Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Faber, V. & Rosendal, K. (1954). *Acta path. microbiol. scand.* **35**, 159.
Jackson, R. F. & Mathews, J. A. (1932). *J. Res. nat. Bur. Stand.* **8**, 403.
Laurent, T. C. & Gergely, J. (1955). *J. biol. Chem.* **212**, 325.
Lowther, D. A. & Rogers, H. J. (1956). *Biochem. J.* **62**, 304.
MacLennan, A. P. (1956*a*). *J. gen. Microbiol.* **14**, 134.
MacLennan, A. P. (1956*b*). *J. gen. Microbiol.* **14**, 143.
McLean, D. & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
Ogston, A. G. (1953). *Trans. Faraday Soc.* **49**, 1481.
Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
Ogston, A. G. & Stanier, J. E. (1951). *Biochem. J.* **49**, 591.
Ogston, A. G. & Stanier, J. E. (1953). *Biochem. J.* **53**, 4.
Ogston, A. G. & Woods, E. F. (1954). *Trans. Faraday Soc.* **50**, 635.
Rogers, H. J. & Spensley, P. C. (1954). *Biochim. biophys. Acta*, **13**, 293.
Rogers, H. J. & Spensley, P. C. (1955). *Biochem. J.* **60**, 635.
Scott, J. E. (1955). *Chem. & Ind.* p. 168.
Snyder, C. F., Isbell, H. S., Dryden, N. R. & Holt, N. B. (1954). *J. Res. nat. Bur. Stand.* **53**, 131.
Tolksdorf, S., McCready, B. A., McCullough, D. R. & Schwenk, B. (1949). *J. Lab. clin. Med.* **34**, 74.
Wright, H. D. (1933). *J. Path. Bact.* **37**, 257.

Initial Stages in the Biosynthesis of Porphyrins

1. THE FORMATION OF δ-AMINOLAEVULIC ACID BY PARTICLES OBTAINED FROM CHICKEN ERYTHROCYTES

BY W. G. LAVER,* A. NEUBERGER AND S. UDENFRIEND†
Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2

(*Received* 28 *January* 1958)

The biosynthesis of porphyrins is thought to occur by the initial condensation of glycine with an unsymmetrical derivative of succinic acid (for review see Shemin, 1955) to form α-amino-β-oxoadipate, the latter being decarboxylated to δ-aminolaevulic acid, which is then converted through porphobilinogen into porphyrins. Observations indicating the formation of δ-aminolaevulic acid as an intermediate have been made with animal tissues (Shemin & Russell, 1953; Shemin, Russell & Abramsky, 1955; Neuberger & Scott, 1953; Berlin, Neuberger & Scott, 1956*a*, *b*; Dresel & Falk, 1953, 1956*a*, *b*) and in micro-organisms by Lascelles (1956, 1957) and Pawelkiewicz & Zodrow (1956). The evidence provided by these investigations for the formation of δ-aminolaevulic acid is quite strong, but somewhat indirect, being based largely on results obtained from isotope-dilution experiments, on a demonstration that this acid was much more active *in vitro* than glycine as a precursor of haem, and on the fact that δ-aminolaevulic acid is converted into porphobilinogen or porphyrins in a wide variety of systems (Gibson, Neuberger & Scott, 1955; Granick, 1954). A net synthesis of δ-aminolaevulic acid has not yet been demonstrated in systems *in vitro* and little evidence as to tha nature of the enzymic mechanisms involved has been obtained.

The difficulty of demonstrating a net synthesis of the acid *in vitro* is mainly due to two causes. In the first place all the animal tissues used so far in studies in biogenesis of porphyrins, such as mammalian reticulocytes, red cells from non-anaemic birds, liver and bone marrow, produce on incubation *in vitro* so little δ-aminolaevulic acid that it cannot easily be measured by available methods without the use of isotopes. Secondly, the tissues mentioned metabolize the acid fairly rapidly, so that it does not accumulate to any appreciable extent. Both these difficulties have been overcome in the present work. It was found that erythrocytes obtained from anaemic chickens synthesize δ-aminolaevulic acid from glycine and succinate or α-oxoglutarate at a fairly high rate. Moreover, a particulate fraction was obtained from the lysates of such cells which retains the capacity to synthesize δ-aminolaevulic acid if suitably fortified with cofactors, but is free of enzymes concerned with the further metabolism of this acid. Chemical work on α-amino-β-oxoadipic acid (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work) showed that this material loses carbon dioxide rapidly under physiological conditions, and in the present work we have therefore concentrated on demonstrating the formation of δ-aminolaevulic acid. For a preliminary account see Laver & Neuberger (1957).

* Holder of Studentship of the Australian Commonwealth Scientific and Industrial Research Organization.
† U.S. Public Health Research Fellow. Present address: Laboratory of Clinical Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.

EXPERIMENTAL

Materials

δ-Aminolaevulic acid (ALA) was prepared by the acid hydrolysis of ethyl α-amino-β-oxoadipate (Neuberger, Scott & Shuster, 1956) and recrystallized from dry methanol–ether. [*carboxy*-^{14}C]Glycine, [α-^{14}C]glycine and [*carboxy*-^{14}C]succinic acid were supplied by the Radiochemical Centre, Amersham, Bucks. Coenzyme A (70–75% pure), adenosine triphosphate (ATP), and triophosphopyridine nucleotide (TPN) were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., pyridoxal 5-phosphate from Roche Products Ltd., Welwyn Garden City, Herts., and diphosphopyridine nucleotide (DPN, 75% pure) from Boehringer und Soehne, Mannheim, Germany. Samples of L-penicillamine and D-penicillamine were kindly supplied by Dr S. Wilkinson of the Wellcome Chemical Laboratories, Beckenham, Kent. All other chemicals were commercial samples of A.R. grade used without further purification.

Methods

Production of anaemia and preparation of haemolysates. Some difficulty was experienced in producing a degree of anaemia required for high activity of the final preparations without losing too many birds. Phenylhydrazine hydrochloride (generally 40 mg./kg. body wt.) gave a mortality rate of about 50%. An equivalent dose of acetylphenylhydrazine gave a mortality rate of about 20%, but the degree of anaemia produced was not sufficiently severe. A combination of phenylhydrazine and of its acetyl compound was found to be most satisfactory, active preparations resulting with a mortality rate of about 10%. A mixture (2·5 ml./kg. body wt.) of equal volumes of aqueous 2% (w/v) acetylphenylhydrazine and 2% phenylhydrazine hydrochloride, neutralized with NaOH, was injected intramuscularly into white Leghorn laying hens weighing 2–3 kg. After a period of 63–64 hr., veterinary Nembutal (Abbott Laboratories, London) (30 mg./kg. body wt.) and heparin (1–5 × 10^3 units/kg.) were injected into the wing vein, the neck feathers were removed and the blood was collected into a chilled container by cutting the external jugular vein. The red cells were separated by centrifuging at 2° and washed twice with cold 0·9% NaCl, care being taken not to remove any of the light-coloured upper layer which appeared to consist largely of immature erythrocytes. The packed, washed red cells were then thoroughly chilled in ice and lysed by the addition with thorough stirring of 2 vol. of water also cooled to 0°. After 4 min. at 0° isotonicity was restored to the haemolysates by the addition of solid KCl (1·15 g./100 ml. of added water) to give a completely fluid preparation free of clots or gels of any kind.

