- Giri, K. V. (1955). *J. Indian Inst. Sci.* 37, 1.
 Giri, K. V. & Krishnaswamy, P. R. (1956). *J. Indian Inst. Sci.* 38, 232.
 Giri, K. V., Krishnaswamy, P. R. & Appaji Rao, N. (1957). *Nature, Lond.*, 179, 1134.
 Hubner, H. & Verzar, F. (1939). *Helv. chim. acta*, 21, 2006.
 Kearney, E. B. & England, S. (1951). *J. biol. Chem.* 193, 821.
 LePage, G. A. (1949). *Biochem. Prep.* 1, 5.
 Pulner, R. & Verzar, F. (1939). *Enzymologia*, 6, 333.
 Schrecker, A. W. & Kornberg, A. (1950). *J. biol. Chem.* 182, 795.
 Snell, E. E. & Strong, F. M. (1939). *Industr. Engng Chem. (Anal.)*, 11, 346.
 Watson, S. A. & Nogelle, G. R. (1947). *Plant Physiol.* 22, 228.

Initial Stages in the Biosynthesis of Porphyrins

2. THE FORMATION OF δ -AMINOLAEVULIC ACID FROM GLYCINE AND SUCCINYL-COENZYME A BY PARTICLES FROM CHICKEN ERYTHROCYTES*

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(Received 5 March 1958)

It has been known for some time that the carbon skeleton of porphyrins can be synthesized *in vivo* entirely from glycine and a C_4 compound which is an intermediate in the oxidative decarboxylation of α -oxoglutarate to succinate (for review see Shemin, 1955). The discovery of the role of δ -aminolaevulinic acid in porphyrin synthesis (Shemin & Russell, 1953; Neuberger & Scott, 1953; Dresel & Falk, 1953) led to the hypothesis that the initial reaction in porphyrin synthesis is the condensation of glycine with this C_4 compound to form δ -aminolaevulinic acid, which is then further metabolized to porphobilinogen and porphyrins. The correctness of this view was demonstrated in Part 1 (Laver, Neuberger & Udenfriend, 1958), which described the isolation and properties of a particulate system from erythrocytes of anaemic chickens that catalyses a net synthesis of δ -aminolaevulinic acid from glycine and α -oxoglutarate or succinate.

However, the nature of the reacting C_4 compound could not be determined in any of these investigations. Shemin & Wittenberg (1951) suggested that it might be succinyl-coenzyme A (succinyl-CoA) even before the isolation of this substance by Sanadi & Littlefield (1953); but little supporting evidence was obtained for this idea. Strong, but indirect evidence that coenzyme A is involved in the synthesis of δ -aminolaevulinic acid comes from the study of haem and porphyrin formation in pantothenate-deficient ducks (Schulman & Richert, 1957) and *Tetrahymena vorax* (Lascelles, 1957). Coenzyme A produces also a stimulation of activity in the system studied by Laver *et al.* (1958); but all

attempts to determine its role in this reaction were unsuccessful, and indeed no δ -aminolaevulinic acid was formed when synthetic succinyl-coenzyme A was substituted for succinate or α -oxoglutarate.

It has now been found that, if particles prepared according to Laver *et al.* are freeze-dried, they lose most of their capacity to form δ -aminolaevulinic acid from glycine and α -oxoglutarate or succinate. However, when synthetic or enzymically generated succinyl-coenzyme A is used as source of the C_4 compound, the formation of δ -aminolaevulinic acid is largely restored and it appears that succinyl-coenzyme A is indeed the reactive derivative of the C_4 compound. Evidence has also been obtained which strongly implicates pyridoxal phosphate as a cofactor in this reaction. A preliminary report has appeared earlier (Gibson, Laver & Neuberger, 1958).

