Biochem. J. (1963) 87, 186

Studies on Ferrochelatase

2. AN INVESTIGATION OF THE ROLE OF FERROCHELATASE IN THE BIOSYNTHESIS OF VARIOUS HAEM PROSTHETIC GROUPS

By R. J. PORRA AND O. T. G. JONES
Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T., Australia

(*Received* 20 *August* 1962)

The preparation, and some of the properties, of pig-liver ferrochelatase, an enzyme system converting porphyrin and Fe^{2+} ions into haem, have been described by Porra & Jones (1963).

Though it is reasonable to assume that ferrochelatase is involved in the enzymic sequence leading to the formation of the haem moiety of haemoproteins other than haemoglobin, there is no direct evidence to support this hypothesis. The adult mammalian liver, which synthesizes haemoglobin only in certain pathological conditions, is rich in cytochromes, and the ferrochelatase activity detected in this tissue may be involved in the biosynthesis of cytochrome prosthetic groups. To obtain more conclusive evidence for the participation of ferrochelatase in the biosynthesis of haemoproteins other than haemoglobin, the occurrence of this enzyme was sought in micro-organisms. We have also tested the substrate specificity of a ferrochelatase preparation from pig liver with a number of porphyrins related to haem prosthetic groups.

EXPERIMENTAL

Materials

Hydrochloric acid solutions. Concentrations of aqueous HCl solutions are given as % (w/v) to conform with the convention in this field.

Emasol 4130. Emasol 4130 (polyoxyethylenesorbitan mono-oleate) was a gift from the Kao Soap Co., Tokyo, Japan.

Ether. Analytical grade reagent was washed with water until it was free of peroxides (cf. Weissberger, Proskauer, Riddick & Toops, 1955).

Porphyrins. The porphyrins used are defined chemically in Table 2. Coproporphyrin III, and the dimethyl esters of deuteroporphyrin IX, of mesoporphyrin IX and of monoformyldeuteroporphyrin IX, were given by Dr J. E. Falk. Chlorocruoroporphyrin, porphyrin *a*, haematoporphyrin IX dimethyl ester and a small sample of mono-β-hydroxyethylmonovinyldeuteroporphyrin dimethyl ester were given by Mr J. Barrett and Dr D. B. Morell, Royal North Shore Hospital, Sydney, Australia. Mesoporphyrin I dimethyl ester was a gift from Professor C. Rimington, University College Hospital Medical School, London, and porphyrin *c* was given by Professor H. Tuppy, Zweites Chemisches Institut, University of Vienna, Austria.

Mono-β-hydroxyethylmonovinyldeuteroporphyrin was isolated from a sample of haematoporphyrin (Fluka A. G., Basel, Switzerland). The haematoporphyrin was dissolved in aq. 0·1 N-NH_3, adjusted to pH 4·0 and extracted into ether. The porphyrins were then fractionated by extraction with 0·1% and 0·5% HCl, the second fraction was collected and a portion of the porphyrin present was esterified with HCl–methanol for comparison with an authentic sample of the dimethyl ester of mono-β-hydroxyethylmonovinyldeuteroporphyrin synthesized by Mr J. Barrett. The spectra in chloroform were identical and both samples behaved identically on paper chromatograms developed by the propanol–kerosene solvent system of Chu, Green & Chu (1951). The yield of mono-β-hydroxyethylmonovinyldeuteroporphyrin was about 27%. The free porphyrin was extracted from ether into aq. 0·5 N-NH_3–Emasol 4130 as described below.

Hydrolysis of porphyrin esters. The dimethyl esters of mesoporphyrin I, of 4-monoformyldeuteroporphyrin and of haematoporphyrin IX were hydrolysed in 20% HCl at 4° overnight. These solutions were then adjusted to pH 4 with sodium acetate and the free porphyrins were extracted into ether. The haematoporphyrin, mesoporphyrin I and monoformyldeuteroporphyrin were extracted into 0·1, 5 and 10% HCl respectively. The porphyrins were re-extracted into ether at pH 4, washed free of acid with water and then extracted into aq. 0·5 N-NH_3–Emasol 4130 as described below.