Fractionation of the haemolysate and preparation of particles. The haemolysate was centrifuged at 3500 *g* at 0° for 30 min.; the supernatant fluid was removed and discarded unless it is otherwise stated. The residue was composed of two distinct layers, both about equal in volume. The light-coloured loosely-packed upper layer of the residue was carefully removed with a pipette and the particles were washed by centrifuging (3500 *g* for 10 min.) with cold 1·15% (w/v) KCl solution containing 2 mM-KH_2PO_4–K_2HPO_4 (pH 7·4), until the washings were free of haemoglobin (about six washings). Unless it is otherwise stated the tightly packed, dense, dark-coloured lower layer of the residue was discarded. The resulting preparation of particles was usually diluted with an equal volume of 1·15% KCl to facilitate pipetting, and any small lumps or clots which may have formed (due to contamination by the lower layer of the residue) were removed by straining through muslin. The washed particles were stored at 0° until use, but often lost their activity if kept for more than a few hours. Freezing and thawing reduced by half their capacity to form ALA from glycine and α-oxoglutarate, and completely destroyed the activity when α-oxoglutarate was replaced by succinate. In ten preparations the average volume of washed particles, packed at 3500 *g* for 10 min. in 1·15% KCl, obtained from 100 ml. of packed red cells was 65 ml.

Incubation. Samples were incubated in unstoppered conical flasks or wide-bore test tubes with shaking at about 100 shakes/min. at 38°. Anaerobic incubations were done *in vacuo* in Thunberg tubes without shaking.

Isolation of protoporphyrin. Protoporphyrin methyl ester was prepared from haemoglobin by the method of Grinstein (1947) and plated for counting as the crystalline copper complex (Dresel & Falk, 1954).

Isolation of radioactive δ-aminolaevulic acid as a pyrrole derivative from reaction mixtures containing [α-^{14}C]*glycine.* Unless it is otherwise stated, the reaction mixture, containing the haemolysate or fractionated haemolysate, was incubated with [α-^{14}C]glycine (5 μC, 0·01 M), potassium succinate (0·01 M), the appropriate cofactors, and carrier ALA (100 mg. of the hydrochloride dissolved in 0·25 M-KH_2PO_4–K_2HPO_4 buffer and adjusted to pH 7·4). When systems in which there was no metabolism of ALA were used, the carrier ALA was added at the end of the incubation; with systems which rapidly metabolized ALA, the amount of ALA remaining after incubation was determined by assay with picric acid.

To the reaction mixture after incubation trichloroacetic acid was added to give a final concentration of 5% (w/v), the precipitate of protein was centrifuged off and the supernatant was evaporated to dryness *in vacuo* over NaOH. The residue was extracted once with ether (150 ml.) and the ether extract was discarded. The residue was then dissolved in 0·25 M-Na_2HPO_4 (10 ml.) and the pH was adjusted to 7·0. ALA was then condensed with ethyl acetoacetate to give a pyrrole (Mauzerall & Granick, 1956). Ethyl acetoacetate (1 ml.) was added and the mixture heated in a boiling-water bath for 15 min. The mixture was then cooled and a small amount of precipitate removed by centrifuging. To the supernatant, non-labelled glycine was added and the mixture acidified with conc. HCl to pH 1. The precipitate of 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole which formed was filtered off and washed with a solution of glycine acidified with HCl. It was then dissolved in 2·5 N-NaOH (2 ml.) and the pyrrole again precipitated in the presence of non-labelled glycine by the addition of conc. HCl. The precipitate was filtered off, washed with a small amount of water, dissolved in methanol and transferred to a sublimation flask. After evaporation of the methanol, the pyrrole was sublimed *in vacuo* (water pump) at 160–175° over a period of about 0·5 hr. The sublimate was dissolved in the minimum amount of hot ethyl acetate, the solution was cooled to 0° and light petroleum (b.p. 40–60°) was added. The white crystalline precipitate which formed was filtered off, washed with light petroleum, air-dried and plated for counting.

Estimation of δ-aminolaevulic acid. ALA was estimated colorimetrically by condensation with acetylacetone (Mauzerall & Granick, 1956) or by reaction with picric acid (Shuster, 1956), the sensitivity and specificity of the latter method being increased by the following modifications. The 5% (w/v) trichloroacetic acid filtrate (2·0 ml.) containing up to 0·5 μmole of ALA was mixed with saturated picric acid (0·2 ml.). 2·5 N-NaOH (0·5 ml.) was then added and the mixture allowed to stand for 7 min. before the addition of 8 N-HCl (1·0 ml.). After 10 min. the excess of picric acid was removed by two extractions with ether (10 ml.) and one extraction with light petroleum (b.p. 40–60°, 10 ml.) and the colour was read in a spectrophotometer (420 mμ) against a reagent blank.

This method allowed ALA to be estimated accurately in the presence of concentrations of α-oxoglutarate up to 0·01 M.

Radioactivity measurements. Counting was done in 1 cm.² disks at infinite thickness with an end-window Geiger counter.

RESULTS

Effect of anaemia on synthesis of haem in vitro

As a system for studying the formation of ALA from glycine and succinate, haemolysates of chicken erythrocytes (Dresel & Falk, 1954) were chosen. The activity of such systems could be greatly increased if the chickens had been previously made anaemic by the injection of phenylhydrazine (Wright & Van Alstyne, 1931; Hammarsten, Thorell, Åqvist, Eliasson & Åkerman, 1953). Haemolysates of these erythrocytes (many of which were immature) synthesized haem from glycine and succinate at a much greater rate than did the haemolysates prepared from normal cells (Table 1).

