

tories by Mr S. W. Thorne. The fluorescence emission of a zero-time sample was equated with the initial amount of mesoporphyrin.

(3) *Spectrophotometric assay of porphyrin disappearance.* The incubation was performed as in method (1) but in Thunberg cuvettes. The reaction was started by tipping the enzyme from the side arm and spectra were recorded periodically between 450 and 650mμ in a Cary model 14R spectrophotometer. Mesohaem formation was then determined by measuring the decrease in $E^{498}_{1\,cm.}$. A difference coefficient ($\Delta\epsilon^{498}_{mM}$) was calculated by allowing an incubation to proceed until all, or nearly all, the porphyrin substrate had been utilized; the observed $\Delta E^{498}_{1\,cm.}$ was then correlated with the amount of porphyrin utilized as measured by the spectrofluorimetric technique. The $\Delta\epsilon^{498}_{mM}$, so calculated, changed with GSH concentration (see Figs. 4 and 5): it was 4·75 and 6·20 at 4mM- and 20mM-GSH respectively. Alternatively, the reaction could be measured continuously by following $\Delta E^{498}_{1\,cm.}$ in the Cary spectrophotometer.

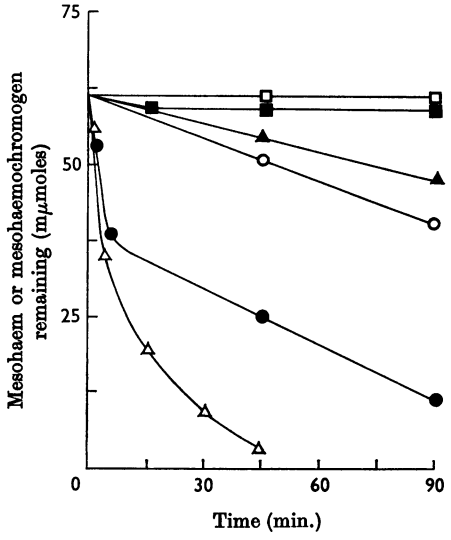

Fig. 1. Degradation of mesohaem and pyridine mesohaemochromogen in the presence of cysteine. Mesohaem (61·2mμmoles) was incubated at 38° with 30μmoles of cysteine, 0·1% Tween 20 and 200μmoles of tris–HCl buffer, pH7·8, in a final volume of 3ml. Aerobic incubations, in test tubes, and anaerobic incubations, in double-based Thunberg tubes, were started by the addition of cysteine. All the reactions were terminated by the addition of iodoacetamide. All incubations were shaken at 180 cycles/min. with an amplitude of 3cm. Residual mesohaem was determined by the pyridine haemochromogen technique after incubating: ○, *in vacuo*; ▲, *in vacuo* after bubbling N_2 through the incubation mixture; ●, under aerobic conditions; ■, aerobically with 0·26μM-catalase. A control (□) was carried out by incubating mesohaem aerobically without cysteine. Pyridine mesohaemochromogen, prepared by dissolving 61·2mμmoles of mesohaem in 3ml. of 0·075N-NaOH containing 2·1M-pyridine, was incubated aerobically with 30μmoles of cysteine; the remaining mesohaemochromogen (△) was determined by the pyridine haemochromogen method.

RESULTS

Effect of thiols on the determination of haems

In these experiments GSH, cysteine and mercaptoethanol were interchangeable with only slight variation in reaction rates.

Aerobic degradation of haems in the presence of GSH. GSH interfered in the determination of mesohaem as its pyridine haemochromogen by the method of Porra & Jones (1963*a*); in the presence of 13mM-GSH the recovery of mesohaem was about two-thirds of that obtained in its absence. The recovery, however, was not diminished if 0·02M-iodoacetamide was added immediately after the addition of the GSH to the mesohaem solution. The destruction of mesohaem and of its pyridine haemochromogen in the presence of cysteine and the enhancement of this degradation by aerobic conditions are illustrated in Fig. 1. The almost