EXPERIMENTAL

Materials

Succinyl-coenzyme A (succinyl-CoA) was synthesized according to Simon & Shemin (1953); the product was kept at 0° until required, and was always used within a few hours. Values quoted for succinyl-CoA concentrations are nominal only and represent the amount of coenzyme A (CoA) used. Acetyl-CoA, propionyl-CoA and glutaryl-CoA were prepared similarly from the corresponding anhydrides and CoA. Crystalline glutamic dehydrogenase [10 mg./ml., suspended in 40% saturated $(NH_4)_2SO_4$] was obtained from Boehringer und Soehne, Mannheim. α -Oxoglutarate dehydrogenase was prepared according to Sanadi, Littlefield & Bock (1952); the preparation used in this work was capable of oxidizing 0.21 μ mole of α -oxoglutarate/min./mg. of protein, as measured by reduction of diphosphopyridine nucleotide (DPN) in the presence of mM-cysteine and excess of succinyl-CoA deacylase (Gergely, Hele & Ramakrishnan, 1952). Crude succinic thiokinase was obtained from *Escherichia coli* (National Collection of Industrial

* Part 1: Laver, Neuberger & Udenfriend (1958).

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Bacteria, No. 8545). A suspension of this organism, grown according to Korkes (1955), except that 0.5% glucose replaced Cerelese in the growth medium, was subjected to ultrasonic disintegration on a Mullard ultrasonic generator, type E7590A (25 kc./sec. for 40 min.); the resulting suspension was clarified by centrifuging to give a pale-yellow solution which formed 3.5 μ moles of succino-hydroxamic acid/hr./mg. of protein in the test system of Kaufman (1955). Phosphate buffers were made by mixing solutions of K_2HPO_4 and KH_2PO_4 as required; triethanolamine buffers were made from triethanolamine hydrochloride (obtained from Boehringer und Soehne) and NaOH. Other buffers were prepared according to Gomori (1955). Liver-coenzyme concentrate was obtained from the Armour Laboratories, Eastbourne. Aminoacetone was prepared by a modification of the stannous chloride reduction (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work) of oximinacetone; it was recrystallized from dry ethanol-ether. D-Cysteine was prepared by reduction with tin and HCl of a sample of D-cystine kindly supplied by Dr H. R. V. Arnstein of the National Institute for Medical Research, Mill Hill, London, N.W. 7. For the sources of other materials see Part I (Laver *et al.* 1958).

Methods

Preparation of freeze-dried particles. The isolation of active particles from erythrocytes of anaemic chickens was described previously (Laver *et al.* 1958). Particles prepared in this manner were freeze-dried by a conventional method and stored at -20° ; under these conditions they appear to maintain their activity for at least a month. In a typical preparation, 446 mg. of freeze-dried material (including 115 mg. of KCl) was obtained from 10 ml. of packed particles. Before use, they were suspended in ice-cold water (approx. 3 ml./100 mg.) and subjected to homogenization for about 1 min. at 0° in a Potter-Elvehjem homogenizer. Amounts of freeze-dried particles used are expressed in terms of dry wt. which includes some KCl.

Incubation. Samples having a final volume of 1.7 ml. were incubated at 37° in unstoppered tubes, or *in vacuo* in Thunberg tubes. After incubation, 0.06 M-iodoacetamide (0.1 ml.) was added, followed after about 1 min. by 20% trichloroacetic acid (0.6 ml.). The precipitate was removed by centrifuging, and the δ -aminolaevulinic acid (ALA) content of the supernatant was estimated as described below. Addition of iodoacetamide stabilizes the colour formed with Ehrlich's reagent, probably by removing interfering thiol compounds.

Estimation of δ -aminolaevulinic acid. ALA was estimated colorimetrically after condensation with acetylacetone, according to the following procedure, which was modified slightly from that of Mauzerall & Granick (1956). The 5% trichloroacetic acid filtrate (1.0 ml.) containing up to 0.1 μ mole of ALA was mixed with M-sodium acetate buffer, pH 4.6 (1.0 ml.), 2.5 N-NaOH (0.1 ml.), and acetylacetone (0.05 ml.). The mixture was heated at 100° for 10 min. and cooled to room temperature, and the colour was developed by addition of 2.0 ml. of the modified Ehrlich's reagent of Mauzerall & Granick (1956). After 15 min. the absorption was read at 553 m μ . Internal standards were used in each experiment. Aminoacetone was assayed by the same method.