Fractionation of the haemolysate

In the initial fractionation experiments it was found that on centrifuging the haemolysate (3500 *g*; 30 min.) the cell solids formed a gelatinous mass which could not readily be resuspended. However, if the haemolysate was homogenized (1–2 min. in a Measuring and Scientific Equipment Ltd. Homogeniser at 0°) before centrifuging, the residue which was then obtained could be resuspended in the supernatant (or in 1·15% KCl), the reconstituted haemolysate having lost only 16% of its activity. In later experiments it was found that by shortening the period of lysis and by keeping the temperature at 0° it was no longer necessary to homogenize the haemolysate before centrifuging. The cell solids obtained in this way could be washed with 1·15% potassium chloride solution with little loss of activity, as measured by incorporation of [α-^{14}C]glycine into haem. When recombined with the original supernatant a 5% drop in activity resulted after two washings. The supernatant alone had only 10% of the activity of the whole haemolysate.

Table 1. *Synthesis of haem in haemolysates from normal and anaemic chickens*

Haemolysates (14 ml.) were incubated in air for 2 hr. at 38° with 12 mM-[α-^{14}C]glycine (2·5 μc), 12 mM-potassium succinate, mM-$MgCl_2$ and 2 mM-$FeCl_3$, final volume of 16 ml. Protoporphyrin methyl ester was prepared from the haemoglobin and its radioactivity determined as described.

Source of haemolysate	Counts of Cu^{2+} protoporphyrin ester/min.
Normal chickens	73
Anaemic chickens	551

However, as a measure of the formation of ALA, a determination of the incorporation of [α-^{14}C]-glycine into haem was unsatisfactory for a number of reasons, and a direct estimate of the amount of ALA synthesized was sought. ALA was rapidly metabolized in the haemolysates and no means of causing its accumulation was known. However, by adding a large excess of carrier ALA before incubation and by re-isolating it at the end of the reaction as 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole (Mauzerall & Granick, 1956) a direct measure of the incorporation of [α-^{14}C]-glycine into ALA could be obtained. Further experiments on the fractionation of the haemolysate showed that the cell solids could be separated into two distinct fractions: an upper, loosely-packed light-coloured fraction (P_1), which could be readily suspended in 1·15% potassium chloride solution; and a lower, tightly-packed dark-coloured fraction (P_2), which was only resuspended with difficulty. These two fractions (P_1 and P_2) were separated and washed, and each was recombined with a portion of the supernatant (S_2). The activity for synthesis of ALA was found to be associated mainly with P_1 (Fig. 1, Table 2). The original supernatant (S_1) was found to have a small amount of activity, but this was lost completely after centrifuging at 105 000 *g* for 1 hr.

Further experiments showed that the washed upper layer of particles (P_1) would form ALA from [α-^{14}C]glycine and succinate without the addition of the supernatant, but that the activity was greatly increased in the presence of the whole supernatant (S_2) and to a less extent by a boiled extract or ultrafiltrate of the supernatant (Table 3). This discovery, that the washed particles would synthesize ALA from glycine and succinate, and that the activity could be increased by the addition of heat-stable and diffusible cofactors, was accompanied by the finding that there was no metabolism of ALA by the particles, as shown by the following experiment. When ALA was incubated at 37° with washed particles in the presence of phosphate, there was no disappearance of ALA. Thus a direct

demonstration of a net synthesis of ALA by colorimetric methods was possible. The nature of the heat-labile, non-diffusible material in the supernatant which stimulated the formation of ALA by the particles was not further investigated.

Requirements of particles for the synthesis of δ-aminolaevulic acid

Although the initial experiments on synthesis of ALA by the washed particles were carried out with glycine and succinate as substrates, it was later found that α-oxoglutarate was much more effective than succinate as a precursor of ALA (especially at low concentration), and most of the work reported here was done with α-oxoglutarate as substrate. At a concentration of 24 mM the amount of ALA formed from α-oxoglutarate was twice that formed from succinate, and at a concentration of 1·7 mM six times as much ALA was formed from α-oxoglutarate as from succinate. At low concentrations of α-oxoglutarate, in the presence of non-limiting concentrations of glycine, nearly half of the added α-oxoglutarate was converted into ALA. The optimum concentration of α-oxoglutarate was 1·7 mM, and at high concentrations synthesis of ALA was inhibited (Fig. 2). This inhibition of formation of ALA by excess of substrate was also observed when succinate was used. The washed particles apparently did not contain any endogenous glycine, α-oxoglutarate or succinate, as there was no synthesis of ALA in the presence of cofactors and in the absence of added substrates. There was also an absolute requirement for oxygen, little or no synthesis of ALA occurring under anaerobic conditions with both succinate and α-oxoglutarate (Tables 5 and 6). The conversion of glycine into ALA was less efficient than that of α-oxoglutarate, but there was no inhibition of ALA

Table 2. *Synthesis of δ-aminolaevulic acid in fractionated haemolysates from anaemic chickens*

The fractions were incubated in air for 2 hr. at 38° with 9 mM-[α-^{14}C]glycine (5 μc), 6 mM-potassium succinate, mM-$MgCl_2$ and ALA hydrochloride (100 mg.) in 0·25 M-KH_2PO_4–K_2HPO_4 (pH 7·0) (2 ml.). P_1 and P_2 were suspended in 1·15% KCl before addition. The final volume of the reaction mixture was 33 ml. At the end of the reaction the crystalline pyrrole derived from ALA was isolated and its radioactivity determined as described.

Fraction	Counts of 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole/min.
S_1 (20 ml.)	29
S_2 (20 ml.)	0
S_2 (16 ml.) + P_2 (13 ml.)	26
S_2 (16 ml.) + P_1 (13 ml.)	125

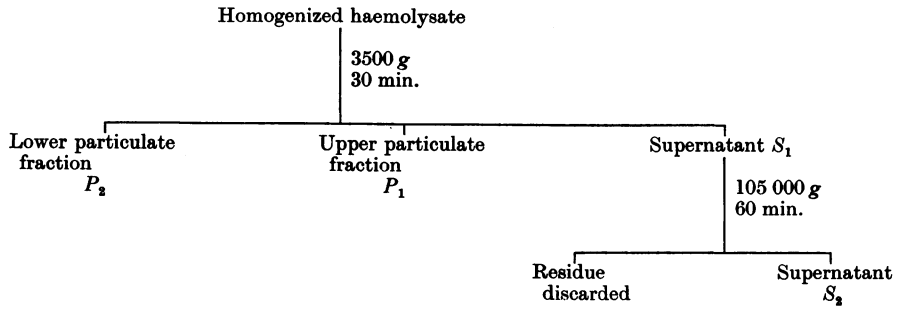

Fig. 1. Fractionation of haemolysates of immature chicken erythrocytes.

