- Henry, K. M., Kon, S. K., Lea, C. H. & White, J. C. D. (1948). *J. Dairy Res.* **15**, 292.
- Henry, K. M., Kon, S. K. & Watson, M. B. (1937). *Milk and Nutrition*, part 1, p. 37. Reading: National Institute for Research in Dairying.
- Henry, K. M., Kosterlitz, H. W. & Quenouille, M. H. (1953). *Brit. J. Nutr.* **7**, 51.
- Hughes, C. W. & Hauge, S. M. (1945). *J. Nutr.* **30**, 245.
- Hungate, R. E. (1942). *Biol. Bull., Woods Hole*, **83**, 303.
- Hungate, R. E. (1943). *Biol. Bull., Woods Hole*, **84**, 157.
- Johnson, B. C., Hamilton, T. S., Robinson, W. B. & Garey, J. C. (1944). *J. Anim. Sci.* **3**, 287.
- Loureiro, A. de (1931). *Arch. Pat., Lisboa*, **3**, 72.
- Louw, J. G. & van der Wath, J. G. (1943). *Onderstepoort J. vet. Sci.* **18**, 177.
- McNaught, M. L. & Smith, J. A. B. (1947). *Nutr. Abstr. Rev.* **17**, 18.
- McNaught, M. L., Smith, J. A. B., Henry, K. M. & Kon, S. K. (1950). *Biochem. J.* **46**, 32.
- Mangold, E. (1929). *Handbuch der Ernährung und des Stoffwechsels der Landwirtschaftlichen Nutztiere*, vol. 2, p. 161. Berlin: Springer.
- Masson, F. M. & Oxford, A. E. (1951). *J. gen. Microbiol.* **5**, 664.
- Mitchell, H. H. (1924a). *J. biol. Chem.* **58**, 873.
- Mitchell, H. H. (1924b). *J. biol. Chem.* **58**, 905.
- Mitchell, H. H. & Carman, G. G. (1926). *J. biol. Chem.* **68**, 183.
- Owen, E. C. (1946). Unpublished observations.
- Owen, E. C. (1947a). *J. Dairy Res.* **15**, 142.
- Owen, E. C. (1947b). *Nature, Lond.*, **160**, 78.
- Owen, E. C. (1951). *J. Dairy Res.* **18**, 113.
- Owen, E. C. (1954). *J. Dairy Res.* (in the Press).
- Oxford, A. E. (1951). *J. gen. Microbiol.* **5**, 83.
- Pearson, R. M. & Smith, J. A. B. (1943). *Biochem. J.* **37**, 153.
- Reed, F. M., Moir, R. J. & Underwood, E. J. (1949). *Aust. J. sci. Res. B*, **2**, 304.
- Schmid, H. (1939). *Z. Tierz. Zücht. Biol.* **43**, 239.
- Schwarz, C. (1925). *Biochem. Z.* **156**, 130.
- Smith, J. A. B. & Baker, F. (1944). *Biochem. J.* **38**, 496.
- Sugden, B. & Oxford, A. E. (1952). *J. gen. Microbiol.* **7**, 145.
- Sure, B. & House, F. (1949). *Arch. Biochem.* **20**, 55.
- Uselli, F. & Fiorini, P. (1938). *Boll. Soc. ital. Biol. sper.* **13**, 11.
- van der Wath, J. G. & Myburgh, S. J. (1941). *Onderstepoort J. vet. Sci.* **17**, 61.
- Whitehouse, K., Zarow, A. & Shay, H. (1945). *J. Ass. off. agric. Chem., Wash.*, **28**, 147.
- Williams, V. J. & Moir, R. J. (1951). *Aust. J. sci. Res. B*, **4**, 377.

Studies on the Biosynthesis of Blood Pigments

1. HAEM SYNTHESIS IN HAEMOLYSED ERYTHROCYTES OF CHICKEN BLOOD

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In recent years, the isotope tracer method has been used extensively to study the biosynthesis of haem. This has shown that glycine nitrogen probably supplies all four haem nitrogens (Wittenberg & Shemin, 1949; Muir & Neuberger, 1949), and that glycine- α -carbon is used for the four methene bridges and for one carbon atom in each pyrrole nucleus (Muir & Neuberger, 1950; Wittenberg & Shemin, 1950). The remainder of the carbon atoms can be furnished by acetate (Shemin & Wittenberg, 1951). For these studies either whole animals or intact avian erythrocytes were used. For detailed studies of the mechanism, a system without cell-permeability barriers would be of obvious advantage. The synthesis can be studied in rabbit bone marrow 'homogenates' (Altman, Salomon & Noonan, 1949) and in rabbit-spleen 'homogenates' (Altman & Salomon, 1950). It appeared to us that, if active preparations could be obtained, haemolysed avian erythrocytes should provide a particularly convenient system.