Reduced formyl derivatives of porphyrin a *and monoformyldeuteroporphyrin.* The formyl groups of these compounds were reduced to alcohol groups by the method of Clezy & Barrett (1961). The alcohol derivatives, possessing lower HCl numbers than the parent formyl compounds, were extracted from the ether in which they were formed, with 5 and 0·5% HCl respectively. After re-extraction into ether at pH 4 the solutions were washed free of acid with water. The neutral spectra were observed to have changed from the oxorhodo and rhodo type, characteristic of the two formyl compounds, to the aetio type of the reduced formyl derivatives (cf. Lemberg & Falk, 1951). The porphyrins were then extracted into aq. 0·5 N-NH_3–Emasol 4130 as described below.

Solutions of porphyrins in aq. 0·5 N-*ammonia–Emasol* 4130. Porphyrins were dissolved in aq. 0·5 N-NH_3 containing Emasol 4130, titrated to pH 7·8 with HCl and diluted with water to a concentration of 132 μm-moles of porphyrin/ml. The final concentration of Emasol 4130 was adjusted to 3% (w/v).

Solutions of the dimethyl esters of mesoporphyrin IX and deuteroporphyrin IX in dilute Emasol 4130. Small quantities of the esters were dissolved in several drops of chloroform and diluted with ether (4 ml.). After the addition of 4 ml. of aq. 1% Emasol 4130, the chloroform–ether solvent was removed under reduced pressure. Spectrophotometric

examination of the solution revealed the presence of approximately 60 μm-moles of ester/ml. in solution.

Haemins. Protohaemin, mesohaemin and deuterohaemin were donated by Dr J. E. Falk.

Micro-organisms. Chromatium D cells, grown in the autotrophic medium of Hendley (1955), were donated by Dr C. A. Appleby. *Thiobacillus X* cells, grown by the method of Trudinger (1961), were donated by Dr P. A. Trudinger. Compressed baker's yeast (*Saccharomyces cerevisiae*) was supplied by the Compressed Yeast Co. (N.S.W.) Pty. Ltd., Sydney, Australia. *Escherichia coli* was grown for 16 hr. at 37° with mechanical shaking in a medium containing 2% of Difco-Bactopeptone and 0·1% of Difco-Yeast extract. The cells were harvested and washed twice with 0·1 M-potassium phosphate buffer, pH 7·4. *Clostridium welchii* (strain no. 230–2), donated by Dr F. Lehmann-Grube, Department of Microbiology, Australian National University, was grown anaerobically for 19 hr. at 37° in a medium described by MacLennan, Mandl & Howes (1953) by using the anaerobic technique of Parker (1955). The bacteria were harvested and washed twice with 0·1 M-potassium phosphate, p 7·4.

Preparation of tissue and cell extracts. Pig-liver extract was prepared as described by Porra & Jones (1963). Disrupted bacterial preparations were prepared by passing thick suspensions of the cells through the apparatus described by Milner, Lawrence & French (1950) at 15 000–20 000 lb./in.^{2}. Unbroken cells and cell debris were removed by low-speed centrifuging. The cell-free preparations were then submitted to various treatments. *E. coli* and *Chromatium D* extracts were the supernatant solutions prepared by centrifuging the cell-free extracts for 60 min. at 16 000 rev./min. (SS-34 rotor, Servall model RC-2 centrifuge). Cell-free extracts from *Thiobacillus X* and baker's yeast were centrifuged for 60 min. at 30 000 rev./min. (30 rotor, Spinco model L centrifuge) and final extracts were prepared by extracting the pellets with Tween 20–$KHCO_3$, centrifuging and dialysing as described for the pig-liver extract (Porra & Jones, 1963). The cell-free extract of *Cl. welchii* was centrifuged for 60 min. at 30 000 rev./min. (30 rotor, Spinco model L centrifuge), and the supernatant liquid was designated the S_1 fraction. The pellet was extracted as described for the pig-liver extract and the resulting solution designated the S_2 fraction.

Methods

Determination of ferrochelatase activity. Ferrochelatase activity was assayed (see Fig. 2) by the pyridine haemochromogen method described by Porra & Jones (1963). Emasol 4130 (final concn. 1%) was added to all incubation mixtures. Except where otherwise specified the assay was used without modification.

Difference spectra of reduced and oxidized pyridine haemochromogens. Enzymically formed haems were identified and determined by reference to the difference spectra of their reduced and oxidized pyridine haemochromogen derivatives (Porra & Jones, 1963). Reference spectra were obtained (Tables 1 and 2) by using haems prepared from the corresponding porphyrins by the method of Morell, Barrett & Clezy (1961).