Table 3. *Formation of δ-aminolaevulic acid by washed particles* (P_1)

The fractions to be tested were incubated in air at 38° for 2 hr. with 13 mM-[α-^{14}C]glycine (5 μc), 10 mM-potassium succinate, 2 mM-$MgCl_2$, and ALA hydrochloride (100 mg.) in 0·25 M-K_2HPO_4–KH_2PO_4 (pH 7·3) (1 ml.). In the first series of experiments 8 ml., and in the second series 10 ml., of washed particles (prepared as described under Methods) were used. The additions made are described in the table. The boiled supernatant was prepared by heating S_2 to 100° for 2 min., centrifuging and taking the supernatant. The ultrafiltrate was prepared by centrifuging S_2 in a cellophan bag supported on a layer of glass beads in a centrifuge tube (3000 g; 1 hr.). The crystalline pyrrole derived from ALA was isolated at the end of the reaction and its radioactivity determined.

Expt.	Additions	Series 1 (ml.)	Series 2 (ml.)	Counts of 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole/min. Series 1	Series 2
1	1·15% KCl	10	15	28	38
2	Supernatant (S_2)	10	15	120	126
3	Ultrafiltrate of S_2	10	—	46	—
4	Boiled S_2	—	15	—	52

synthesis by excess of glycine. The optimum concentration of glycine was above 67 mM (Fig. 3) and for most experiments a concentration of 33 mM was used. The effect of concentration of enzyme on formation of ALA is shown in Fig. 4, and the effect of time of incubation in Fig. 5.

The optimum pH for the system with oxoglutarate was 7·2 (Fig. 6). In the presence of borate the optimum pH was similar, but activity was greatly reduced. A similar decrease in activity was also obtained, when phosphate was replaced by tris buffer (Table 4). With succinate as substrate, substitution of phosphate by tris buffer reduced activity by 80–90%, whereas replacement of phosphate by barbitone produced a less severe inhibition. With barbitone the inhibition was almost completely reversed by addition of phosphate, but addition of phosphate to mixtures

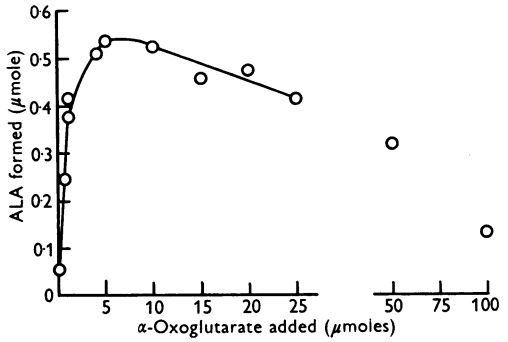

Fig. 2. Effect of concentration of α-oxoglutarate on synthesis of δ-aminolaevulic acid. Particles (1·5 ml.) were incubated in air for 1·5 hr. at 38° with 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-K_2HPO_4–KH_2PO_4 (pH 7·4), coenzyme A (0·05 mg.), pyridoxal phosphate (0·05 mg.) and potassium α-oxoglutarate. The volume was made up to 3·0 ml. with 1·5% KCl. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

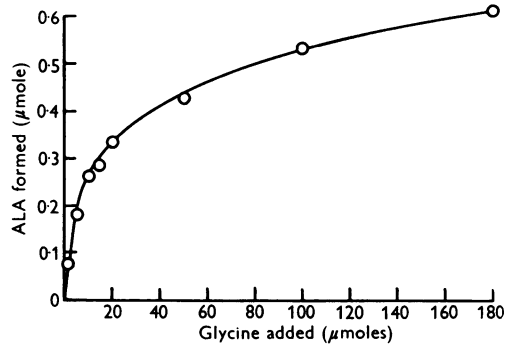

Fig. 3. Effect of concentration of glycine on synthesis of δ-aminolaevulic acid. Particles (1·5 ml.) were incubated in air for 1·5 hr. at 38° with 0·0017 M-potassium α-oxoglutarate, 0·0017 M-$MgCl_2$, 0·05 M-K_2HPO_4–KH_2PO_4 (pH 7·4), coenzyme A (0·05 mg.), pyridoxal phosphate (0·05 mg.) and glycine. The volume was made up to 3·0 ml. with 1·15% KCl. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

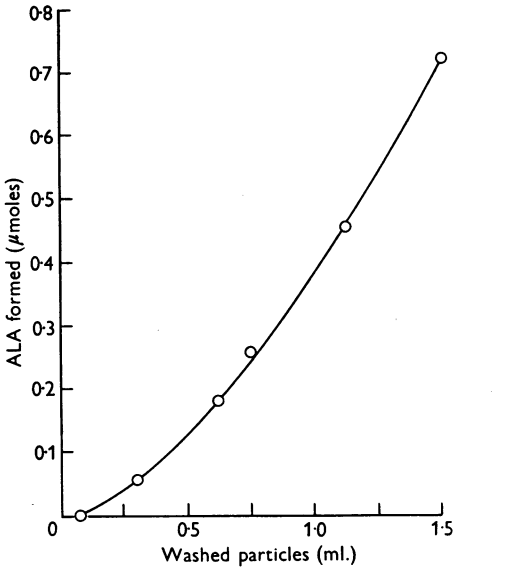

Fig. 4. Effect of concentration of enzyme on formation of δ-aminolaevulic acid. Particles were incubated in air for 1·5 hr. at 38° with 0·0017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·05 mg.). The volume was made up to 3·0 ml. with 1·15% KCl. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

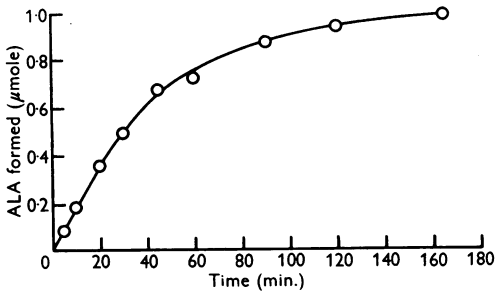

Fig. 5. Effect of time of incubation on formation of δ-aminolaevulic acid. Particles (1·44 ml.) were incubated in air at 38° with 0·0017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·05 mg.). The volume was made up to 3·0 ml. with 1·15% KCl. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

containing tris buffer resulted in only 50% reactivation (Table 4).

The effects of various cofactors on the formation of ALA from glycine and oxoglutarate or succinate by the particles are shown in Tables 5 and 6. Duplicates for experiments carried out with the same enzyme preparations agreed within ±4% but different preparations did not behave in an identical manner with regard to activation by pyridoxal phosphate, coenzyme and $MgCl_2$. The omission of coenzyme A, pyridoxal phosphate or magnesium chloride caused a decrease in synthesis of ALA by the particles. The effects of other substances are shown in Table 7. Ethylenediaminetetra-acetic acid (EDTA) in the absence of magnesium also caused a considerable activation. Cyanide and the sulphydryl reagents, *p*-chloromercuribenzoate and iodoacetamide were very strongly inhibitory, but 2:4-dinitrophenol and sodium azide in concentrations which are stated to uncouple oxidative phosphorylation completely (Hunter, 1955) were virtually without effect. Glutathione, in both the presence and the absence of coenzyme A, was slightly inhibitory and nicotinamide and sodium fluoride were without significant effect.