In the present work, conditions are described for the preparation, from the blood of domestic fowls,

of completely haemolysed erythrocytes which retain a high proportion of the synthetic activity of the intact cells, as measured by the use of [α - 14 C]-glycine. The rate of haem synthesis has been studied under various conditions, including the presence of some haemopoietic factors. It has also been possible to show a net increase of free porphyrin on incubation. A preliminary report of this work has been made (Falk & Dresel, 1952). Haem synthesis has been obtained in similar preparations by London & Yamasaki (1952).

MATERIALS

The blood used was taken from normal adult domestic fowls (*Gallus domesticus*) pooled for marketing. Approximately 500 ml. blood was collected into 2 ml. 0.9% NaCl, containing 15 mg. penicillin G, 15 mg. streptomycin and 20 mg. heparin, and was used the same day. In one experiment, blood from vitamin B₁₂-deficient, 4-week-old chicks, and from the corresponding controls, was used. The parent hens had been on a diet containing all-vegetable protein for about 18 months. The 'B₁₂-deficient' chicks were reared on a B₁₂-free diet (Coates, Harrison & Kon, 1949), whereas the

controls were fed 4 μ g. vitamin B₁₂/100 g. food throughout their lives. The mean weights of the ten B₁₂-deficient and ten B₁₂-supplemented chicks were 164 and 262 g., respectively. We are grateful to Dr M. Coates (National Institute for Research in Dairying, Reading) for this material.

Boiled yeast extract. Moist baker's yeast (150 g.) was added to 300 ml. boiling water, the mixture kept at boiling point for 3 min., cooled rapidly, and centrifuged. The 220 ml. supernatant were stored at -10° .

Chemicals. Succinic acid was crystallized twice from water, and the sodium salt prepared from this was crystallized twice from aqueous ethanol. α -Oxoglutaric acid and adenosine 5-phosphate (AMP) were commercial samples (Light and Co. Ltd.). Vitamin B₁₂ was 'Cytamen' (Glaxo Laboratories Ltd.). We are grateful to Dr J. Judah for samples of glutathione, diphosphopyridine nucleotide and adenosine triphosphate (ATP), and to Lederle Laboratories (New York) for generous gifts of synthetic citrovorum factor (Leucovorin), folic acid and aminopterin. All other chemicals used were of A.R. grade. Solutions were made in glass-distilled water.

METHODS

Preparation of haemolysate. The blood was centrifuged at about 2000 rev./min., and the cells were washed twice with 0.9% NaCl at 2° . After the first washing, the leucocyte layer was removed by suction. The erythrocytes from 25 ml. blood were cooled to 0° and 21 ml. water at 0° added with hand-stirring. The stirring was continued and the mixture maintained between 0° and 1° for 5 min., when 5.25 ml. 0.15 M-KH₂PO₄/Na₂HPO₄ buffer, pH 7.4, containing 0.483 M-KCl, were added. These concentrations were calculated to restore approximate isotonicity.

Microscopic examination of this preparation, including the use of phase contrast, revealed free nuclei and ghosts, but no intact erythrocytes. The absence of the latter was confirmed by centrifugation at about 2000 rev./min.; under conditions chosen for incomplete haemolysis, remaining whole erythrocytes pack down as a dense, dark layer, but after haemolysis in the way described above, no trace of this dark layer is observed. The cell solids pack into a rather diffuse opaque layer under a clear red supernatant; the latter contains occasional fragments of ghost cells and clumps of minute granules of less than 0.5 μ . diameter, which can be shown up by phase contrast or by staining with Sudan black or pyronin. We are indebted to Dr G. Christie for the microscopic examinations. The following two preparations were used: (A) The haemolysate obtained by the above procedure (34 ml. approx.) was centrifuged at 2000 rev./min. and 2° , and sufficient clear supernatant removed to leave a total volume including the solids of 21 ml. (B) 21 ml. of the whole haemolysate. This corresponds, in terms of erythrocytes, to approximately 15.5 ml. of the original whole blood. The activity of this preparation was about 15% lower than that of preparation A.

Incubation. The haemolysate was placed in 100 ml. conical flasks plugged with cotton wool, and incubated with shaking at 90–100 shakes/min. at 38° . Each flask contained 21 ml. preparation A or B; additions were made so as to maintain approximate isotonicity, and the volume made up to 33 ml. with 0.122 M-KCl. When whole washed cells were incubated, 0.154 M-NaCl (0.9%, w/v) was used for making up to volume.