Solutions of meso-, proto- and deutero-haemochromogen were prepared by the method of Paul, Theorell & Åkeson (1953) from the corresponding haems and assayed by reference to the following values of ϵ_{mM} of the α-peaks: for protohaemochromogen, $\epsilon_{mM}^{557} = 32$ (de Duve, 1948); for mesohaemochromogen, $\epsilon_{mM}^{547} = 33{\cdot}2$ (Lemberg & Legge, 1949); for deuterohaemochromogen, $\epsilon_{mM}^{545} = 24{\cdot}0$. The last value was obtained by using weighed amounts of deuterohaem that had been recrystallized to give a constant ϵ_{mM}^{545}. Values for $\Delta\epsilon_{mM}$, as defined by Porra & Jones (1963), were then determined from solutions of known concentration (Tables 1 and 2). All spectra were recorded with a Bausch and Lomb Spectronic 505 spectrophotometer calibrated by reference to the Hg emission spectrum.

Table 1. *Difference spectra of reduced and oxidized pyridine haemochromogens*

Pyridine haemochromogen derived from	Absorption maxima and minima (mμ)			$\Delta\epsilon_{mM}$
	α	min.	β	($\epsilon_{mM}^{\alpha} - \epsilon_{mM}^{min.}$)
Protohaem	557	541	526	20·7
Mesohaem	547	531	518	21·7
Deuterohaem	545	530·5	515	15·3
Haematohaem	549	535	519	—
Monoformyldeuterohaem	581	553	532	—
Monohydroxymethyldeuterohaem	546	534	515	—
Mono-β-hydroxyethylmonovinyl-deuterohaem	552	536	520	—
Chlorocruorohaem	583·5	562	543	—

Table 2. *Spectra of pyridine haemochromogens*

Pyridine haemochromogen derived from	Absorption maxima (mμ)	
	α	β
Diformyldeuterohaem*	584·3	549·7
Haem *a**	587	—
Haem *c*†	551	522
Coprohaem‡	545	516

* From Lemberg & Falk (1951).
† From the pyridine haemochromogen spectrum of cytochrome *c* (cf. Morton, 1958).
‡ From Orlando (1958).

RESULTS

Effect of Emasol 4130 *on porphyrin solubility.* Many of the porphyrins used in this study are poorly soluble in aqueous media at the pH of the incubation mixture. The addition of the non-ionic detergent Emasol 4130 greatly increases porphyrin solubility. The spectra of such 'solutions' of protoporphyrin are shown in Fig. 1 (*a*). At pH 7·8 (spectrum 3) the porphyrin was only partly dissolved and close inspection revealed the presence of fine particles suspended in a highly coloured solution. In the presence of 1 % Emasol the solution was clear and the spectrum (spectrum 2) was similar to that in alkaline pyridine (spectrum 1), in which porphyrins are completely soluble. The solubility of protoporphyrin at pH 7·8 increased as the Emasol 4130 concentration increased from 0 to 1 % (Fig. 1*b*), but was unaffected in the presence of 1 % Emasol 4130 by changes of pH between 7·8 and 9·0.

Effect of Emasol 4130 *on haem formation.* The effect of various concentrations of Emasol 4130 on the formation of mesohaem and of protohaem by the pig-liver extract is shown in Fig. 2. Low (0·1 %) detergent concentrations slightly increased mesohaem formation, presumably by increasing the concentration of porphyrin in solution. At higher concentrations there is little or no stimulation. Protohaem formation was partly inhibited at the concentrations of Emasol 4130 required to increase protoporphyrin solubility substantially. Nevertheless, this detergent was useful in the specificity studies described in Table 2 since porphyrin *a*, chlorocruoroporphyrin, monoformyl-