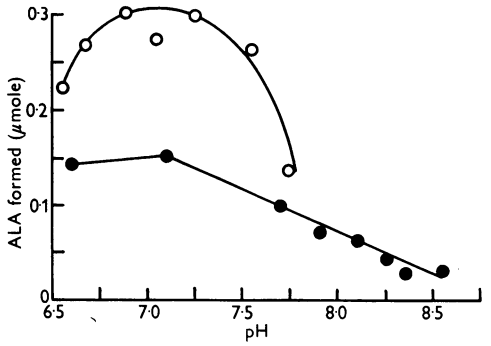

Fig. 6. Effect of pH on formation of δ-aminolaevulic acid. Particles (1·0 ml.) were incubated in air at 38° for 1·5 hr. with 0·017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, coenzyme A (0·05 mg.), pyridoxal phosphate (0·05 mg.) and 0·05 M-KH_2PO_4–K_2HPO_4 (○) or a mixture of 0·05 M-boric acid–potassium borate and 0·01 M-KH_2PO_4–K_2HPO_4 (●). The volume was made up to 3 ml. with 1·15% KCl. The pH of the reaction mixtures were checked with the glass electrode before incubation. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

The effects of other substances on the formation of ALA from glycine and succinate or α-oxoglutarate was also studied. Under aerobic conditions and in the presence of phosphate the addition of DPN, TPN, glucose, glucose 6-phosphate, fructose 1:6-diphosphate, D-3-phosphoglycerate and ATP produced very slight and variable results. In the absence of oxygen DPN had a slight, but definite, activating effect.

Effect of penicillamine

Because of the finding of du Vigneaud, Kuchinskas & Horvath (1957) that L-penicillamine inhibited rat-liver alanine–glutamic and aspartic–glutamic transaminase activities *in vitro* and that this inhibition could be reversed by the addition of pyridoxal phosphate, the effect of L-penicillamine and D-penicillamine on the synthesis of ALA by the washed particles was investigated. The results

Table 4. *Effects of various buffers on synthesis of δ-aminolaevulic acid from glycine and succinate or α-oxoglutarate by washed particles*

The system contained suspension of particles (1 ml.), 16 mM-potassium succinate or 16 mM-potassium α-oxoglutarate, 40 mM-glycine, 2 mM-$MgCl_2$, coenzyme A (0·05 mg.) and pyridoxal phosphate (0·1 mg.). The buffers used were: (*a*) 0·05 M-KH_2PO_4–K_2HPO_4, pH 7·4; (*b*) sodium barbitone–HCl, pH 7·4; (*c*) 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl, pH 7·6. The incubations were carried out at 38° for 1 hr. in air.

Expt. no.	Substrate	Buffer	Activity (μmole of ALA/ml. of particles/hr.)
1	Succinate	0·05 M-Phosphate	0·077
	Succinate	0·05 M-Tris	0·010
2	Succinate	0·05 M-Phosphate	0·12
	Succinate	0·05 M-Barbitone	0·07
3	Succinate	0·05 M-Phosphate	0·14
	Succinate	0·05 M-Barbitone	0·04
	Succinate	0·05 M-Barbitone + 0·05 M-phosphate	0·12
4	Succinate	0·05 M-Phosphate	0·11
	Succinate	0·05 M-Tris	0·013
	Succinate	0·05 M-Tris + 0·05 M-phosphate	0·063
5	Succinate	0·05 M-Phosphate	0·091
	Succinate	0·05 M-Tris	0·021
	α-Oxoglutarate	0·05 M-Phosphate	0·174
	α-Oxoglutarate	0·05 M-Tris	0·071

Table 5. *Cofactor requirements for synthesis of δ-aminolaevulic acid from glycine and α-oxoglutarate by washed particles*

Control tubes contained particles, 17 mM-potassium α-oxoglutarate, 33 mM-glycine, 17 mM-$MgCl_2$, 50 mM-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·05 mg.). The volume was made up to 3·0 ml. with 1·15% KCl. The tubes were incubated in air at 38° for 1·5 hr. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid. Substances omitted from the control are shown below.

Omissions	Activity (μmole of ALA/ml. of packed particles/1·5 hr.) Test system	Control system	Test system as percentage of control (averaged where applicable)	
Pyridoxal phosphate	0·300	0·359	83	
Pyridoxal phosphate and coenzyme A	0·211	0·359	59	
Coenzyme A	0·303	0·359	84	85
	0·474	0·520	91	
	0·647	0·799	81	
$MgCl_2$	0·435	0·570	76	71
	0·522	0·799	65	
Air	0·014	0·432	3	5
	0·057	0·799	7	

Table 6. *Cofactor requirements for synthesis of δ-aminolaevulic acid from glycine and succinate by washed particles*

The complete system contained suspension of particles (1 ml.), 16 mM-potassium succinate, 40 mM-glycine, 2 mM-$MgCl_2$, 50 mM-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·1 mg.). The volume was made up to 2·5 ml. with 1·15% KCl. The tubes were incubated at 38° for 1 hr. Omissions are indicated below. The reaction was stopped by adding 20% trichloroacetic (1 ml.) and ALA estimated by the picric acid method.

Omissions	Activity (μmole of ALA/ml. of particles/hr.) Test system	Control system	Test system as percentage of control
Pyridoxal phosphate	0·065	0·080	81
Coenzyme A	0·052	0·080	65
Coenzyme A	0·11	0·16	69
$MgCl_2$	0·026	0·080	33
$MgCl_2$	0·10	0·16	63
Succinate	0·000	0·080	0
Succinate	0·03	0·16	17
Air	0·015	0·14	11

(Table 8) showed that L-penicillamine caused a marked decrease in the amount of ALA formed from glycine and α-oxoglutarate, whereas D-penicillamine had only a slight effect. du Vigneaud *et al.* (1957) had also observed that D-penicillamine had only slight activity as inhibitor on transaminases compared with the L-isomeride. The addition of increasing amounts of pyridoxal phosphate to the reaction mixture containing L-penicillamine caused a slight reversal of this inhibition. The reversing effect of pyridoxal phosphate is slight and is smaller than that found by du Vigneaud *et al.* (1957) for transaminase.