Isolation and purification of porphyrin. Protoporphyrin

dimethyl ester and its Cu²⁺ complex. Protoporphyrin ester, mainly derived from the haem of haemoglobin (see discussion), was prepared by the method of Grinstein (1947) with two crystallizations from CHCl₃:methanol; further recrystallization did not alter the radioactivity. Crystalline protoporphyrin ester is often difficult to pack into planchets to give a smooth surface; preparation of the copper complex overcame this difficulty. For preparation of the Cu²⁺ complex, once-crystallized protoporphyrin ester (about 30 mg.) was dissolved in about 1 ml. hot pyridine, and about 0.5 ml. of a boiling, saturated solution of copper acetate in pyridine was added. The copper complex, which crystallized on cooling, was filtered at the pump and washed with methanol, boiling water and methanol again. It was found repeatedly that recrystallization of the Cu²⁺ complex from CHCl₃:methanol did not alter the radioactivity. For routine radioactivity determinations the crystalline Cu²⁺ complex prepared from once-crystallized porphyrin ester was used.

In one experiment, active glycine (10.6 μ C) was added to the incubated haemolysate just before precipitation with acetone (cf. Grinstein, 1947). The resulting protoporphyrin ester, crystallized once only, had zero activity, showing that free glycine was completely removed during the Grinstein procedure. Further, when boiled haemolysate was incubated with glycine (12.7 μ C), the isolated protoporphyrin ester (as the Cu²⁺ complex) had zero activity, as against 61 counts/min. in the unboiled control.

Determination of free (non-haem) porphyrins. Free copro- and proto-porphyrins were extracted essentially by the method of Schwartz & Wikoff (1952) and determined with the Beckman spectrophotometer.

Assay of radioactivity. Crystalline materials were packed in polythene planchets (1 cm.²), and the radioactivity was determined with a Geiger-Müller counter having a thin mica window. Counts were made at infinite thickness as determined experimentally for the various materials. Corrections for thickness were made where necessary, and standard errors calculated as described by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949).

Extraction of radioactive uncombined glycine from haemolysate. The protein of the haemolysate was precipitated with cold trichloroacetic acid (final concentration of acid 5%, w/v), and the supernatant obtained after centrifugation was passed through an anionic ion-exchange resin (De-acidite E). The eluate was treated with 1-fluoro-2,4-dinitrobenzene; the 2:4-dinitrophenylglycine was isolated by chromatography on Celite, essentially by the method of Perrone (1951), and crystallized from ethyl acetate-cyclohexane.

Dilution of 2:4-dinitrophenylglycine for assay of radioactivity. Dinitrophenylglycine was determined spectrophotometrically at 360 m μ . in 0.5% (w/v) aqueous NaHCO₃ (Krol, 1952). Suitable samples of carrier and radioactive materials were then mixed, the solution was acidified and the dinitrophenylglycine returned to ether for crystallization. No appreciable hydrolysis occurred during the short exposure to alkali, since subsequent chromatography on Celite showed only one band, identified as 2:4-dinitrophenylglycine. Dilution of 2:4-dinitrophenylglycine of known radioactivity by this method gave the expected result.

Microbiological assays. Vitamin B₁₂ was kindly assayed by Dr G. M. Ross, using *Euglena gracilis*; folic acid and citrovorum factor assays, with *Lactobacillus casei* and *Leuconostoc citrovorum*, respectively, were kindly performed by Dr J. Lascelles.

RESULTS

Effect of time of incubation and of glycine concentration. Fig. 1 illustrates the effect of time of incubation on the extent of incorporation of labelled glycine in haemolysates. In the presence of boiled yeast extract (see below), the synthesis fell off gradually until at 8 hr., it had practically ceased. Up to 4 hr., the mixture remained clear but after this time the formation of a gel became progressively greater. Both in the presence and absence of yeast extract the synthesis was virtually linear for 2 hr. Clearly, the amount of incorporation in 2 hr. serves as an approximate index of the initial rate of synthesis, and further effects were therefore studied on this basis.

The effect of glycine concentration on the initial rate of synthesis is shown in Fig. 2. Maximal activity was obtained with concentrations of added glycine above 0.015 M.

Endogenous glycine levels. The concentration of free glycine in a haemolysate was determined by measuring the extent of dilution of added radioactive glycine. All radioactivity measurements were made after conversion of glycine into its pure 2:4-dinitrophenyl derivative (see Methods section).

The initial endogenous glycine level in 21 ml. haemolysate *B* was found to be of the order of 3 mg. (Table 1, flask 1); after 2 hr. incubation there was no appreciable change in the apparent endogenous glycine, whether radioactive glycine was added at relatively low or high concentration (0.0013 M and 0.0065 M, cf. Fig. 2), as will be seen from flasks 2 and 3 (Table 1). Thus the radioactivity of available glycine remains virtually constant, at least during 2 hr. of incubation.

Recalculation of the results shown in Fig. 2, to allow for dilution by endogenous glycine, displaced the curve somewhat, but the saturation level was not altered appreciably.

Effect of variations in preparation on activity of haemolysates. Leucocytes were usually removed before haemolysis (see Methods); in one experiment, where they were allowed to remain, a 30% decrease in the glycine incorporation was found.