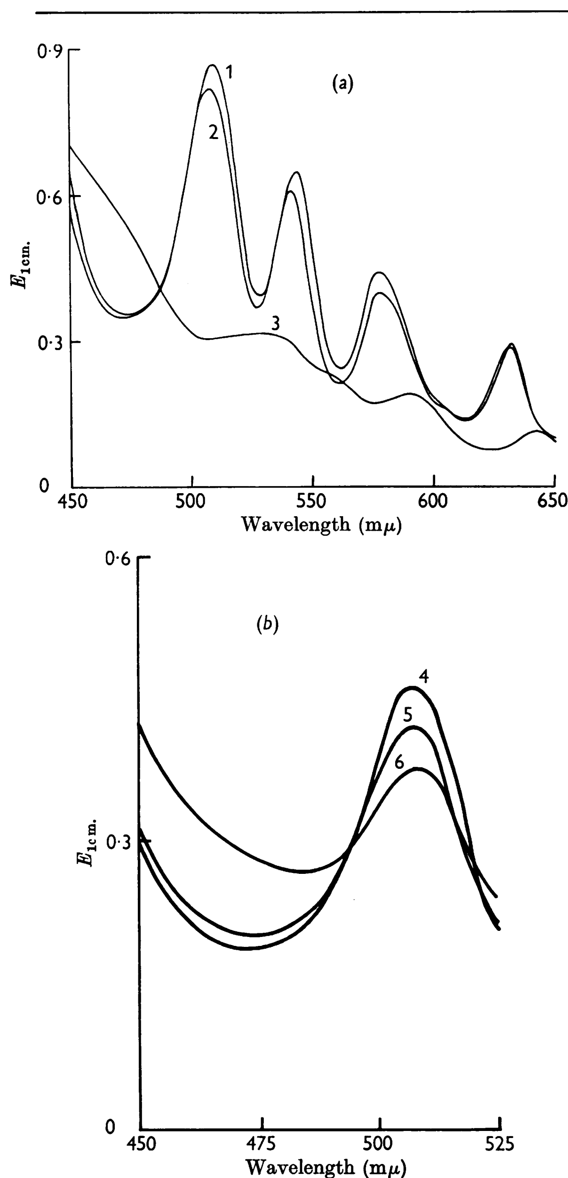

Fig. 1. Effect of Emasol 4130 on protoporphyrin spectra. (*a*) Spectra of protoporphyrin solutions (52·3 μm-moles/ml.): 1, in alkaline pyridine reagent (Paul *et al.* 1953); 2, in 1 % Emasol 4130 at pH 7·8; 3, in 0·05 M-potassium phosphate buffer, pH 7·8. (*b*) Spectra of protoporphyrin solutions (30·5 μm-moles/ml.) at pH 7·8: 4, in 1 % solution; 5, in 0·5 % solution; 6, in 0·1 % solution of Emasol 4130.

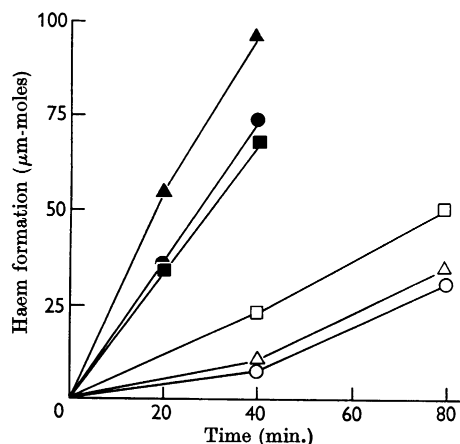

Fig. 2. Effect of Emasol 4130 concentration on enzymic haem formation. Pig-liver extract was incubated *in vacuo* in Thunberg tubes at 37° with 200 μm-moles of substrate porphyrin, 400 μm-moles of $FeSO_4$, 40 μmoles of GSH and 200 μmoles of potassium phosphate at pH 7·8, in a final volume of 4·2 ml. Ferrochelatase activity was measured by the pyridine haemochromogen assay (Porra & Jones, 1963). Mesohaem formation was measured in the presence of 1·0 ml. of extract: ■, 0 %; ▲, 0·1 %; ●, 1·0 % Emasol 4130. Protohaem formation was measured in the presence of 2·0 ml. of extract: □, 0 %; △, 0·3 %; ○, 1·0 % Emasol 4130.

deuteroporphyrin and diformyldeuteroporphyrin are even less soluble than protoporphyrin.