RESULTS

Synthesis of δ -aminolaevulinic acid by freeze-dried particles

When the freeze-dried particles were incubated with glycine and α -oxoglutarate and the cofactors, i.e. pyridoxal phosphate, CoA and magnesium chloride, previously found to activate ALA synthesis (Laver *et al.* 1958), there was negligible synthesis of ALA. The activity of the freeze-dried particles could be restored to a slight extent by the addition of DPN and to a greater extent by a mixture of DPN and ammonium chloride. When α -oxoglutarate was replaced by synthetic succinyl-CoA, the ability of the freeze-dried particles to synthesize ALA was greatly increased (Table 1), the amount of ALA formed probably being limited by the stability of the added succinyl-CoA at the pH and temperature of incubation. When whole particles were incubated with glycine and α -oxoglutarate under the standard conditions (Laver *et al.* 1958, Table 5), 0.240 μ mole of ALA was formed by 36 mg. dry wt. of particles. When particles from the same preparation were freeze-dried, and succinyl-CoA (1 μ mole) substituted for α -oxoglutarate under the conditions given in Fig. 1, 36 mg. of particles produced 0.173 μ mole of ALA. Particles prepared from erythrocytes of normal, non-anaemic chickens, when freeze-dried and incubated with succinyl-CoA, glycine and pyridoxal phosphate, formed a negligible amount of ALA.

Requirements of the freeze-dried particles for synthesis of δ -aminolaevulinic acid

Freeze-dried particles required the addition of both glycine and succinyl-CoA for the synthesis of ALA. There was no ALA synthesis if either of these two substrates was omitted. Fig. 1 shows the effect on ALA formation of adding increasing amounts of succinyl-CoA. It seems likely that at

Table 1. *Formation of δ -aminolaevulinic acid by freeze-dried particles*

Each tube contained freeze-dried particles (30 mg.), glycine (0.06 M), pyridoxal phosphate (0.2 μ mole), $MgCl_2$ (3 mM), CoA (0.045 μ mole), phosphate, pH 7.4 (75 mM) and other additions as described below. The final volume was 1.7 ml.; tubes were incubated for 60 min. at 37° and ALA was estimated as described.

Additions	ALA formed (μ mole)
α -Oxoglutarate (0.03 M)	0.004
α -Oxoglutarate (0.03 M) + DPN (0.1 μ mole)	0.015
α -Oxoglutarate (0.03 M) + NH_4Cl (7 mM) + DPN (0.1 μ mole)	0.022
α -Oxoglutarate (0.03 M) + NH_4Cl (7 mM) + DPN (0.3 μ mole)	0.031
Succinyl-CoA (0.3 μ mole)	0.111

still higher concentrations of succinyl-CoA more ALA would have been formed, and it is probable that only the enzymes concerned in the formation of succinyl-CoA were affected by freeze-drying, while the enzyme(s) which condenses glycine with succinyl-CoA is stable. The effect of varying glycine concentration on formation of ALA (Fig. 2) is similar to that described with whole particles (Laver *et al.* 1958).

Unlike whole particles, freeze-dried particles did not require oxygen for ALA synthesis from succinyl-CoA and glycine, and no activation was obtained

Fig. 1. Effect of succinyl-CoA concentration on ALA formation. Freeze-dried particles (32 mg.) were incubated for 60 min. at 37° with glycine (0.06M), pyridoxal phosphate (0.2 μmole), phosphate, pH 7.4 (75 mM), and succinyl-CoA as indicated in a final volume of 1.7 ml. ALA was estimated as described.

Fig. 2. Effect of glycine concentration on ALA formation. Freeze-dried particles (28 mg.) were incubated for 60 min. at 37° with succinyl-CoA (approx. 0.3 μmole), pyridoxal phosphate (0.2 μmole), phosphate, pH 7.4 (75 mM), and glycine as indicated in a final volume of 1.7 ml. ALA was estimated as described.