Effect of S-*succinyl-coenzyme A*

Succinyl-CoA was prepared from 5 mg. of coenzyme A and 0·43 mg. of succinic anhydride according to Simon & Shemin (1953). A solution containing nominally 1·5 μmoles/ml. was used; on incubation at 0° for 30 min. of 0·5 ml. of this solution with 0·1 ml. of neutralized 2·4 M-hydroxylamine hydrochloride a strongly positive hydroxamic acid reaction with ferric chloride was obtained. A control sample containing succinic anhydride, but no CoA, gave a negative hydroxamic acid reaction under these conditions. Incubation of a suspension of particles (1 ml.) with succinate, glycine, magnesium chloride, pyridoxal phosphate and coenzyme A under standard conditions (Table 6) gave an ALA synthesis of 1·8 μmoles/ml.; addition of succinyl-CoA solution (0·5 ml.) for this system resulted in a slight reduction in synthesis of ALA (0·165 μmole/ml.). When succinate and coenzyme A were replaced by succinyl-CoA there was no significant amount of ALA synthesis. In another experiment there was slight synthesis of ALA under such conditions.

Decarboxylation of glycine by the washed particles

In order to investigate the association of the decarboxylation of glycine with its condensation with the active succinyl derivative, the liberation of radioactive carbon dioxide from [*carboxy*-^{14}C]-glycine by the particles was examined in the presence and absence of α-oxoglutarate.

Table 7. *Effects of various substances on the synthesis of δ-aminolaevulic acid from glycine and α-oxoglutarate by washed particles*

Control tubes contained particles, 0·0017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4), coenzyme A (0·05 mg.) and pyridoxal phosphate (0·05 mg.). The volume was made up to 3·0 ml. with 1·15% KCl. The tubes were incubated in air at 38° for 1·5 hr. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid. Substances added to the control are shown below.

Additions	Activity (μmole of ALA/ml. of packed particles/1·5 hr.) Test system	Control system	Test system as percentage of control
KCN (0·5 mM)	0·000		0
KCN (0·1 mM)	0·122		23
KCN (0·01 mM)	0·467		90
2:4-Dinitrophenol (0·33 mM)	0·446		86
2:4-Dinitrophenol (0·66 mM)	0·419		81
2:4-Dinitrophenol (mM)	0·426		82
Sodium azide (2 mM)	0·467	0·520	90
Sodium azide (4 mM)	0·412		79
Sodium azide (6 mM)	0·412		79
$CaCl_2$ (0·2 mM)	0·419		81
Glutathione (10 mM)	0·495		95
Sodium fluoride (10 mM)	0·537		103
Sodium fluoride (mM)	0·579		111
EDTA (mM; $MgCl_2$ omitted)	0·826	0·522*	158
EDTA (0·25 mM; $MgCl_2$ omitted)	0·840		161
p-Chloromercuribenzoate (mM; CoA omitted)	0·016		2
p-Chloromercuribenzoate (0·25 mM; CoA omitted)	0·064	0·647†	10
Iodoacetamide (2 mM; CoA omitted)	0·164		25
Iodoacetamide (0·5 mM; CoA omitted)	0·340		53
Nicotinamide (10 mM)	0·624	0·605	103
None	—	0·392‡	—

* $MgCl_2$ omitted from control system.
† Coenzyme A omitted from control system.
‡ ALA estimated by condensation with acetylacetone (pyridoxal phosphate omitted in this experiment).

Table 8. *Effect of* L-*penicillamine and* D-*penicillamine on the formation of δ-aminolaevulic acid*

Each tube contained particles, 0·0017 M-potassium α-oxoglutarate, 0·033 M-glycine, 0·0017 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4) and coenzyme A (0·05 mg.), and the additions made are shown below. The volume was made up to 3·0 ml. with 1·15% KCl. The tubes were incubated at 38° for 1·5 hr. The reaction was stopped by adding 20% trichloroacetic acid (1 ml.) and ALA estimated by assay with picric acid.

Expt. no.	Additions	μmole of ALA/ml. of packed particles/1·5 hr.	Inhibition (%)
1	None	0·389	—
	L-Penicillamine (2·67 mM)	0·071	82
	L-Penicillamine (2·67 mM) + pyridoxal phosphate (0·134 mM)	0·081	79
	L-Penicillamine (2·67 mM) + pyridoxal phosphate (0·268 mM)	0·094	76
	D-Penicillamine (2·67 mM)	0·333	15
2	None	0·513	—
	L-Penicillamine (2·25 mM)	0·086	83
	L-Penicillamine (2·25 mM) + pyridoxal phosphate (0·95 mM)	0·145	72

Table 9. *Decarboxylation of* [carboxy-^{14}C]*glycine by particles in the presence and absence of* α*-oxoglutarate*

Flasks contained washed particles (15 ml.), [*carboxy*-^{14}C]glycine (5 μc, 38·5 mg.), coenzyme A (0·5 mg.), pyridoxal phosphate (0·5 mg.), 0·002 M-$MgCl_2$, 0·05 M-KH_2PO_4–K_2HPO_4 (pH 7·4), and $NaHCO_3$ (5 mg.). The volume was made up to 30 ml. with 1·15% KCl. The additions made are shown below.

A slow stream of CO_2-free air was passed through the reaction mixture during incubation for 1·5 hr. at 38°, and the CO_2 evolved in the reaction was collected in two 25 ml. portions of CO_2-free N-NaOH.

At the end of the incubation $NaHCO_3$ (10 mg.) in water (1 ml). was injected into the reaction flask through the rubber tubing, followed by 3 N-HCl (6 ml.). Air was passed through the system for a further 10 min., and then 5 M-NH_4Cl (6 ml.) was injected into each of the sodium hydroxide vessels. The contents of these two vessels were then mixed and an excess of $BaCl_2$ was added. The $BaCO_3$ precipitate was filtered off, washed with water and acetone and dried, and its radioactivity determined. At the end of the experiment carrier ALA (100 mg.) was added to flask 2 and re-isolated by reaction with acetoacetate and its radioactivity determined as described under Methods.

The amount of ALA synthesized by the particles under these conditions was estimated in a parallel small-scale experiment by assay with picric acid.

Additions	μmole of ALA formed	Counts of barium carbonate/min.	Counts of 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole/min.
Flask 1 None	0·13	257	—
Flask 2 1·7 mM-potassium α-oxoglutarate	7·89	2227	15

The washed particles were incubated with [*carboxy*-^{14}C]glycine and the carbon dioxide evolved was collected as barium carbonate and its radioactivity determined. The ALA formed was also measured. The results showed that in the presence of α-oxoglutarate there was nine times as much labelling in the carbon dioxide as when α-oxoglutarate was not added (Table 9).