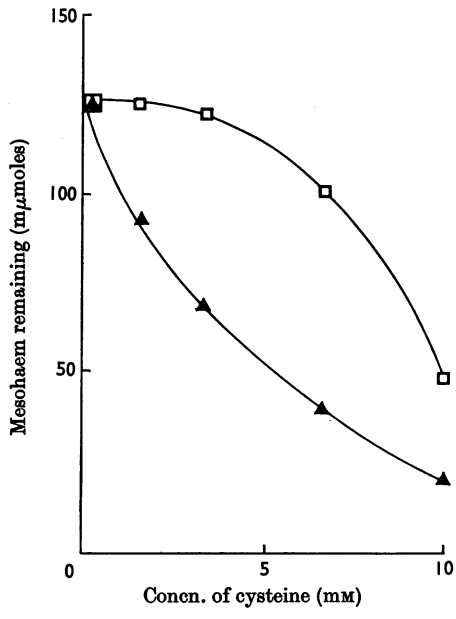

Fig. 2. Effect of cysteine concentration on mesohaem degradation. Mesohaem (125mμmoles) was incubated with various concentrations of cysteine in a final volume of 3ml. for 45min. at 38° with shaking as described in Fig. 1. All reactions were started by the addition of cysteine and terminated by the addition of iodoacetamide. The residual haem was determined by the pyridine haemochromogen method. Incubations were performed either *in vacuo* in double-based Thunberg tubes (□) or aerobically in open test tubes (▲).

complete absence of this reaction in the presence of catalase (Fig. 1) suggests the participation of free peroxide; this was confirmed when degradation was observed in similar experiments in which the thiol was replaced by hydrogen peroxide. The effect of thiol concentration on the rate of mesohaem degradation under aerobic and anaerobic conditions is shown in Fig. 2; little or no degradation was observed under anaerobic conditions at cysteine concentrations below 2·5mM. Ascorbic acid, which some workers have used in place of thiols to stimulate ferrochelatase activity, caused a similar degradation of haems. With both reducing agents there was destruction of the tetrapyrrole ring since the Soret band, common to the haems and their parent porphyrins, was absent at the completion of the reaction. This degradation is specific for haems; mesoporphyrin was not degraded under identical conditions.

To minimize this aerobic degradation of haems, which affects the pyridine haemochromogen assay, incubations were carried out under anaerobic conditions in Thunberg tubes and at the end of the incubation the remaining GSH was inactivated by adding an excess of iodoacetamide. This treatment prevented the aerobic degradation of mesohaem in the subsequent manipulations of the assay.

Interference by thiols in the determination of protohaem. When protohaem was incubated for 20min. in the presence of mercaptoethanol or cysteine at pH 8·2 and 38°, the pyridine haemochromogen subsequently prepared showed an α-peak at 552mμ instead of the usual 556mμ. No displacement occurred when protohaem was replaced by mesohaem. The shift of the α-peak to a lower wavelength could be due to an addition reaction with the vinyl groups; the α-peak of the pyridine haemochromogen of a dithiol adduct of protohaem, namely cytochrome *c*, is at 551mμ (Morton, 1958). However, the formation of such adducts under similar conditions was not detected in more extensive studies by Sano, Nanzyo & Rimington (1964). Consequently the nature of the interfering reaction is still in doubt. However, to avoid this interference we have used mesoporphyrin as substrate; this substrate has the additional advantage that it is more readily converted into the corresponding haem than is protoporphyrin (Porra & Jones, 1963*b*; Labbe *et al.* 1963).

Assay of ferrochelatase

Effect of oxygen on the formation of haem by ferrochelatase. Using a pyridine haemochromogen technique, Porra & Jones (1963*a*) found that ferrochelatase was completely inhibited in the presence of oxygen. Our present findings suggest that this so-called inhibition might reflect the total destruction of the newly formed haem under these conditions, rather than an effect of oxygen on enzyme activity. To test this hypothesis we ran the spectrophotometric assay of porphyrin disappearance under aerobic conditions and found that at the standard GSH concentration (4mM) activity was very low (Fig. 3). Some activity was observed with 20mM-GSH, but this was considerably diminished if the tubes were aerated by vigorous shaking. Consequently it is now certain that oxygen has a definite inhibitory effect on enzyme activity as well as a destructive action on the product. Three possible causes of this inhibition may be suggested: inactivation of the enzyme due to oxidation of essential thiol groups; oxidation of Fe^{2+} ion to the unusable ferric form (Porra & Jones, 1963*a*); or a more indirect effect through the production of peroxide, which, according to Mazanowska *et al.* (1966), may oxidize a lipid fraction essential for enzyme activity.