Table 2. Effect of possible cofactors on the synthesis of δ -aminolaevulic acid

The control tubes contained freeze-dried particles (approx. 30 mg.), glycine (0.06M), pyridoxal phosphate (0.2 μmole), $MgCl_2$ (3 mM), phosphate, pH 7.4 (75 mM), and succinyl-CoA (approx. 0.3 μmole). The final volume was 1.7 ml. Omissions from the control are shown below. Tubes were incubated for 30 min. at 37°, and ALA was estimated as described.

Omissions	ALA formed (μmoles)		Test system as % of control
	Control system	Test system	
$MgCl_2$	0.096	0.093	97
Pyridoxal phosphate	0.068	0.038	56
	0.089*	0.058	65
Air	0.096*	0.087	91

* $MgCl_2$ omitted from control.

Table 3. Effect of various buffers on synthesis of δ -aminolaevulic acid

Each tube contained freeze-dried particles (28.5 mg.), glycine (100 μmoles), succinyl-CoA (approx. 0.3 μmole), pyridoxal phosphate (0.2 μmole) and buffer as shown, in a volume of 1.7 ml. After incubation for 30 min. at 37°, the tubes were deproteinized and ALA was estimated as described.

Buffer, pH 7.4	ALA synthesized (μmole)
Phosphate (0.09M)	0.099
Triethanolamine (0.09M)	0.079
2-Amino-2-hydroxymethylpropane-1:3-diol (0.09M)	0.079
Barbitone (0.03M)	0.047

Fig. 3. Effect of time of incubation on the formation of ALA and aminoacetone. Freeze-dried particles (30 mg.) were incubated with glycine (0.06M), pyridoxal phosphate (0.2 μmole), phosphate, pH 7.4 (75 mM), and succinyl-CoA (0.3 μmole) (●) or acetyl-CoA (0.3 μmole) (○), in a final volume of 1.7 ml. at 37° for the time indicated. ALA and aminoacetone were estimated as described.

by the addition of magnesium chloride (Table 2). The activation by phosphate observed previously with whole particles was not so marked with freeze-dried particles, but activity was still greatest when phosphate buffers were used (Table 3). The effect of time of incubation is shown in Fig. 3. The short period required for complete reaction to occur was probably the result of the instability of the added succinyl-CoA, since pre-

incubation of the reaction mixture for 30 min. before the addition of succinyl-CoA did not result in any lowering of activity. The optimum pH for the reaction was found to be at about 7.4 in phosphate (Fig. 4). The requirement for pyridoxal phosphate was more marked with freeze-dried particles than previously found with whole particles (Table 2; see also Tables 6 and 8). Complete activation was obtained at a concentration of $23.5 \mu\text{M}$ of added pyridoxal phosphate, higher concentrations having no effect on the

Fig. 4. Effect of pH on ALA formation. Freeze-dried particles (22 mg.) were incubated for 30 min. at 37° with glycine (0.06 M), pyridoxal phosphate ($0.2 \mu\text{mole}$), succinyl-CoA (approx. $0.2 \mu\text{mole}$), and phosphate (0.13 M) (pH as indicated) in a final volume of 1.7 ml. ALA was estimated as described.

Fig. 5. Inhibition of ALA formation by cyanide. Freeze-dried particles (25 mg.) were incubated for 30 min. at 37° with glycine (0.06 M), pyridoxal phosphate (0.12 M), succinyl-CoA (approx. $0.25 \mu\text{mole}$), phosphate, pH 7.4 (75 mM), and KCN as indicated in a final volume of 1.7 ml. ALA was estimated as described.

Table 4. *Effect of various substances on the formation of δ -aminolaevulinic acid*

Control tubes contained freeze-dried particles (approx. 30 mg.), glycine (0.06 M), pyridoxal phosphate (0.12 M), succinyl-CoA (approx. $0.3 \mu\text{mole}$) and phosphate, pH 7.4 (75 mM). The final volume was 1.7 ml. Additions made to the control are shown below. In the experiment with CO, the soln. was saturated with the gas which had been generated from formic acid and H_2SO_4 . The tubes were incubated for 30 min. at 37° and ALA was estimated as described.

