- Schachman, H. K. (1957). In *Methods in Enzymology*, vol. 4, p. 32. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Shettles, L. B., Dische, Z. & Osnos, M. (1951). *J. biol. Chem.* **192**, 589.
- Smith, W., Westwood, J. C. N. & Belyavin, G. (1951). *Lancet*, ii, 1189.
- Svedberg, T. & Pederson, K. O. (1940). *The Ultracentrifuge*. Oxford University Press.
- Svennerholm, L. (1956). *Acta Soc. Med. Upsalien.* **57**, 230.
- Svennerholm, L. (1957). *Biochim. biophys. Acta*, **24**, 604.
- Tamm, I., Bugher, J. C. & Horsfall, F. L. (1955). *J. biol. Chem.* **212**, 125.
- Tamm, I. & Horsfall, F. L. (1952). *J. exp. Med.* **95**, 71.
- Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
- Watkins, W. M. (1953). *Biochem. J.* **54**, xxxiii.
- Watkins, W. M. & Morgan, W. T. J. (1957). *Nature, Lond.*, **180**, 1037.
- Weimer, H. E., Mehl, J. W. & Winzler, R. J. (1950). *J. biol. Chem.* **185**, 561.
- Werner, I. (1953). *Acta Soc. Med. Upsalien.* **58**, 1.
- Wilson, C. M. (1959). *Analyt. Chem.* **31**, 1199.
- Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.
- Yamashina, I. (1956). *Acta chem. scand.* **10**, 1666.

Biochem. J. (1961) **78**, 146

The Enzymic Formation of Haem by Human and Rat Tissues

By ANN C. LOCHHEAD AND A. GOLDBERG

University Department of Medicine, Gardiner Institute, Western Infirmary, Glasgow

(Received 25 July 1960)

Previous studies have shown that chicken erythrocytes and human reticulocytes contain iron-incorporating enzyme or enzymes, which incorporate iron into protoporphyrin to form haem (Goldberg, 1959). In the adult, the tissues predominantly concerned in haem synthesis are the bone marrow and liver. In the foetus, and in pathological states of extramedullary erythropoiesis in the adult, the liver and spleen play a major role in haem synthesis. Gibson, Neuberger & Scott (1955) have shown that the enzyme δ -amino-laevulinic dehydrase is present not only in bone marrow and liver, but also in the kidney and spleen of the rabbit. The experiments described in this paper were undertaken to determine the distribution and activities of the iron-incorporating enzyme in human and rat tissues.

Bessis & Breton-Gorius (1957) have shown by electron microscopy that iron is preferentially located in the mitochondria of the haemoglobin-forming erythroblast. These histological studies have been a stimulus to determine which intracellular component is the richest source of iron-incorporating enzyme. Ascorbic acid and reduced glutathione potentiate the enzyme system when it is derived from chicken blood. Further investigations were therefore carried out to determine the effect of these substances on the iron-incorporating enzyme derived from human and rat tissues.

MATERIALS

Radio iron ($^{59}\text{FeCl}_3$; specific activity 1–3 mc/mg. of iron) was obtained from A.E.R.E., Harwell. Protoporphyrin methyl ester, prepared from blood by the method of

Grinstein (1947), was a gift from Professor C. Rimington, F.R.S. The ester was hydrolysed with 25% HCl for 16 hr., the free protoporphyrin was dried in a desiccator, suspended in 0.142 M-sodium bicarbonate and the final solution adjusted to pH 7.4. Ascorbic acid and reduced glutathione (GSH; British Drug Houses Ltd.) were dissolved in water and the resultant solutions were adjusted to pH 7.4 before use. The water used throughout the experiments was purified by passage through an Elgastat deionizer. The glassware was washed with chromic acid, then with 2N-HCl and finally with water.

METHODS

Rat tissues. Young female albino rats, Wistar strain, were anaesthetized with ether and killed by exsanguination. Rat intestine was washed free of its contents by repeated washes with 0.9% sodium chloride solution. Each tissue was washed with water, dried on filter paper, weighed and homogenized with three or seven times its volume of 0.25 M-sucrose in an ice-cooled Potter glass homogenizer. To the homogenate was added one-quarter of its volume of 0.45 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.4. With liver homogenates, intracellular constituents were fractionated (from a 1:8 homogenate), by the method of Schneider (1948), into nuclei, mitochondria and microsomes plus cell sap. Each fraction was suspended in 0.25 M-sucrose to the same volume as the original homogenate, and 0.25 vol. of 0.45 M-tris buffer, pH 7.4, was added.