Synthesis of δ-aminolaevulic acid by particles obtained from non-anaemic chickens

Particles obtained from lysates of erythrocytes from normal chickens were tested for their capacity to form ALA from glycine and α-oxoglutarate or succinate by using the same methods as those employed when the cells from phenylhydrazine-treated birds were used. With glycine and α-oxoglutarate as substrates, 0·060 μmole of ALA/ml. of particles/1·5 hr. was formed, as compared with a value of 0·547 μmole of ALA/ml. of particles/1·5 hr. obtained with particles from phenylhydrazine-treated birds (average from six experiments). With glycine and succinate as substrates, 0·003 μmole of ALA/ml. of particles/hr. was formed with non-anaemic birds, as compared with a value of 0·120 μmole of ALA/ml. of particles/hr. obtained with birds which had been treated with phenylhydrazine (average of nine preparations).

DISCUSSION

The experiments reported in the present paper show that particles obtained from the lysates of erythrocytes of chickens, which had been made anaemic, can synthesize ALA from glycine and succinate or α-oxoglutarate. The identification of the product of the enzymic reaction as ALA rests on the following observations. The material gives with picric acid a colour having the same spectral characteristics and under the same conditions as does ALA; the colour, like that produced with authentic ALA, is partially extracted into ethyl acetate. The substance produced by incubation also gives, after condensation with acetylacetone or ethyl acetoacetate, the pyrrole reaction with Ehrlich's reagent. Observations that are reported in Part 2 (Gibson, Laver & Neuberger, 1958*b*) show quantitative agreement for the amount of ALA produced in enzymic experiments, if measured by the modified method of Shuster (1956) or by the pyrrole method of Mauzerall & Granick (1956). Moreover, when the system was incubated with [α-^{14}C]glycine and non-radioactive succinate and a large amount of non-radioactive ALA was then added, a crystalline pyrrolecarboxylic acid was obtained, after condensation with ethyl acetoacetate, which was radioactive (Table 3). In another experiment, not reported in detail, the incubation was done with non-radioactive glycine and radioactive succinate; carrier ALA was again added and the ALA was isolated after chromatography on paper with butanol–acetic acid as solvent. The aminolaevulic acid hydrochloride was recrystallized several times to constant radioactivity and repeatedly extracted with ether to remove possible traces of succinic acid. On oxidation with periodate (Shemin *et al.* 1955) radioactive succinic acid was produced. Evidence showing that the formation of the substance behaving as ALA is associated with the decarboxylation of glycine was also obtained (Table 9). These observations taken together provide convincing evidence that ALA is indeed formed. But these experiments do not indicate whether ALA is the initial product of the condensation or whether it arises by enzymic or spontaneous decarboxylation (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work) of α-amino-β-oxoadipate.

The fraction which was found to be active in the synthesis of ALA contains nuclei which can be seen under the microscope, and probably also cell debris arising from the cell membrane. On differential centrifuging of the supernatant fluid no evidence could be obtained that the erythrocytes contain mitochondria. The active particulate fraction is rich in deoxyribonucleic acid but also contains some ribonucleic acid. No conclusion can at present be drawn as to whether the enzymic activity is associated with nuclei, the cell membrane or some other cell constituents, such as mitochondria, which for unknown reasons may be obtained in the 'nuclear' fraction. Rubinstein & Denstedt (1953, 1954) found that in lysates of erythrocytes from non-anaemic chickens the succinic dehydrogenase, succinic oxidase and cytochrome oxidase activities are associated with nuclei. These authors also comment on the apparent absence of mitochondria in these cells.

The particles obtained by lysis of the red cells can synthesize ALA, provided that glycine, succinate or α-oxoglutarate and oxygen are supplied. There is no absolute requirement for phosphate, but the amount of ALA produced is markedly or even greatly decreased if phosphate is replaced by tris, barbitone or borate buffer (Table 4, Fig. 6). Addition of magnesium chloride increased the formation of ALA from oxoglutarate (Table 5) by about 50% and to an even greater extent from succinate (Table 6). The addition of pyridoxal phosphate and of coenzyme A, both separately and together, activated the formation of ALA. The effects were not very large, the activation varying between 15 and 50%; but they were consistently found in a large number of experiments. The belief that these two substances are cofactors for the enzymic reactions studied in the particles is based mainly on circumstantial evidence. The marked inhibition by L-penicillamine observed here is similar to that observed by du Vigneaud *et al.* (1957) with a liver transaminase which is known to be an enzyme containing pyridoxal phosphate as prosthetic group. When the particles are broken up by freeze-drying or homogenization, succinyl-CoA, but not α-oxoglutarate or succinate, can be used as substrate for the formation of ALA (Gibson, Laver & Neuberger, 1958*a*, *b*). It seems reasonable to assume that succinyl-CoA is also the active intermediate in intact particles. With whole particles, however, succinyl-CoA was not active. This must be ascribed to the existence of permeability barriers or to the action of a deacylase.

The requirement of pyridoxal phosphate for haem synthesis and the effect of pantothenic acid deficiency have been investigated by Schulman & Richert (1957). These authors obtained evidence that pyridoxal phosphate is concerned in the formation of ALA and they also suggest that the effect of pantothenic acid is explicable in terms of a requirement for coenzyme A. Similar results have been reported on cofactor requirements for porphyrin synthesis in *Tetrahymena vorax* by Lascelles (1957). In this organism synthesis of porphyrin from glycine is greatly reduced by deficiency of pyridoxal or pantothenate, whereas synthesis from ALA is not affected. The inhibitory effect of *p*-chloromercuribenzoate and iodoacetamide is also readily explained, if CoA is a necessary cofactor in the condensation. The action of the other inhibitors and activators and the detailed mechanism of the reaction are discussed in Part 2.

SUMMARY

1. Particles have been obtained, from lysates of erythrocytes of anaemic chickens, which catalyse a net synthesis of δ-aminolaevulic acid from glycine and succinate or α-oxoglutarate. δ-Aminolaevulic acid was not further metabolized by these particles. Preparations from non-anaemic chickens had little or no activity.

2. Synthesis of δ-aminolaevulic acid required the addition of glycine, succinate or α-oxoglutarate and the presence of oxygen, and was increased by the addition of pyridoxal phosphate, coenzyme A, phosphate and magnesium chloride. Inhibition of synthesis of δ-aminolaevulic acid was produced by addition of cyanide, iodoacetamide and *p*-chloromercuribenzoate. Ethylenediaminetetra-acetic acid showed a marked stimulating action. A number of other substances such as sodium azide, 2:4-dinitrophenol and sodium fluoride had no significant effect.