Specificity of the pig-liver extract. The porphyrin specificity of the ferrochelatase system in the pig-liver extract is shown in Table 3. In addition to synthetic porphyrins, several porphyrins of biological interest were tested. The extract converted protoporphyrin into protohaem, which is the prosthetic group of haemoglobins, leghaemoglobins, myoglobins, catalases, cytochromes *b* and some peroxidases. The extract acted on protoporphyrin much less rapidly than on many of the non-natural porphyrins. Likewise the extract catalysed the slow conversion of chlorocruoroporphyrin into chlorocruorohaem, which is the prosthetic group of chlorocruorin, the oxygen-carrying pigment of certain polychaete worms. Neither haem *a*, the prosthetic group of cytochrome *a*, nor haem *c*, which is related to the prosthetic group of cytochromes *c*, was formed when porphyrin *a* or porphyrin *c* was tested as substrate.

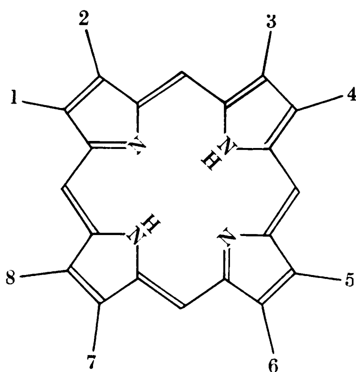

Fig. 3. Porphyrin nucleus.

Haem formation by aged extracts. Aged pig-liver extract did not convert monoformyldeuteroporphyrin into monoformyldeuterohaem (pyridine haemochromogen maxima at 581 and 532 mμ; see Tables 1 and 2) but into another haem with pyridine haemochromogen peaks at 562·5 and 526 mμ. Several explanations appear possible: there may be formation of Schiff bases between haem formyl groups and amino groups of some denatured protein, or some other modification of the side chains may occur before or after the insertion of iron into the porphyrin. The spectra obtained resemble those recorded by Lemberg & Newton (1961) for Schiff bases of formyl haems with denatured protein.

Ferrochelatase activity in micro-organisms. The results in Table 4 demonstrate the presence of

Table 3. *Porphyrin-specificity of a pig-liver extract*

Incubations were performed with pig-liver extract in the presence of 1% Emasol 4130 as described in Fig. 2. The haems formed were identified and determined by reference to the difference spectra of the reduced and oxidized pyridine haemochromogens. Haem formation is expressed as μm-moles/ml. of extract/20 min. Side-chain abbreviations: E, $-C_2H_5$; EOH, $-CH(OH)\cdot CH_3$; F, $-CHO$; H, $-H$; M, $-CH_3$; MOH, $-CH_2\cdot OH$; P, $-CH_2\cdot CH_2\cdot CO_2H$; PMe, $-CH_2\cdot CH_2\cdot CO_2CH_3$; V, $-CH{:}CH_2$; X, $-CH(OH)\cdot CH_2R'$; Y, $-CH{:}CHR''$ (Clezy & Barrett, 1961); Z, $-CH(CH_3)\cdot S\cdot CH_2\cdot CH(NH_2)\cdot CO_2H$.

	Substituents at positions 1–8*									
Porphyrin	1	2	3	4	5	6	7	8	Haem formation	pK_3†
Deutero-	M	H	M	H	M	P	P	M	182·8	5·63
Deutero- dimethyl ester	M	H	M	H	M	PMe	PMe	M	0	—
Meso- (IX)	M	E	M	E	M	P	P	M	59·3	5·94
Meso- (IX) dimethyl ester	M	E	M	E	M	PMe	PMe	M	0	—
Meso- (I)	M	E	M	E	M	P	M	P	48·2	—
Haemato-	M	EOH	M	EOH	M	P	P	M	29·0‡	5·22
Monoformyldeutero-	M	H	M	F	M	P	P	M	12·5‡	3·9
Monohydroxymethyldeutero-	M	H	M	MOH	M	P	P	M	9·5‡	—
Monohydroxyethylmonovinyldeutero-	M	EOH	M	V	M	P	P	M	6·0‡	—
Proto-	M	V	M	V	M	P	P	M	3·9	4·89
Chlorocruoro-	M	F	M	V	M	P	P	M	+§	3·04‖
Diformyldeutero-	M	F	M	F	M	P	P	M	0	2·9
Porphyrin *a*	M	X	M	Y	M	P	P	F	0	3·4
Porphyrin *a* alcohol	M	X	M	Y	M	P	P	MOH	0	—
Copro-	M	P	M	P	M	P	P	M	0	—
Porphyrin *c*	M	Z	M	Z	M	P	P	M	0	—
Pyrro-	M	E	M	E	M	H	P	M	0	—

* See Fig. 3.
† Phillips (1963).
‡ The haem formed was calculated assuming ΔE_{mM} 21·7, this being the value for the meso- derivative.
§ Accurate estimation of the small amount of chlorocruorohaem formed was not possible in the presence of endogenous protohaem.
‖ Falk & Phillips (1963).