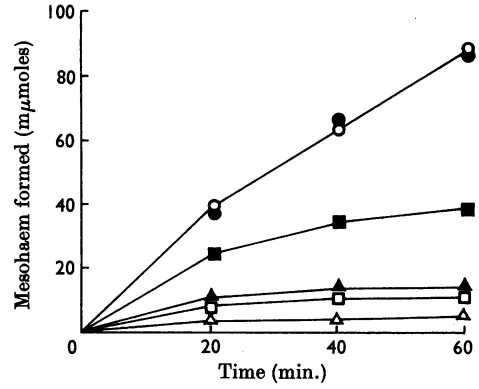

Fig. 3. Effect of GSH concentration and aerobiosis on ferrochelatase activity. Activity of a rat-liver ferrochelatase preparation (0·65mg. of protein) was measured by the spectrophotometric assay of porphyrin disappearance (see the Experimental section), but at various GSH concentrations and under anaerobic and aerobic conditions: ○, 4mM-GSH, anaerobic; □, 4mM-GSH, in open but unshaken test tubes; △, 4mM-GSH, in open shaken test tubes; ●, 20mM-GSH, anaerobic; ■, 20mM-GSH, in open but unshaken test tubes; ▲, 20mM-GSH, in open shaken test tubes.

Attention was now directed towards the possibility that residual oxygen might influence the assay of ferrochelatase by the pyridine haemochromogen method under standard anaerobic conditions (see the Experimental section). Oxygen can cause the degradation of haem compounds, and catalase can inhibit this degradation (see Fig. 1). The addition of 0·26μM-catalase did not increase the recovery of the mesohaem, but the ferrochelatase preparation itself, when assayed by the method of Sumner &

Dounce (1955), was found to contain 0·022 μM-catalase. Thus, if the concentration of residual oxygen present under standard assay conditions is high enough to cause peroxidative destruction of mesohaem, then it is clear that the endogenous catalase is sufficient to prevent such degradation, since progress curves obtained with the pyridine haemochromogen assay were almost identical with those of porphyrin disappearance (see below). It was still necessary to remove free thiol with iodoacetamide at the end of the incubation, as the endogenous catalase would be inactivated by the addition of pyridine and alkali and thus be unable to protect the mesohaemochromogen during the subsequent aerobic manipulations of the assay.

Effect of the concentration of GSH on ferrochelatase activity. When the effect of GSH concentration was measured by means of the assay of porphyrin disappearance, the spectrum of the final product varied with the initial GSH concentration. Spectra 10 in Figs. 4 and 5 are those of the final product at 20mM- and 4mM-GSH respectively and are clearly different. In each assay, however, isosbestic points were well-defined. With 20mM-GSH (Fig. 4) the spectrum of the product closely resembles that of ferrous mesohaem. At the lower concentration of GSH (Fig. 5) the spectrum is not identifiable, but it does bear some resemblance to that of ferric haemoglobin. It therefore seems likely that the product is, in this case, a complex of ferric mesohaem with some non-specific protein in the enzyme preparation. Evidence for this was obtained by incubating mesohaematin with a sample of enzyme preparation and observing an immediate shift in spectrum from that of free mesohaematin to one resembling the final product obtained in the enzyme assays. A similar shift was not obtained when plasma albumin was used in place of enzyme. It is clear from the above results that difference coefficients ($\Delta\epsilon_{mM}^{498}$) must be calculated for each concentration of GSH. Therefore, to study the effect of GSH concentrations up to 40mM on ferrochelatase activity, we employed the pyridine haemochromogen assay and the spectrofluorimetric assay of porphyrin disappearance. Two facts emerged: first, that low but variable activity was observed in the absence of GSH; secondly, that this activity increased rapidly with increase of GSH concentration until a plateau was reached. A concentration of 4mM was selected for routine assays because it was