Fig. 1. Effect of time of incubation on incorporation of $[\alpha\text{-}^{14}\text{C}]$ glycine. $\bigcirc\text{---}\bigcirc$, Haemolysate *A* (21 ml.) incubated at 38° with 0.0036 M MgCl_2 , 0.01 M sodium succinate, 0.0055 M sodium acetate, 0.00036 M AMP, 0.013 M glycine ($8.54\ \mu\text{C}$) and 3.0 ml. boiled yeast extract; $\times\text{---}\times$, haemolysate *B* (21 ml.) incubated with MgCl_2 , sodium succinate and glycine as above.

Fig. 2. Effect of concentration of glycine on its incorporation. Haemolysate *B* (21 ml.) incubated 2 hr. at 38° with 0.01 M sodium succinate, 0.0036 M MgCl_2 and glycine ($0.4\ \mu\text{C}/\text{mg.}$).

Table 1. *Dilution of added radioactive glycine by endogenous glycine*

(Flasks contained 21 ml. haemolysate *B*, 0.0036 M MgCl_2 and 0.01 M sodium succinate in a final volume of 33 ml. The $[\alpha\text{-}^{14}\text{C}]$ glycine added had 570×10^3 counts/min./cm.² at infinite thickness ($0.801\ \mu\text{C}/\text{mg.}$). Flask 1 was treated immediately with trichloroacetic acid, and flasks 2 and 3 were treated similarly after incubation for 2 hr. at 38° . Glycine was isolated and its radioactivity determined as described under Methods.)

Flask	$[\alpha\text{-}^{14}\text{C}]$ Glycine added (mg.)	Time incubated (hr.)	Radioactivity of isolated glycine as % of added glycine	Calculated endogenous glycine (mg.)
1	3.2	0	57	2.41
2	3.2	2	51	3.07
3	16.0	2	85	2.82

Storage of the haemolysate at 2° for 24 hr. before incubation resulted in a loss of only 10% of the initial activity.

Different methods of restoring approximate isotonicity after haemolysis were investigated. The activities of haemolysates were similar, whether the water added for haemolysis was brought to a final concentration of 0.122M potassium chloride or 0.25M sucrose on the one hand, or 0.097M potassium chloride with 0.03M phosphate of pH 7.4, or 0.20M sucrose with 0.03M phosphate on the other. At the end of incubation, the pH of these haemolysates varied between 7.45 and 7.48, indicating that the buffering capacity of the system was sufficient even in the absence of added phosphate buffer.

Effect of various additions. Neither sodium succinate (0.01M) nor sodium α -oxoglutarate (0.01M), added separately or together, affected the incorporation of glycine significantly. The addition

of sodium acetate (0.0055M) appeared to cause slight inhibition, but the purity of this sample was not studied (Table 2). No appreciable effect was obtained with magnesium chloride (0.0036M), ferrous sulphate (0.005M), or diphosphopyridine nucleotide (0.0005M) in the presence of 0.04M nicotinamide. AMP inhibited slightly, but the purified sample of ATP gave approximately 30% stimulation.

The effect of adding boiled yeast extract is shown in Table 3. Whereas some inhibition was found with intact washed erythrocytes, the synthesis in haemolysates was stimulated about sixfold at the optimum concentration of boiled yeast extract. With further increases in concentration the stimulation decreased, the yeast preparation apparently containing a substance which inhibited at higher concentrations. The effect was not entirely consistent, and with some preparations maximum stimulation was not more than twofold.

Table 2. *Effect of various additions on incorporation of [α - 14 C]glycine in haemolysates*

(Control flasks contained 21 ml. haemolysate A or B, 0.0036M-MgCl₂, 0.01M sodium succinate, 0.0055M sodium acetate and glycine (0.0065M (6.4 μ C) in Expts. 30 and 32, 0.0065M (12.8 μ C) in Expt. 80 and 0.013M (12.8 μ C) in Expts. 42 and 44). Incubation 2 hr. at 38°. Other conditions as described in Methods section.)

Expt. no. (Preparation)	Conditions	Counts/min. of Cu ²⁺ -protoporphyrin ester	Activity as % of control (averages taken where necessary)
42 (B)	Control	56 $\pm$ 2.9	100
	MgCl ₂ omitted	48 $\pm$ 2.7, 51 $\pm$ 2.8	88
44 (B)	Control	40 $\pm$ 2.7, 49 $\pm$ 2.8	100
	Sodium acetate omitted	62 $\pm$ 3.1	139
	Sodium succinate omitted	43 $\pm$ 2.7	97
30 (A)	Control	77 $\pm$ 3.2	100
	Control + AMP (0.00036M)	73 $\pm$ 3.2	95
	Control + ATP (0.00085M)	99 $\pm$ 3.6	129
32 (A)	Control	65 $\pm$ 3.1	100
	Control + AMP (0.00036M)	46 $\pm$ 2.8, 49 $\pm$ 2.8	73
	Control + AMP + FeSO ₄ (0.005M)	47 $\pm$ 2.8	72
80 (B)	Control*	84 $\pm$ 3.4, 91 $\pm$ 3.5	100
	Control + α -oxoglutarate (0.01M)	80 $\pm$ 3.3	91
	Control + α -oxoglutarate (0.01M), succinate omitted	94 $\pm$ 3.5	107
	Control + diphosphopyridine nucleotide (0.0005M) + nicotinamide (0.04M)	87 $\pm$ 3.4	99