Human tissues. Samples of spleen and kidney removed from patients undergoing surgery for splenectomy or nephrectomy were treated similarly. Biopsies of normal liver were obtained from patients undergoing upper abdominal surgery. Bone-marrow samples were obtained in the following way: a 10 ml. sample of mixed bone marrow and blood was withdrawn from the iliac crest by the method of Rheingold, Weifuse & Dameshek (1949) with a heparin-

ized syringe and transferred to an ice-cooled heparinized measuring cylinder. The blood-bone-marrow tissue was haemolysed by addition of 20 ml. of water and iso-osmotic concentration was restored with 5 ml. of 0.45 M-tris, pH 7.4. The incubation was performed in 50 ml. Erlenmeyer flasks, each of which contained a portion of the material whose enzyme activity was to be measured (in 1–5 ml.), 20 μ M-protoporphyrin, 1 μ C of $^{59}\text{FeCl}_3$, 1 ml. 0.45 M-tris, pH 7.4, 2 mg. each of penicillin G and streptomycin sulphate, to prevent bacterial contamination, and 0.9% sodium chloride solution to 10 ml. Ascorbic acid and GSH when used were each contained in a volume of 1 ml. Ascorbic acid was L-ascorbic acid, unless otherwise stated. The pH of the flask contents rose during the incubation by about 0.2–0.3 pH unit. After incubation at 37° for 3 hr. with mechanical shaking (90 shakes/min.) the reaction was stopped by addition of 1 ml. of M-sodium cyanide.

Carrier haemoglobin (25 ml.) from human blood was added to each flask to facilitate isolation of haem (Goldberg, Ashenbrucker, Cartwright & Wintrobe, 1956). This carrier haemoglobin solution was prepared by haemolysing 1 vol. of washed human red cells with 1 vol. of water. Iso-osmotic concentration was restored by addition of sodium chloride to a concentration of 0.9% (w/w). The flask contents were homogenized for 3 min. in a Waring Blender and centrifuged at 1600 g for 20 min. to remove tissue debris. Haem was isolated from the supernatant fluid by the method of Fischer (1941) and purified and re-crystallized by the method of Shemin, London & Rittenberg (1950). Weighed samples of the isolated haem were counted

in a well-type scintillation counter with a counting efficiency for iron of 22%. The standard error of the counting procedure was $\pm 2\%$ and the reproducibility of the system as a whole was $\pm 5\%$.

Fig. 1. Effect of concentration of ascorbic acid on the iron-incorporating-enzyme activity of dialysed rat-liver homogenate at pH 7.4.

Fig. 2. Effect of varying concentration of reduced glutathione on the iron-incorporating-enzyme activity of dialysed rat-liver homogenate at pH 7.4.

Table 1. Ascorbic acid and reduced glutathione concentrations in rat-liver homogenate before and after dialysis against water at 4° for 22 hr.

Ascorbic acid ($\mu\text{g./g. of tissue}$)		GSH ($\mu\text{g./g. of tissue}$)	
Before dialysis	After dialysis	Before dialysis	After dialysis
161	8	2200	400
177	6	2200	600
159	4	1800	300

Table 2. Comparison of iron-incorporating-enzyme activities of intracellular constituents of rat-liver homogenate in sucrose and the effect of ascorbic acid and reduced glutathione on these activities

Incubations were carried out under standard conditions at pH 7.4 (see Methods section) with 1 μ C of $^{59}\text{FeCl}_3$ and 20 μ M-protoporphyrin, with and without the addition of ascorbic acid (2 mM) or GSH (15 mM). Control flasks were made up in the same way as the test flasks but contained no enzyme source.

Expt. no.	Tissue source	Uptake of $^{59}\text{Fe/g. of wet tissue}$ (%)		
		No addition	With ascorbic acid	With GSH
1	Homogenate	6.3	8.7	8.4
	Nuclei	0.6	1.2	0.6
	Mitochondria	4.8	12.7	6.6
	Microsomes and cell sap	0.9	3.3	1.8
	Control	<0.1	0.4	<0.1
2	Homogenate	3.5	3.8	4.0
	Nuclei	0.5	2.2	1.9
	Mitochondria	2.5	6.2	4.2
	Microsomes and cell sap	0.3	1.8	1.5
	Control	—	0.2	<0.1

Ascorbic acid and GSH contents of the homogenate were determined by the methods of Roe & Kuether (1943) and Woodward & Fry (1932) respectively. Haemoglobin concentration was measured by the alkaline-haematin method of Clegg & King (1942).