Table 4. *Distribution of ferrochelatase*

The formation of meso- and monoformyldeutero-haem by various extracts (see the Materials section) was determined in the presence of 1% Emasol 4130 as described in Fig. 2. Only the volume of the extracts used and the period of incubation were modified as indicated in the Table. Specific activity is expressed as μm-moles of haem formed/mg. of protein/20 min. Monoformyldeuterohaem was calculated as mesohaemochromogen; $\Delta\epsilon_{mM} = 21{\cdot}7$.

Source of extract	Porphyrin substrate	Time (min.)	Enzyme (ml.)	Haem formed (μm-moles)	Concn. of protein (mg./ml.)	Specific activity
Baker's yeast	Meso-	40	1·0	89·9	15·0	3·0
E. coli	Meso-	60	2·0	23·2	18·3	0·21
Pig liver	Meso-	40	1·0	97·3	18·0	2·43
	Monoformyldeutero-	60	2·0	74·7	18·0	0·69
Thiobacillus X	Meso-	60	2·0	61·4	5·5	1·86
(stored cells)	Monoformyldeutero-	60	2·0	0	5·5	0
Thiobacillus X	Meso-	90	2·0	65·0	6·2	1·22
(fresh cells)	Monoformyldeutero-	90	2·0	32·2	6·2	0·73
Chromatium D	Meso-	60	2·0	103·8	12·9	2·68
	Monoformyldeutero-	60	2·0	0	12·9	0
Cl. welchii						
S_1 fraction	Meso-	60	1·5	0	13·5	0
S_2 fraction	Meso-	60	1·5	0	2·3	0

ferrochelatase activity in several bacterial extracts. Strictly quantitative comparisons of the activities are not possible since optimum assay conditions were not determined for each extract. Both mesoporphyrin and monoformyldeuteroporphyrin were tested as substrates for extracts from several organisms (Table 4). The results obtained with *Thiobacillus X* extracts suggest the existence in this micro-organism of two ferrochelatase systems which differ in lability and porphyrin specificity. Extracts of fresh *Thiobacillus X* cells were able to synthesize both meso- and monoformyldeuterohaem, but extracts of cells stored for 3 months at $-16°$ were able to form mesohaem only. The photosynthetic micro-organism *Chromatium D* lacks cytochromes of the *a* type (cf. Newton & Kamen, 1961) in which the prosthetic group has a formyl side chain. Although *Chromatium D* extracts catalysed the rapid insertion of iron into mesoporphyrin no incorporation into monoformyldeuteroporphyrin occurred. However, this correlation of failure to form monoformyldeuterohaem with the absence of haem *a* may not be relevant since in the synthetic porphyrin the formyl group is at position 4 and in porphyrin *a* at position 8 (Table 3).

DISCUSSION

The presence of a system for the insertion of iron into protoporphyrin in micro-organisms was implied by the demonstration of the synthesis of protohaem in *Rhodopseudomonas spheroides* (Lascelles, 1956). The previous finding of Granick & Gilder (1946) that protoporphyrin could replace protohaem as a growth requirement of *Haemophilus influenzae* could be explained by postulating a ferrochelatase reaction in this organism. Our results demonstrate the presence of ferrochelatase in *E. coli*, baker's yeast, *Thiobacillus X* and *Chromatium D*. No ferrochelatase activity was detected in *Cl. welchii*, an organism which is reputed to contain no cytochromes and possibly no other haemoproteins (cf. Newton & Kamen, 1961), although we made a more rigorous examination of extracts from this organism than of extracts from others. Extracts of *Chromatium D* and stored *Thiobacillus X* cells were able to form mesohaem but, unlike those from pig liver, were unable to convert 4-monoformyldeuteroporphyrin into the corresponding haem. It thus seems likely that ferrochelatases with different specificities occur in Nature. The effects of pH on the enzyme activity of pig-liver extract also suggest that more than one ferrochelatase is present (Porra & Jones, 1963). Protoporphyrin is less soluble than mesoporphyrin and detergent might be expected to increase its availability as substrate. However, our results show that Emasol 4130 inhibits the formation of protohaem but stimulates that of mesohaem. This may be due to the presence of two enzymes differing in susceptibility to the detergent.