* Sodium acetate was omitted throughout Expt. 80.

Table 3. *Effect of boiled yeast extract on incorporation of [α - 14 C]glycine*

(Washed cells from 25 ml. blood made up to 25 ml. with 0.9% (w/v) NaCl, or 21 ml. haemolysate A, incubated 18 hr. at 38° with MgCl₂, sodium succinate and sodium acetate as in Table 2, and 0.0016M glycine (2.13 μ C). Expt. 18 also contained 0.00036M AMP. Final volume was 33 ml.)

Expt. no.	Preparation	Counts/min. of Cu ²⁺ -protoporphyrin ester	Activity as % of control
14	Washed cells	353 $\pm$ 3.8	100
	Washed cells + boiled yeast extract (1.0 ml.)	270 $\pm$ 3.4	77
18	Haemolysate	24 $\pm$ 2.3	100
	Haemolysate + boiled yeast extract (2.0 ml.)	105 $\pm$ 3.7	438
	Haemolysate + boiled yeast extract (4.0 ml.)	135 $\pm$ 4.1	563
	Haemolysate + boiled yeast extract (6.0 ml.)	80 $\pm$ 3.3	334
	Boiled yeast extract alone (2.0 ml.)	0 $\pm$ 1.7	0

2,4-Dinitrophenol (DNP) was added both to washed erythrocytes and to haemolysate preparations under conditions similar to those described in Table 3. In intact washed erythrocytes, the synthesis was inhibited 73% by 0.0004M DNP. In haemolysates, 78 and 93% inhibitions were found with 0.0001M and 0.0005M DNP, respectively.

Calcium chloride at 0.0002M inhibited 46% in haemolysates.

Changes in free porphyrin. Studies were undertaken of the changes in free (non-haem) porphyrin in haemolysates incubated with non-labelled glycine. The total free porphyrin, extractable from ethyl acetate with 10% (w/v) aqueous hydrochloric acid, increased on incubation for 2 hr. to about twice the initial level (Table 4). It was mainly protoporphyrin, coproporphyrin amounting to not more than 10%.

Effect of fluoride. Sodium fluoride was found by Gajdos & Gajdos-Török (1951) to cause a large

increase in free protoporphyrin formation in blood from phenylhydrazine-anaemic rabbits *in vitro*. We have tested its effect on haem synthesis and on free porphyrin formation. Free porphyrins were extracted and determined as described under Methods, and the porphyrin-free haemin, which remained in the ethyl acetate solution, was converted to pure protoporphyrin ester copper complex for radioactivity determinations.

In haemolysates, haem synthesis was inhibited 33 and 48% by 0.02M and 0.04M sodium fluoride, respectively (Table 5). At the low concentrations found in haemolysates, free porphyrin determinations are subject to rather large experimental error. Thus small inhibitions cannot be detected with sufficient accuracy, and the values obtained with 0.02M and 0.04M sodium fluoride were probably not significantly different from the control values. With 0.12M sodium fluoride (the concentration used by Gajdos & Gajdos-Török, 1951) there was marked inhibition (Table 5).

Table 4. *Free porphyrin synthesis in haemolysates*

(21 ml. haemolysate B incubated 2 hr. at 38° with 0.013M glycine, 0.01M sodium succinate and 0.0036M-MgCl₂; Expts. 47 and 95 also contained 0.0055M sodium acetate.)

Expt. no.		No. of replicates	Free porphyrin as protoporphyrin (μg.)	
			Mean	Range
95	Initial value	5	4.6	4.1-5.1
	After incubation	7	10.2	8.1-11.7
47	Initial value	2	5.2	4.0-6.3
	After incubation	2	10.6	10.2-11.0
54	Initial value	2	2.2	1.8-2.6
	After incubation	2	5.1	3.9-6.3

Table 5. *Effect of fluoride on haem and porphyrin synthesis*

(Whole blood: 25 ml. incubated 18 hr. at 37° with 0.055M-[α-¹⁴C]glycine (10.68 μC), final vol. 30 ml. Haemolysates: in Expt. 32, 21 ml. haemolysate A incubated 2 hr. at 38° with 0.00036M AMP, as well as additions shown in Table 2; in Expt. 60, 21 ml. haemolysate B incubated 2 hr. at 38° with 0.01M sodium succinate and 0.013M glycine.)