RESULTS

All results are expressed as the percentage of added radio iron which was incorporated into the haem. The total number of counts in the haem was obtained by multiplying the counts per milligram of haem by the weight of haem (mg.) in each flask after the addition of the carrier haemoglobin. In the absence of added protoporphyrin there was no significant incorporation of iron into haem by any tissue system.

Rat tissues

Liver. Ten rat-liver homogenates gave an average uptake of ^{59}Fe of 4.8 %/g. wet wt. of tissue (s.d. ± 2.4 , range 1.2–9.3 %). Addition of ascorbic acid and GSH stimulated iron incorporation into protoporphyrin. After dialysis against water at 4° for 24 hr., the enzymic activity of the homogenate was decreased to about 15 % of the original value, but addition of ascorbic acid or GSH restored the dialysate to its former activity. The GSH and ascorbic acid contents of three liver homogenates before and after dialysis are shown in Table 1. The effects of varying concentrations of ascorbic acid and reduced glutathione on dialysed rat-liver preparations are shown in Figs. 1 and 2 respectively. Maximum potentiation occurred at about 2 mm-ascorbic acid and 15 mm-GSH.

The enzyme activities of the intracellular constituents of liver isolated from a sucrose homogenate are shown in Table 2. Most of the activity is present in the mitochondrial fraction. Ascorbic acid and GSH increased the activity of all intracellular constituents.

Kidney and spleen. Kidney homogenates showed an average uptake of ^{59}Fe of 0.8 %/g. wet wt. in eight samples tested (s.d. ± 0.4 , range 0.2–1.2 %) and spleen homogenates gave a similar incorporation with a mean value of 0.6 % in six samples (s.d. ± 0.3 , range 0.3–1 %). Another tissue that showed marked enzyme activity was heart muscle, which had an average uptake of 1.6 % (s.d. ± 0.3 %) in six samples. Small intestine, brain and leg muscle had very little enzymic activity (0.1 % incorporation). Heat-treatment, i.e. placing in a boiling-water bath for 4 min., caused almost complete loss of activity in each of the tissue homogenates.

Human tissues

Bone marrow. Bone marrow showed small but significant iron-incorporating-enzyme activity in nine samples tested (Table 3). Normal human

Table 3. Iron-incorporating-enzyme activity of iliac-crest 'bone marrow-blood' tissue in normal and pathological states and the effect of ascorbic acid and reduced glutathione on this activity

Patient no.	Diagnosis	Age (years)	Sex	Hb (g./100 ml.)	P.C.V. (%)	Uptake of ⁵⁹ Fe/g. of wet tissue (%)			Marrow-blood analysis		
						Marrow alone	Marrow + ascorbic acid (1.4 mm)	Marrow + GSH (6.5 mm)	Ascorbic acid (mg./100 ml.)	GSH (mg./100 ml.)	Iron (μg./100 ml.)
1	Osteoarthritis	57	M	15.9	49.5	0.3	0.7	0.7	1.8	14	—
2	Rickets	14	M	13.0	44.5	0.5	0.5	0.5	1.2	23	108
3	Thyrototoxicosis	51	M	14.4	47.0	0.2	0.5	0.6	2.5	13	—
4	Cerebral thrombosis	57	M	15.9	47.5	0.2	0.2	—	1.2	—	83
5	Combined deficiency of vitamin B ₁₂ and folic acid	54	M	10.7	34.5	0.3	0.5	0.5	2.1	22	—
6	Bronchial carcinoma; deposits in bone marrow	62	F	13.9	42.5	0.3	1.4	1.5	0.5	6	—
7	Scurvy (treated)	58	M	8.4	25.0	0.3	0.4	0.3	0.97	19	100
8	Haemochromatosis	57	M	13.6	43.0	0.4	0.3	0.5	1.6	16	164
9	Thyrototoxicosis; iron-deficiency anaemia	59	F	11.1	39.0	0.1	0.3	—	0.96	—	70

Standard incubation procedure was followed with $1 \mu\text{C}$ of $^{59}\text{FeCl}_3$ and 20 μM -protoporphyrin with the addition where stated of 1.4 mm-ascorbic acid or 6.5 mm-GSH. Samples of the blood-bone-marrow tissue were analysed chemically for ascorbic acid, GSH and iron. Hb, Haemoglobin; P.C.V., packed-cell volume.

blood does not have significant iron-incorporating activity (Goldberg, 1959). In only one case (no. 6) did the addition of ascorbic acid or glutathione significantly increase the activity of the bone-marrow samples. In this case (a patient with bronchial carcinoma and bone-marrow metastases) the marrow ascorbic acid and glutathione concentrations were very low. The marrow from a patient with treated scurvy and a patient with haemochromatosis showed values that were within the normal range.