Since more than one ferrochelatase may be present in the pig-liver extract, the initial rates of haem formation (Table 3) may reflect the concentrations of different enzymes with overlapping specificity, and not only the effect of various β-substituents on the mechanism of incorporation of metal ions. Thus conclusions involving comparisons of these reaction rates must be regarded as tentative pending isolation of the individual enzymes.

It is possible, however, with the information in Table 3 to deduce certain qualitative structural requirements. Only dicarboxylic porphyrins were converted into haems. Not all the dicarboxylic compounds tested, however, were active substrates. Negative results were obtained with pyrroporphyrin XV, which has only one propionic acid side chain (see Table 3), and also with the dimethyl esters of meso- and deutero-porphyrin. The tetracarboxylic porphyrins, coproporphyrin III and porphyrin *c*, were not utilized. The conversion of the series I isomer of mesoporphyrin into mesohaem shows that the vicinal grouping of the propionic acid side chains is not essential. Examination of molecular models shows, however, that the carboxyl groups of the series I and III isomers can be so orientated as to occupy identical spatial relationships,

The failure of the pig-liver extract to form haem *a* may indicate that porphyrin *a* is not involved in the biosynthesis of this prosthetic group; iron may be inserted before all side-chain modifications have occurred. Such modifications are known to occur after the insertion of Mg^{2+} ions into protoporphyrin (Tait & Gibson, 1961).

Porphyrin *c* (Neilands & Tuppy, 1960) is not utilized by the pig-liver extract. This compound could only serve as a model and it is not likely to be a true intermediate in cytochrome *c* biosynthesis. The insertion of iron would result in the formation of haem *c*, an adduct of protohaem and 2 mol.prop. of free cysteine. In cytochrome *c*, however, the two cysteine moieties form part of a polypeptide chain in the apoprotein.

The correlation between non-enzymic metalloporphyrin formation and the pK_3 of the porphyrins (Phillips, 1963) supports the view that an intermediate metal ion–porphyrin complex is formed before the removal of the two protons from the porphyrin molecule. Increased electronegativity of substituents at the periphery of the porphyrin ring increases the positive charge at the centre and diminishes the tendency to form a complex with metal ions. In Table 3 no correlation between pK_3 and enzymic haem formation is apparent, which indicates that other factors are involved in the formation of haem by pig-liver extract. One of these is the possible presence of two or more enzymes in the pig-liver extract (Porra & Jones, 1963).

SUMMARY

1. Emasol 4130, which increases the solubility of porphyrins in aqueous media at pH 7·8, was used to facilitate a study of enzymic haem formation.

2. Some reduced pyridine haemochromogens were prepared and details of the difference spectra of their reduced and oxidized forms were recorded.

3. Fresh pig-liver extract with Fe^{2+} ions converted deutero-, meso- (IX), meso- (I), haemato-, monoformyldeutero-, monohydroxymethyldeutero-, mono-β-hydroxyethylmonovinyldeutero-, proto- and chlorocruoro-porphyrin into the corresponding haems. Of the other dicarboxylic compounds tested, porphyrin *a* and diformyldeuteroporphyrin were not used. Both the tetracarboxylic compounds, coproporphyrin and porphyrin *c*, and the monocarboxylic pyrroporphyrin were likewise inactive. The dimethyl esters of mesoporphyrin IX and deuteroporphyrin were not substrates.

4. Haem formation by aged pig-liver extracts differed from that in fresh extracts.

5. Ferrochelatase activity has been found in extracts of several micro-organisms and its significance in the biosynthesis of haem prosthetic groups has been discussed.

6. Evidence has been obtained for the existence of several forms of ferrochelatase in different organisms; pig-liver extract itself may contain more than one form of the enzyme.

We thank Dr J. E. Falk for his interest and Professor A. H. Ennor and other members of the Department of Biochemistry, Australian National University, for helpful discussions. We are grateful to those who supplied us with rare chemicals and micro-organisms; we thank especially Mr J. Barrett, who undertook several difficult syntheses on our behalf. O.T.G.J. thanks Fisons Pest Control Ltd., Harston, Cambs., for financial support.