Expt. no.	Sodium fluoride (M)	Counts/min of Cu ²⁺ -protoporphyrin ester prepared from haem	% Inhibition of haem synthesis (averages taken where necessary)	Free porphyrin as protoporphyrin			% Inhibition of porphyrin synthesis
				No. of replicates	Mean (μg.)	Range	
Whole blood							
60	—	125 ± 3.9, 128 ± 4.0	—	3	462	435-485	—
	0.12	40 ± 2.6, 41 ± 2.7	68	2	34	—	93
62	—	155 ± 4.5	—	2	530	515-543	—
	0.12	37 ± 2.6	76	2	66	61-72	88
Haemolysate							
32	—	46 ± 2.8, 49 ± 2.8	—	—	—	—	—
	0.02	32 ± 2.5	33	—	—	—	—
	0.04	25 ± 2.4	48	—	—	—	—
60	—	—	—	1	7.2	—	—*
	0.02	—	—	3	6.1	5.6-6.3	21
	0.04	—	—	3	6.3	4.2-7.7	17
	0.12	—	—	3	2.5	2.2-2.7	90

* No initial value for free protoporphyrin was determined, and a figure of 2 μg. is assumed from experience with other experiments.

In whole blood, fluoride again inhibited both free porphyrin and haem synthesis (Table 5). The fluoride inhibition of free porphyrin formation in normal chicken blood has been confirmed by Ajdos (personal communication), the behaviour of this tissue thus standing in direct contrast to that of blood from phenylhydrazine-anaemic rabbits.

Haemopoietic factors. Vitamin B₁₂, from 0.15 to 0.6 µg./ml., made no difference to the rate of incorporation of [α -¹⁴C]glycine into the porphyrin nucleus in haemolysates. A similar result was found with haemolysates from the blood of B₁₂-deficient chicks and from control chicks fed supplementary B₁₂ (Table 6). The blood of these chicks had similar synthetic activity to that of normal adult fowls.

Haemolysates from normal chicken erythrocytes contained no measurable B₁₂ (i.e. less than 0.3 µmg./ml.). Added B₁₂ (0.1 µg./ml.) was not broken down on incubation for 2 hr.

Folic acid, in relatively high concentrations, stimulated as shown in Fig. 3. The results of the two experiments shown here agreed well, but the effect was nevertheless variable, the stimulation at 0.002M ranging in different experiments from 20 to 70 %. Ascorbic acid caused 20 % stimulation at 0.0057M, but did not potentiate folic acid (Table 7). Reduced glutathione (0.001M) had no effect.

Folinic acid, supplied as the calcium salt (Leucovorin) was found to be inhibitory, and it was found that calcium chloride at the same molarity (0.0002M) inhibited to precisely the same extent (see above). Free folinic acid was prepared from Leucovorin after precipitating the calcium ions as calcium oxalate, and was shown by microbiological assay to have the same activity as the original Leucovorin. This material had no effect whatever on the incorporation of [α -¹⁴C]glycine by haemolysates at concentrations from 1×10^{-6} M to 2×10^{-4} M.

Table 6. *Effect of vitamin B₁₂ on incorporation of [α -¹⁴C]glycine in haemolysates from B₁₂-deficient chick blood*

(For description of experimental material see Methods. 21 ml. haemolysate B incubated 2 hr. at 38° with 0.01M sodium succinate, 0.0036M-MgCl₂ and 0.013M glycine (12.8 µc).)

	Counts/min. of Cu ²⁺ -protoporphyrin ester	
	No addition	Added vitamin B ₁₂ (0.3 µg./ml.)
Blood from B ₁₂ -deficient chicks	74 ± 3.5	77 ± 3.5
Blood from control chicks	60 ± 2.7	53 ± 3.2
(B ₁₂ added to diet)	56 ± 3.2

Fig. 3. *Effect of folic acid on incorporation of [α -¹⁴C]glycine. Haemolysate A incubated under conditions described in Table 2, including 0.00036M AMP and 0.0065M glycine (6.4 µc). Results from two experiments indicated by (○) and (×).*

Table 7. *Effect of folic acid, aminopterin and ascorbic acid, on incorporation of [α -¹⁴C]glycine in haemolysates*

(21 ml. haemolysate B incubated 2 hr. at 38° with 0.01M sodium succinate, 0.0036M-MgCl₂ and 0.0065M glycine (8.54 µc).)