3. The pH optimum was found to be near 7·2, when phosphate buffer was used. Replacement of phosphate by borate, barbitone or 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer reduced the amount of δ-aminolaevulic acid formed.

4. L-Penicillamine, but not D-penicillamine, was strongly inhibitory. It is suggested that this inhibition is caused by interaction with pyridoxal phosphate.

5. No δ-aminolaevulic acid was formed when succinate or α-oxoglutarate was replaced by synthetic succinyl-coenzyme A. It is suggested that this is the result of permeability barriers.

6. The decarboxylation of glycine by the particles was found to be greatly increased by addition of α-oxoglutarate.

Grateful acknowledgement is made to the Rockefeller Foundation, New York, for the gift of a Spinco Ultracentrifuge and Roche Products Ltd., Welwyn Garden City, Herts, for supplying us with pyridoxal phosphate. The authors also wish to thank Dr Norman Kretchmer for the work he did on the characterization of the particles used in the experiments described.

REFERENCES

Berlin, N. I., Neuberger, A. & Scott, J. J. (1956*a*). *Biochem. J.* **64**, 80.
Berlin, N. I., Neuberger, A. & Scott, J. J. (1956*b*). *Biochem. J.* **64**, 90.
Dresel, E. I. B. & Falk, J. E. (1953). *Nature, Lond.*, **172**, 1185.
Dresel, E. I. B. & Falk, J. E. (1954). *Biochem. J.* **56**, 156.
Dresel, E. I. B. & Falk, J. E. (1956*a*). *Biochem. J.* **63**, 80.
Dresel, E. I. B. & Falk, J. E. (1956*b*). *Biochem. J.* **63**, 388.
du Vigneaud, V., Kuchinskas, E. J. & Horvath, A. (1957). *Arch. Biochim. Biophys.* **69**, 130.
Gibson, K. D., Laver, W. G. & Neuberger, A. (1958*a*). *Biochem. J.* **68**, 17P.
Gibson, K. D., Laver, W. G. & Neuberger, A. (1958*b*). *Biochem. J.* **70**, 71.
Gibson, K. D., Neuberger, A. & Scott, J. J. (1955). *Biochem. J.* **61**, 618.
Granick, S. (1954). *Science*, **120**, 1105.
Grinstein, M. (1947). *J. biol. Chem.* **167**, 515.
Hammarsten, E., Thorell, B., Åqvist, S., Eliasson, N. & Åkerman, L. (1953). *Exp. Cell Res.* **5**, 404.
Hunter, F. E. (1955). In *Methods in Enzymology*, vol. 2, p. 610. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Lascelles, J. (1956). *Biochem. J.* **62**, 78.
Lascelles, J. (1957). *Biochem. J.* **66**, 65.
Laver, W. G. & Neuberger, A. (1957). *Biochem. J.* **67**, 22P.
Mauzerall, D. & Granick, S. (1956). *J. biol. Chem.* **219**, 435.
Neuberger, A. & Scott, J. J. (1953). *Nature, Lond.*, **172**, 1093.
Neuberger, A., Scott, J. J. & Shuster, L. (1956). *Biochem. J.* **64**, 137.
Pawelkiewicz, J. & Zodrow, K. (1956). *Acta biochim. polon.* **3**, 225.
Rubinstein, D. & Denstedt, O. F. (1953). *J. biol. Chem.* **204**, 623.
Rubinstein, D. & Denstedt, O. F. (1954). *Canad. J. Biochem. Physiol.* **32**, 548.
Schulman, J. P. & Richert, D. A. (1957). *J. biol. Chem.* **226**, 181.
Shemin, D. (1955). In *A Symposium on Amino Acid Metabolism*, p. 727. Ed. by McElroy, W. D. & Glass, H. B. Baltimore: The Johns Hopkins Press.
Shemin, D. & Russell, C. S. (1953). *J. Amer. chem. Soc.* **75**, 4893.
Shemin, D., Russell, C. S. & Abramsky, T. (1955). *J. biol. Chem.* **215**, 613.
Shuster, L. (1956). *Biochem. J.* **64**, 101.
Simon, E. J. & Shemin, D. (1953). *J. Amer. chem. Soc.* **75**, 2520.
Wright, G. P. & Van Alstyne, M. (1931). *Folia haemat., Lpz.*, **46**, 26.

The Association of Adrenaline and Noradrenaline with Blood Platelets

By H. WEIL-MALHERBE* and A. D. BONE
Research Laboratory, Runwell Hospital, Wickford, Essex

(*Received* 18 *October* 1957)

It is becoming apparent that blood platelets, in addition to their classical role in blood coagulation, have other important functions. Among these their function as carriers of pharmacologically active amines is of particular interest. Weil-Malherbe & Bone (1954*a*) have briefly reported that platelets contain adrenaline and noradrenaline, and more extensive data will now be presented. Experiments on the nature of this association will also be described.

The results were obtained with a fluorimetric method which, though not strictly specific for adrenaline and noradrenaline, is believed to be specific for the catechol grouping. Of other biologically occurring catechol compounds those possessing a carboxylic or ketonic group in the side chain give rise to much smaller fluorescence intensities than adrenaline and noradrenaline and would interfere only if they were present in great excess. Experiments with ion-exchange resins, paper chromatography and paper electrophoresis have shown that interfering substances do not significantly contribute to the fluorescence obtained when the method is applied to plasma (Weil-Malherbe & Bone, 1957*b*). Although we have so far been unable to demonstrate the presence of adrenaline by bioassay in plasma of resting human subjects, Born & Hornykiewicz (1957) were successful in demonstrating the presence of adrenaline, though not noradrenaline, by bioassay in pig platelets.

* Present address: Clinical Neuropharmacology Research Center, National Institute of Mental Health, St Elizabeth's Hospital, Washington 20, D.C.

METHODS

Collection of blood

When human blood was used, the donors were hospital patients suffering from mental disorders. Blood was collected from an antecubital vein with a transfusion needle (16 s.w.g.) mounted on a short length of polyvinyl chloride tubing and was run into a measuring cylinder containing anticoagulant solution. Unless stated otherwise, a solution containing 1% (w/v) of neutralized sodium ethylenediaminetetra-acetate (EDTA) and 2% (w/v) of $Na_2S_2O_3,5H_2O$ (Renton & Weil-Malherbe, 1956) was used in the proportion of 1 vol to 3 vol. of blood. This anticoagulant is referred to below as 'EDTA–thio solution'. Solid heparin, containing 107 units/mg. (Evans Medical Supplies Ltd.), was dissolved in a minimum volume of 0·9% NaCl and