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Studies on Rat-Liver Glycylglycine Dipeptidase

BY H. G. WILCOX AND M. FRIED
Department of Biochemistry, University of Florida College of Medicine, Gainesville, Fla., U.S.A.

(*Received* 27 *August* 1962)

The metal-ion-activated enzyme found in various mammalian tissues which catalyses the hydrolysis of glycylglycine (Smith, 1948*a*; Adams, McFadden & Smith, 1952; Robinson & Shaw, 1960; Rademaker & Soons, 1957; Uzman, Rumley & Van Den Noort, 1960) has been named 'glycylglycine dipeptidase' (Smith, 1948*a*). Analogous enzymic activity has also been demonstrated in micro-organisms (Crewther & Lennox, 1953; Nishi, 1958). The glycylglycine-splitting activity demonstrable in all preparations studied can be activated by Co^{2+} ions, and in some cases by Mn^{2+} ions (Smith, 1948*a*). In contrast with the marked lability of most of the preparations described, the extracts from mammalian uterus (Smith, 1948*a*) and rat liver (M. Fried & S. M. Cain, unpublished work) are relatively stable when frozen.

The purpose of the present paper is to study and compare the glycylglycine-hydrolysing activities of rat liver in the presence and absence of bivalent metal ions, as well as in the presence of some organic substances. Such a comparison provides evidence for at least two separate glycylglycine-hydrolysing activities.

MATERIALS AND METHODS

Assay procedure. The enzymic hydrolyses were carried out at 35° in a volume of 2 ml. The solutions were 50 mM with respect to substrate, 1 mM with respect to the bivalent metal ion, and 50 mM with respect to tris buffer, giving a final pH of 8·2–8·4 throughout the activity measurement. Solutions of other substances, added in various experiments, were adjusted to pH 7 and included in the assay mixture. The enzyme was added last after preincubation of the other constituents for 20 min. at 35°. Longer preincubation did not alter the reaction rate.

The extent of hydrolysis was determined by the method of Grassman & Heyde (1929); samples of the reaction mixture were titrated at various time-intervals until hydrolysis was about 60% completed. As reported by Smith (1948*a*), the reaction was zero order to this point. The results were plotted and the rate of hydrolysis was determined graphically from the slope of the line drawn by the method of least mean squares (Brey, 1958).

Protein concentrations were determined by the biuret method (Gornall, Bardawill & David, 1949), with bovine serum albumin as the protein standard. Specific activities are expressed as μmoles of substrate hydrolysed/min./mg. of protein.

Three enzymic systems were studied: the 'residual system', assayed without added bivalent metal ions, the 'Co^{2+} ion system', assayed in the presence of Co^{2+} ions at an initial concentration of 1 mM, and the 'Mn^{2+} ion system', assayed in the presence of Mn^{2+} ions at an initial concentration of 1 mM.

Tris buffer was used because its buffer capacity is high in the pH ranges studied and because it forms metal complexes to an extent too small to affect these studies (Wehber, 1959).

Enzyme preparation. White rats (300–500 g. each) were killed by decapitation. After exsanguination, the livers were removed and homogenized for 2 min. with 3–5 vol. of 5 mM-phosphate buffer, pH 7·5, at 4°. This homogenate was then centrifuged for 20 min. at 7000g to remove cell debris. Crude homogenates prepared in this manner had specific activities ranging from 0·20 to 0·25 when measured in the Co^{2+} ion system.

Since serum contains some glycylglycine-dipeptidase activity (Smith, 1948*a*; Adams *et al.* 1952), most of the blood was removed by perfusing the liver with 0·9% sodium chloride. This was done by inserting a polyethylene tube into the portal vein of the freshly excised liver and forcing cold 0·9% sodium chloride through the tissue until it assumed an even blanched colour. After homogenization, differential centrifuging (Hogeboom, 1955) was employed to obtain nuclear, mitochondrial, microsomal and supernatant fractions. All activity was found in the supernatant, as has been reported by Rademaker & Soons (1957). The supernatant fraction, obtained after centrifuging for 3–4 hr. at 104 000g, represented a 2·5–3·0-fold increase in