Expt. no.	Additions	Counts/min. of copper protoporphyrin ester	Activity as % of control (averages taken where necessary)
56	None	63 ± 3.0, 65 ± 3.0	100
	Folic acid (0.002M)	90 ± 3.4	141
	Aminopterin (0.002M)	83 ± 3.3	130
	Folic acid (0.002M) + aminopterin (0.002M)	72 ± 3.2	113
	Ascorbic acid (0.0057M)	77 ± 3.3	120
	Folic acid (0.002M) + ascorbic acid (0.0057M)	95 ± 3.5	149
58	None	66 ± 3.1, 69 ± 3.2	100
	Folic acid { (0.002M)	81 ± 3.4	120
	(0.004M)	80 ± 3.4	119
	Aminopterin { (0.002M)	62 ± 3.1	92
	(0.004M)	43 ± 2.8	64
	Folic acid (0.002M) + aminopterin (0.002M)	66 ± 3.2	98

The folic acid content of haemolysates from normal chicken erythrocytes was $0.3 \mu\text{mg./ml.}$ ($6 \times 10^{-10} \text{M}$); about 90 % of this had disappeared after 2 hr. incubation. The folinic acid content of the haemolysate was below the limit of assay. After 2 hr. incubation, recoveries of added folic acid (0.002M) and folinic acid (0.0001M) were 70 and 30 %, respectively.

Aminopterin, which is a specific antagonist of folic acid in some reactions, might have been expected to decrease the folic acid stimulation. It was found in one set of experiments that, where folic acid stimulated 40 %, aminopterin at the same concentration (0.002M) stimulated 30 %; when both were added together, 13 % stimulation was found. In another experiment, 0.002M aminopterin alone caused 8 % inhibition, increasing to 30 % at 0.004M (Table 7). The effects with aminopterin were thus very variable, and possible interpretations of the findings with this and the pterin factors are discussed below.

DISCUSSION

The study presented here is a preliminary survey of the properties of the tissue preparation; these haemolysates of chicken erythrocytes retain a considerable proportion of the capacity of the whole cells for haem synthesis. Perhaps the most interesting effect we have found is the remarkable stimulation by some samples of boiled yeast extract. We have not yet identified the responsible factor, but we calculate that at optimum boiled yeast extract concentration the activity of the haemolysate was approximately 80 % of that of whole cells. It is interesting to note the finding of Rimington (1943), that the formation of coproporphyrin during incubation of fresh baker's or brewer's yeast is stimulated by a boiled, concentrated extract of baker's yeast.

The radioactivity of protoporphyrin ester isolated by the procedure of Grinstein (1947) has been used extensively, in the experiments reported here, as an index of haem synthesis. The method does, in fact, measure mainly haem synthesis, but a small amount of the free porphyrin synthesized is also included. For critical comparison of haem and free porphyrin synthesis, a method such as that described in the experiment on the effect of fluoride (above) must be used. In a subsequent paper such methods of differential analysis will be discussed in detail.

The free porphyrin formed in our haemolysates during 2 hr. incubation amounted to $2\text{--}5 \mu\text{g./15 ml.}$ blood. In this range our experimental error was too large to allow a detailed study of the effects of inhibitors, and attempts to elucidate the effect of lead on haemopoiesis were unsuccessful. It could be shown that the synthesis of both haem and free

porphyrin was almost totally inhibited at $1 \times 10^{-4} \text{M}$ lead acetate. At $1 \times 10^{-5} \text{M}$ lead acetate, haem synthesis was inhibited by about 50 %. It would be of interest to know whether free porphyrin synthesis was inhibited to the same extent (cf. Watson, 1950), but we were unable to decide this point because of the inaccuracy of the results. Attention must be drawn to an erroneous statement in our earlier publication (Falk & Dresel, 1952). The apparent stimulation of free porphyrin formation by lead observed in preliminary experiments was later shown to have been due to an artifact, as indeed was stated when the paper was read.

The results obtained on the addition of vitamin B_{12} make it quite clear that this has no effect on haem synthesis in haemolysates of erythrocytes from either normal or B_{12} -deficient birds. The effects found with folic acid and the other pterin compounds are much more difficult to interpret. It appears that in some tissues folic acid is converted to folinic acid (citrovorum factor), which is the metabolically active factor; this conversion has been reported to be accelerated by ascorbic acid and to be inhibited by aminopterin (cf. Welch & Heinle, 1951). In our experiments, haem synthesis was stimulated by folic acid at relatively very high concentrations, but this stimulation was not increased by ascorbic acid. Aminopterin, when added with folic acid, inhibited the stimulation due to the latter, but aminopterin added alone caused quite significant stimulation in some experiments. The effect of aminopterin was variable, however, and in other experiments inhibition to below the control level was found. While the inhibition by aminopterin of the effect of added folic acid speaks in favour of the folic stimulation being due to its primary conversion to a substance either identical to, or related to folinic acid, there is a good deal of evidence against such an interpretation. The concentrations of both folic acid and aminopterin necessary to show marked effects were relatively very high, ascorbic acid did not potentiate folic acid, folinic acid did not stimulate at all, and lastly, aminopterin added alone was found to stimulate on a number of occasions.