Additions	ALA formed (μmole)		Test as % of control
	Test	Control	
p-Chloromercuribenzoate (0.44 mM)	0	0.089	0
KCN (0.18 mM)	0.004	0.084	5
KCN (0.18 mM) + pyridoxal phosphate (0.36 mM)	0.003	0.084	4
KCN (35 μM)	0.021	0.103	20
KCN (35 μM) + pyridoxal phosphate (0.36 mM)	0.021	0.103	20
L-Penicillamine (2.35 mM)	0.013	0.096	13
L-Cysteine (2.35 mM)	0.019	0.096	20
L-Cysteine (2.35 mM)*	0.021	0.115	18
D-Penicillamine (2.35 mM)	0.068	0.096	71
D-Cysteine (2.35 mM)*	0.087	0.115	76
Glutathione (2.35 mM)	0.073	0.089	82
Iodoacetamide (3.5 mM)	0.109	0.111	98
Sodium azide (1.76 mM)†	0.075	0.074	101
Sodium azide (5.28 mM)	0.073	0.074	99
$\alpha\alpha'$ -Dipyridyl (0.2 mM)	0.098	0.103	95
Carbon monoxide	0.075	0.074	101
8-Hydroxyquinoline (6 mM)	0.083	0.074	112
EDTA (mM)	0.083	0.068	122

* EDTA (2.6 mM) was added to both test and control tube in these expts.

† Reaction mixture was incubated with sodium azide for 30 min. before addition of succinyl-CoA.

amount of ALA synthesized. As found with whole particles, the amount of activation varies somewhat with different preparations.

Effects of various substances on δ -aminolaevulic acid formation

The effects of various inhibitors and activators on ALA synthesis from succinyl-CoA and glycine by freeze-dried particles are shown in Table 4. Most striking was the strong inhibition by very low concentrations of cyanide, 50 % inhibition occurring at a concentration of 14 μ M (Fig. 5). This inhibition could not be overcome by the addition of a large excess of pyridoxal phosphate (Table 4). Other substances which were found to inhibit the reaction were L-penicillamine and L-cysteine; D-penicillamine, D-cysteine and glutathione had a much smaller effect. Inhibition by varying concentrations of L-penicillamine is shown in Fig. 6. Sodium azide, $\alpha\alpha'$ -dipyridyl and carbon monoxide had no significant effect on ALA formation, while 8-hydroxyquinoline and ethylenediaminetetra-acetic acid (EDTA) activated slightly (Table 4).

It can also be seen from Table 4 that, although iodoacetamide does not inhibit ALA formation to any significant extent, *p*-chloromercuribenzoate inhibits completely at a low concentration. It appeared possible that *p*-chloromercuribenzoate might act by splitting succinyl-CoA rather than by combining with the enzyme. This possibility was investigated in the manner described in Table 5. In the first experiment of Table 5, freeze-dried particles were incubated with *p*-chloromercuribenzoate for 5 min., after which molar quantities of succinyl-CoA varying from two-thirds to more than twice the amount of *p*-chloromercuribenzoate were added. If *p*-chloromercuribenzoate reacts stoichiometrically with succinyl-CoA and not with the enzyme, more ALA should be formed when the amount of succinyl-CoA exceeds the amount of *p*-chloromercuribenzoate than when it does not.

As can be seen, the formation of ALA was no greater in the former case than it was in the latter. In the second experiment of Table 5, succinyl-CoA was incubated with an equal amount of *p*-chloromercuribenzoate for 1 min., after which excess of glutathione was added, followed by particles. If *p*-chloromercuribenzoate acts only by splitting succinyl-CoA, there should have been a large decrease in ALA formation as compared with a control which had no *p*-chloromercuribenzoate; however, it is clear that the degree of inhibition was very small. It is concluded that, if *p*-chloromercuribenzoate does

Fig. 6. Inhibition of ALA formation by L-penicillamine. Freeze-dried particles (29 mg.) were incubated for 30 min. at 37° with glycine (0.06M), pyridoxal phosphate (0.04 μ mole), succinyl-CoA (approx. 0.3 μ mole), phosphate, pH 7.4 (75 mM), and L-penicillamine as indicated in a final volume of 1.7 ml. ALA was estimated as described.