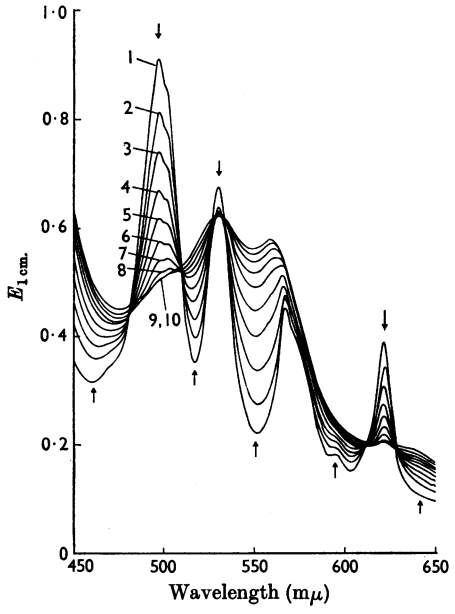

Fig. 4. Spectrophotometric demonstration of ferrochelatase activity in the presence of 20mM-GSH. Activity of a ferrochelatase preparation (0·65mg. of protein) was measured at 38° under anaerobic conditions by the spectrophotometric assay of porphyrin disappearance (see the Experimental section). Spectra 1–10 were recorded at 20min. intervals on a Cary model 14R spectrophotometer. Arrows indicate the direction of change of peaks and troughs of the porphyrin spectrum.

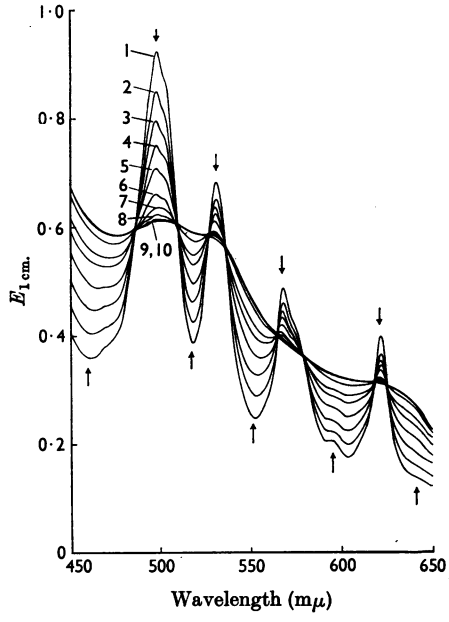

Fig. 5. Spectrophotometric demonstration of ferrochelatase in the presence of 4mM-GSH. The incubation was carried out as described in Fig. 4 with the GSH concentration lowered to 4mM. Spectra 1–10 were recorded at 20min. intervals. The significance of the arrows is explained in Fig. 4.

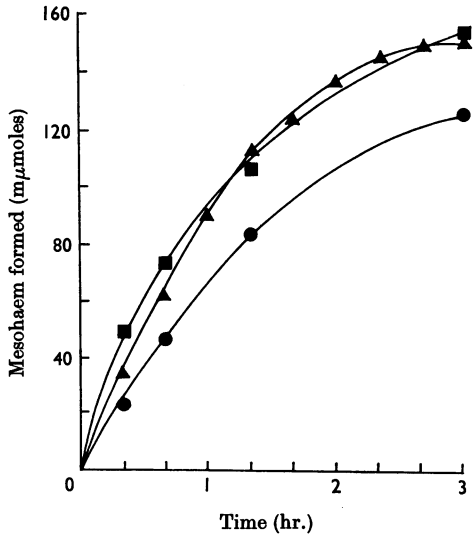

Fig. 6. Comparison of assay methods. A ferrochelatase preparation (0·65mg. of protein) was assayed by the three methods described in the Experimental section in the presence of 20mM-GSH. ■, Spectrofluorimetric assay of porphyrin disappearance; ▲, spectrophotometric assay of porphyrin disappearance; ●, pyridine haemochromogen assay.

the lowest concentration to occur consistently on the plateau.