We cannot offer a satisfactory explanation of these effects, but feel that they are artifacts, rather than suggestive of a coenzyme requirement met by any of the pterin factors. There is, of course, no certainty that the endogenous concentrations of these factors are limiting in our system, and it is possible that such requirements might show up in a more sensitive preparation. However, we incline to the view that the increased haemoglobin formation caused *in vivo* by vitamin B_{12} and folic acid (cf. Welch & Heinle, 1951) is the simple consequence of the generation of more cells capable of producing haemoglobin, and is not due to direct stimulation of the synthesis of the haemoglobin molecule.

SUMMARY

1. A preparation of completely haemolysed erythrocytes from the blood of domestic fowls, which actively incorporates [α - ^{14}C]glycine into haem, is described.

2. The effects of time and of glycine concentration on the rate of synthesis were studied.

3. Stimulation of the synthesis up to sixfold was obtained with some preparations of boiled yeast extract. 2:4-Dinitrophenol inhibited the synthesis almost completely at $5 \times 10^{-4}\text{M}$.

4. Synthesis of free protoporphyrin also occurs during incubation of the haemolysate.

5. Contrary to the findings of Gajdos & Gajdos-Török (1951), using the blood of phenylhydrazine-anaemic rabbits, sodium fluoride was found to inhibit free porphyrin synthesis both in haemolysates and in whole blood of fowls.

6. The effects of various other substances, including some erythropoietic agents, are also described.

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REFERENCES

- Altman, K. I. & Salomon, K. (1950). *Science*, **111**, 117.
 Altman, K. I., Salomon, K. & Noonan, T. R. (1949). *J. biol. Chem.* **177**, 489.
 Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*. New York: Wiley; London: Chapman and Hall.
 Coates, M. E., Harrison, G. F. & Kon, S. K. (1949). *Biochem. J.* **46**, vii.
 Falk, J. E. & Dresel, E. I. B. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 8.
 Gajdos, A. & Gajdos-Török, M. (1951). *Expos. ann. Biochim. méd.* **13**, 201.
 Grinstein, M. (1947). *J. biol. Chem.* **167**, 515.
 Krol, S. (1952). *Biochem. J.* **52**, 227.
 London, I. M. & Yamasaki, M. (1952). *Fed. Proc.* **11**, 250.
 Muir, H. M. & Neuberger, A. (1949). *Biochem. J.* **45**, 163.
 Muir, H. M. & Neuberger, A. (1950). *Biochem. J.* **47**, 97.
 Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.
 Rimington, C. (1943). *Nature, Lond.*, **151**, 393.
 Schwartz, S. & Wikoff, H. M. (1952). *J. biol. Chem.* **194**, 563.
 Shemin, D. & Wittenberg, J. (1951). *J. biol. Chem.* **192**, 315.
 Watson, C. J. (1950). *Trans. Ass. Amer. Physns*, **63**, 219.
 Welch, A. D. & Heinle, R. W. (1951). *Pharmacol. Rev.* **3**, 345.
 Wittenberg, J. & Shemin, D. (1949). *J. biol. Chem.* **178**, 47.
 Wittenberg, J. & Shemin, D. (1950). *J. biol. Chem.* **185**, 103.

Synthesis of Cellulose by *Acetobacter xylinum*

1. MICROMETHOD FOR THE DETERMINATION OF CELLULOSES*

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The use of micro-organisms in studies of cellulose synthesis is dependent on the development of suitable methods for the quantitative determination of cellulose in small samples. For this purpose, several types of approach might be considered; more especially, methods based on: (i) the colour reactions shown by carbohydrate on heating with a chromogen in the presence of strong acid, (ii) total combustion, and (iii) reductometry of a hydrolysate. In view of the relative specificity and precision of reductometric methods of sugar assay, it seemed preferable in principle to base the micromethod for cellulose on the last of the general possibilities listed above. However, the direct quantitative conversion

of cellulose into glucose by acid hydrolysis under conditions tolerated by glucose requires many hours (cf. Heuser, 1946). It will be shown in this communication that the difficulty inherent in the direct hydrolysis of cellulose fibre can be avoided if the material is first subjected to acetolytic degradation. With this means the fibre is rapidly made soluble and a product is obtained which can be hydrolysed quantitatively with relative ease. A microprocedure for the estimation of cellulose has been based on this principle.

The method detailed below has been applied extensively only to the study of the formation of cellulose by washed cell suspensions of *Acetobacter xylinum* in glucose solution (Hestrin, Aschner & Mager, 1947). It is anticipated that the procedure will also prove suitable for use with other celluloses.

* Experiments presented in this communication were performed by one of us (M.S.) in part fulfilment of requirements for a Ph.D. degree.