Liver. A sucrose homogenate of liver contained significant iron-incorporating-enzyme activity, most of which was present in the mitochondria (Table 4). The addition of ascorbic acid (0.14 mM) and GSH (6.5 mM) potentiated the activity of all the cellular constituents, whereas heat-treatment caused complete loss of activity of

the homogenate alone and of the homogenate to which ascorbic acid (0.14 mM) and GSH (6.5 mM) had been added.

Spleen. Iron-incorporating activity was found in homogenates of two human spleens (Table 5) removed from patients with acquired haemolytic anaemia and myelofibrosis respectively. Dialysis of the homogenates against water for 24 hr. decreased the activities to 10% and 22% respectively. Addition of ascorbic acid or GSH greatly potentiated the activity of the dialysate in both cases. There was histological evidence of extramedullary erythropoiesis in the spleen from the patient with myelofibrosis, but not in the spleen from the patient with acquired haemolytic anaemia. The enzymic activity per unit weight of the former was about four times that of the latter.

Table 4. *Comparison of the iron-incorporating-enzyme activities of intracellular constituents of human-liver homogenate in sucrose and the effect of ascorbic acid and reduced glutathione on these activities*

Standard procedure for incubation was followed at pH 7.4 with $1\mu\text{C}$ of $^{59}\text{FeCl}_3$ and $20\mu\text{M}$ -protoporphyrin. Ascorbic acid and GSH were added as indicated. Control flasks contained 0.9% sodium chloride solution in place of a source of enzyme.

Expt. no.	Tissue source	Uptake of $^{59}\text{Fe/g.}$ of wet tissue (%)		
		No addition	With L-ascorbic acid (0.14 mM)	With GSH (6.5 mM)
1	Homogenate	1.7	2.3*	44.4
	No enzyme source	<0.1	—	<0.1
2	Homogenate	1.2	3.5	10.9
	Mitochondria	1.0	1.0	2.8
	No enzyme source	<0.1	<0.1	—
3	Homogenate	3.4	6.8	15.3
	Nuclei	0.1	—	—
	Mitochondria	2.6	3.0	4.0
	Microsomes and cell sap	0.3	—	—
	No enzyme source	<0.1	<0.1	—

* D-Ascorbic acid gave similar incorporation.

Table 5. *Iron-incorporating-enzyme activities of samples of spleen from a case of haemolytic anaemia and from a case of myelofibrosis and of samples from two kidneys; effect on the activity of these samples of dialysis and of the addition of ascorbic acid and reduced glutathione to dialysed and undialysed samples*

Tissue samples were homogenized in sucrose and incubated with $1\mu\text{C}$ of $^{59}\text{FeCl}_3$ and $20\mu\text{M}$ -protoporphyrin under standard conditions. Control flasks containing no enzyme source gave an uptake of ^{59}Fe into haem of less than 0.1%.

Case	Tissue	Uptake of $^{59}\text{Fe/g.}$ of wet tissue (%)		
		No addition	With ascorbic acid (0.14 mM)	With GSH (6.5 mM)
Haemolytic anaemia	Spleen homogenate	1.3	1.8	2.7
	Dialysed spleen homogenate	0.1	2.0	1.2
Myelofibrosis	Spleen homogenate	5.4	12.8	15.1
	Dialysed spleen homogenate	1.2	19.6	31.1
Nephrectomy (renal calculus)	Kidney homogenate	0.7	5.7	42.2
Nephrectomy (renal neoplasm)	Kidney homogenate*	1.3	4.0	12.8

* Sample obtained from non-tumour-bearing area.

Kidney. Two samples of kidney showed iron-incorporating-enzyme activities of 0.7% and 1.3% respectively. The addition of ascorbic acid and GSH potentiated these activities (Table 5).