Comparison of the assay methods. A ferrochelatase preparation was assayed simultaneously by the three methods described in the Experimental section; in addition, the assays were repeated at 20mM-GSH. Because of the instability of haem in the presence of thiols, the results obtained with the haemochromogen assay at high (20mM) GSH concentrations were still lower than those obtained with the two porphyrin disappearance assays (Fig. 6). At the lower concentration of GSH (4mM), which is used in the standard assay procedure, agreement between the haemochromogen method and the porphyrin disappearance techniques was much closer and, in fact, almost identical progress curves were obtained. Moreover, in an experiment to compare this modified assay with the original technique of Porra & Jones (1963*a*,*b*) we found the results produced by the latter method were 30% lower. However, it is noteworthy that, without taking any precautions to minimize the aerobic destruction of haem by thiols, Oyama *et al.* (1961) and Yoneyama *et al.* (1965) demonstrated a stoicheiometric relationship between porphyrin disappearance and haem formation. It is especially surprising that no haem destruction was observed by the latter workers, since their incubations were performed aerobically. It is possible that they took no precautions to prevent rapid oxidation of thiols in the presence of the Fe^{2+} ion substrate before commencing their assays (cf. Blauer & Zvilichovsky, 1965). Alternatively, it is possible that their enzyme preparations contained concentrations of catalase high enough to protect the haem from peroxidative destruction.

DISCUSSION

Degradation of haem by thiols

The protective action of catalase suggests that the aerobic destruction of free haem in the presence of thiols involves free peroxide (Fig. 1). This degradation may be related to the conversion of haemoglobin into choleglobin and bile pigments by a coupled oxidation with thiols or ascorbic acid (Lemberg & Legge, 1949). Considerable caution should be exercised in the use of thiols in conjunction with haem derivatives in aerobic conditions, and the stability of other haemoproteins, such as the cytochromes, should not be assumed in the presence of these reducing agents.

Human blood contains about 1·3mM-GSH (Jellinek & Looney, 1939) and this concentration could interfere with the determination of the haem content of blood (cf. Fig. 2). Though iodoacetamide would prevent decomposition due to thiols, it would not prevent degradation catalysed by ascorbic acid in the blood. The concentration of ascorbic acid in human blood, however, is low, in the range 0·02–0·06mM (Long, 1961).

Initial product of the ferrochelatase reaction

Though the nature of the haem derivative that accumulates in ferrochelatase incubations differs according to GSH concentration, it seems reasonable to assume that the nature of the initial product, formed at the active site of the enzyme, will be independent of thiol concentration. The presence of isosbestic points in the assays at both concentrations of GSH (Figs. 4 and 5) suggests that the initial form of haem, which might arise directly from ferrochelatase activity, is immediately converted into a final form that is characteristic of the GSH concentration. The reduction of mesohaematin to the ferrous state by GSH is an extremely slow reaction; 4μM-mesohaematin in the presence of 20mM-GSH first turns pink and the absorption spectrum reveals a broad peak near 500mμ, but the spectrum of reduced mesohaem is only obtained 2hr. later. Thus the initial product of ferrochelatase activity is probably a ferrous derivative; otherwise isosbestic points would not be expected in Fig. 4. It is suggested that this ferrous derivative is maintained in the reduced form in the presence of 20mM-

GSH, whereas with 4mM-GSH it is immediately converted into the ferric form by some oxidant present in the incubation mixture.

Evaluation of the three assays

All three assays are equally reliable under the standard conditions described in the Experimental section; however, on departure from these conditions it is necessary, when selecting the most suitable assay for a particular purpose, to consider the merits and defects of each.

Assays of porphyrin disappearance. A major advantage of all such assays is that they are unaffected by the subsequent degradation of haem in the presence of thiols. The spectrophotometric assay of porphyrin disappearance, however, suffers the disadvantage that a different $\Delta\epsilon_{mM}^{498}$ must be calculated for each GSH concentration; this disadvantage does not arise in the spectrofluorimetric assay. Other disadvantages of these methods include the failure to identify the haem formed and interference by turbidity, which limit the use of these assays to reasonably clear ferrochelatase preparations.

Assays involving measurement of haem formation. The major advantage of the pyridine haemochromogen method is that the haem formed is not only measured but also identified. This is the only method available to detect side-chain modifications that might occur, for instance, in the biosynthesis of haem *a*, haem *c* and chlorocruorohaem. Further, this assay does not require the purification of the newly formed haem, as is the case with the spectrophotometric techniques of Oyama *et al.* (1961) or with the $^{59}Fe^{2+}$-incorporation assay of Krueger *et al.* (1956) and Goldberg *et al.* (1956). The pyridine haemochromogen assay can be used with relatively crude and turbid enzyme preparations because the turbidity makes an equal contribution to both the reduced and oxidized cuvettes when the difference spectrum is being recorded. The main disadvantage of this method is the instability of haem in the presence of thiols or ascorbic acid, but this can be overcome by three precautions: by ensuring that an adequate amount of catalase is present, by using thiols rather than ascorbic acid since they can be inactivated by iodoacetamide at the end of the reaction, and by using a low GSH concentration (4mM). Although the $^{59}Fe^{2+}$-incorporation assay has not been studied in this paper, the present work suggests that these incubations should also be performed in the presence of thiols and terminated with iodoacetamide. The $^{59}Fe^{2+}$-incorporation assay, though tedious to perform, still has one advantage not shared by any of the methods described here; it can be employed in studies of haem formation by preparations, such as avian haemolysates, that contain high concentrations of endogenous haem.

The authors thank Dr J. E. Falk for encouragement and advice, Mr S. W. Thorne for carrying out spectrofluorimetric examinations and Mrs L. Grindvalds and Mrs R. Thompson for skilled technical assistance. R.F.L. was supported by a U.S. Public Health Service Special Fellowship (no. 1-F3-CM-2S,118-01) from the National Institute of General Medical Sciences and a Research Grant (no. G-19213) from the National Science Foundation.

REFERENCES

Blauer, G. & Zvilichovsky, B. (1965). *Biochim. biophys. Acta*, **110**, 215.

Falk, J. E. (1964). *Porphyrins and Metalloporphyrins*. Amsterdam, London and New York: Elsevier Publishing Co.

Goldberg, A., Ashenbrucker, H., Cartwright, G. E. & Wintrobe, M. M. (1956). *Blood*, **11**, 821.

Jellinek, E. M. & Looney, J. M. (1939). *J. biol. Chem.* **128**, 621.

Johnson, A. & Jones, O. T. G. (1964). *Biochim. biophys. Acta*, **93**, 171.

Krueger, R. C., Melnick, I. & Klein, J. R. (1956). *Arch. Biochem. Biophys.* **64**, 302.

Labbe, R. F. (1959). *Biochim. biophys. Acta*, **31**, 589.

Labbe, R. F. & Hubbard, N. (1961). *Biochim. biophys. Acta*, **52**, 130.

Labbe, R. F., Hubbard, N. & Caughey, W. S. (1963). *Biochemistry*, **2**, 372.

Labbe, R. F. & Nishida, G. (1957). *Biochim. biophys. Acta*, **26**, 437.

Lemberg, R. & Legge, J. W. (1949). In *Hematin Compounds and Bile Pigments*, p. 475. London and New York: Interscience Publishers Inc.

Long, C. (1961). In *Biochemists' Handbook*, p. 864. Ed. by Long, C. London: E. and F. N. Spon Ltd.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Mazanowska, A. M., Neuberger, A. & Tait, G. H. (1966). *Biochem. J.* **98**, 117.

Morton, R. K. (1958). *Rev. pure appl. Chem.* **8**, 161.

Oyama, H., Sugita, Y., Yoneyama, Y. & Yoshikaya, H. (1961). *Biochim. biophys. Acta*, **47**, 413.

Paul, K.-G., Theorell, H. & Åkeson, Å. (1953). *Acta chem. scand.* **7**, 1284.

Porra, R. J. & Jones, O. T. G. (1963*a*). *Biochem. J.* **87**, 181.

Porra, R. J. & Jones, O. T. G. (1963*b*). *Biochem. J.* **87**, 186.

Porra, R. J. & Jones, O. T. G. (1963*c*). In *Proc. 9th Congr. Europ. Soc. Haemat., Lisbon*, vol. 2, part 1, p. 611. Ed. by Lüdin, H. Basel and New York: S. Karger.

Porra, R. J. & Ross, B. D. (1965). *Biochem. J.* **94**, 557.

Riethmüller, G. & Tuppy, H. (1964). *Biochem. Z.* **340**, 413.

Rimington, C. (1958). *Rev. pure appl. Chem.* **8**, 129.

Sano, S., Nanzyo, N. & Rimington, C. (1964). *Biochem. J.* **93**, 270.

Sumner, J. B. & Dounce, A. L. (1955). In *Methods in Enzymology*, vol. 2, p. 780. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Yoneyama, Y., Tamai, A., Yasuda, T. & Yoshikawa, H. (1965). *Biochim. biophys. Acta*, **105**, 100.