DISCUSSION

These experiments have demonstrated the presence of the iron-incorporating enzyme in human and rat liver, spleen and kidney and in rat-heart muscle as well as in human bone marrow. The enzyme activity is present in human tissues that synthesize haemoglobin in adult or foetal life or in pathological states of extramedullary erythropoiesis; the spleen in which this was present contained four times the enzyme activity per unit weight of that in which extramedullary erythropoiesis was absent. The distribution of the iron-incorporating enzyme in human and rat tissues is similar to the distribution of δ -aminolaevulinic dehydrase. Thus Gibson, Neuberger & Scott (1955) found the latter enzyme in the bone marrow, liver, kidney and spleen of the rabbit.

Fractionation of the cellular contents of human and rat liver demonstrated that the mitochondria were the most active source of the iron-incorporating enzyme. This finding is important in view of the work of Bessis & Breton-Gorius (1957), who showed that ferritin iron is mainly concentrated in the mitochondria of the erythroblast. Minikami (1958), using techniques substantially different from those used by us, was able to show the presence of the iron-incorporating enzyme in rat liver, kidney, spleen, heart, brain and muscle and in rat-liver mitochondria. On the other hand, Rabinowitz & Olsen (1958) have suggested that in rabbit reticulocytes the microsomes are responsible for incorporation of iron into haemoglobin.

Goldberg (1959) demonstrated that ascorbic acid and GSH in concentrations of the order present in human or chicken blood potentiate the iron-incorporating enzyme when it is derived from chicken blood. It has now been shown that these physiological reducing substances potentiate incorporation of iron in several human and rat tissues. Dialysis of tissue homogenates not only caused a marked reduction of their ascorbic acid and GSH contents (Table 1) but also significantly decreased their enzymic activities, whereas addition of ascorbic acid and GSH to the dialysed homogenates often more than restored the activities. Thus the initial loss of activity was directly

related to the loss of reducing substances. Mazur, Baez & Shorr (1955) have suggested that ascorbic acid and GSH mobilize ferritin iron for haemoglobin synthesis and the present study has certainly confirmed the important bearing of ascorbic acid and GSH on iron incorporation.

SUMMARY

1. An enzyme system that incorporates iron into protoporphyrin has been demonstrated in human and rat liver, kidney and spleen and in human bone marrow. Studies on the intracellular components of human and rat liver showed that whereas iron incorporation occurred with all fractions, those containing the mitochondria were most active.

2. Ascorbic acid and reduced glutathione potentiated the iron-incorporating-enzyme activity of all tissues. Dialysis of tissue homogenates led to loss of activity which was restored by addition of ascorbic acid or glutathione. The results confirm the important role which these substances play in the incorporation of iron.

We wish to acknowledge the technical assistance of Mr D. Middleton. This work was supported by a generous grant from the British Empire Cancer Campaign.

REFERENCES

- Bessis, M. & Breton-Gorius, J. (1957). *C.R. Acad. Sci., Paris*, **245**, 1271.
Clegg, J. & King, E. J. (1942). *Brit. med. J.* ii, 329.
Fischer, H. (1941). *Organic Synthesis*, vol. 2, p. 53. New York: John Wiley and Sons Inc.
Gibson, K. D., Neuberger, A. & Scott, J. J. (1955). *Biochem. J.* **61**, 618.
Goldberg, A. (1959). *Brit. J. Haematol.* **5**, 150.
Goldberg, A., Ashenbrucker, H., Cartwright, G. E. & Wintrobe, M. M. (1956). *Blood*, **11**, 821.
Grinstein, M. (1947). *J. biol. Chem.* **167**, 515.
Mazur, A., Baez, S. & Shorr, E. (1955). *J. biol. Chem.* **213**, 147.
Minikami, S. (1958). *J. Biochem., Tokyo*, **45**, 833.
Rabinowitz, M. & Olsen, M. E. (1958). *Nature, Lond.*, **181**, 1665.
Rheingold, J. J., Weifuse, L. & Dameshek, W. (1949). *New Engl. J. Med.* **240**, 54.
Roe, J. H. & Kuether, C. A. (1943). *J. biol. Chem.* **147**, 399.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
Shemin, D., London, I. M. & Rittenberg, D. (1950). *J. biol. Chem.* **183**, 757.
Woodward, G. E. & Fry, E. G. (1932). *J. biol. Chem.* **97**, 465.