Table 5. *Inhibition of the synthesis of δ -aminolaevulic acid by *p*-chloromercuribenzoate*

Each tube contained phosphate, pH 7.4 (0.09M), glycine (100 μ moles), succinyl-CoA as indicated, freeze-dried particles (25.2 mg. in 0.6 ml.) and pyridoxal phosphate (0.2 μ mole); *p*-chloromercuribenzoate and glutathione were added as shown below. The final volume was 1.7 ml. In Expt. no. 1 the reaction mixture, complete except for succinyl-CoA, was incubated at 37° for 5 min., after which the reaction was started by addition of succinyl-CoA. In Expt. no. 2, all the additions except particles and glutathione were mixed and incubated at 37° for 1 min.; at the end of this time the glutathione was added, and after a further minute the reaction was started by addition of the particles. In both experiments the reaction was stopped after 10 min., and ALA was estimated as described.

Expt. no.	<i>p</i> -Chloromercuribenzoate (μ mole)	Glutathione (μ mole)	Succinyl-CoA (μ mole)	ALA formed (μ mole)
1	0.3	—	0.2	0.016
1	0.3	—	0.4	0.018
1	0.3	—	0.8	0.011
2	0.3	0.8	0.4	0.047
2	—	0.8	0.4	0.052

Table 6. *Effect of dialysis*

In Expt. no. 1 freeze-dried particles (296 mg.) were suspended in 10 ml. of 1% KCl containing EDTA (mM) and phosphate buffer, pH 7.4 (0.01M). A portion of this suspension (5 ml.) was dialysed against this solution (1.5 l.) for 20 hr. at 0°. Pyridoxal phosphate (0.12 mM) was added to another portion of the suspension (1.5 ml.) which was then dialysed against the same solution (100 ml. for 2 hr.; followed by 1.5 l. for 18 hr.) at 0°. The remainder of the suspension was kept at 0° for the same period.

In Expt. no. 2 freeze-dried particles (110 mg.) were suspended in distilled water containing EDTA (mM). A portion of the suspension (3 ml.) was dialysed against this solution (1 l.) for 20 hr. at 0°, while the remainder was kept at 0° for the same period.

In both experiments the dialysed and undialysed particles were incubated for 30 min. at 37° with glycine (0.06M), phosphate, pH 7.4 (75 mM), and succinyl-CoA (approx. 0.3 μ mole) in a final volume of 1.7 ml. Additions are described below; ALA was estimated as described.

Expt. no.	Freeze-dried particles	Additions	ALA formed (μ mole)
1	Undialysed	None	0.046
	Undialysed	Pyridoxal phosphate (0.12 mM)	0.091
	Dialysed	None	0.056
	Dialysed	Pyridoxal phosphate (0.12 mM)	0.101
	Dialysed (pyridoxal phosphate 0.12 mM added before dialyses)	None	0.102
2	Undialysed	Pyridoxal phosphate (0.24 mM)	0.089
	Dialysed	None	0.009
	Dialysed	Pyridoxal phosphate (0.24 mM)	0.020

react with succinyl-CoA, the rate of this reaction is not sufficient to account for its inhibitory action on ALA formation. *p*-Chloromercuribenzoate therefore probably acts by direct combination with the enzyme responsible for ALA synthesis.

Effect of dialysis

The effect of dialysis on the freeze-dried particles is shown in Table 6. When a suspension of freeze-dried particles was dialysed at 0° against buffered potassium chloride containing EDTA there was no loss of activity, and the amount of activation obtained by the addition of pyridoxal phosphate was about the same as that obtained with an undialysed portion kept for the same period as control. It was also found that addition of Armour's liver coenzyme concentrate did not increase the activity of the dialysed particles. These results suggest that no diffusible cofactor is involved in the reaction. Since no loss of activity occurred on dialysis, it appeared that the endogenous pyridoxal phosphate was very strongly bound. When pyridoxal phosphate was added before dialysis, the particles were slightly more active after dialysis than they were before, although no further addition of pyridoxal phosphate was made before incubation. When the particles were dialysed against distilled water, containing EDTA, instead of against buffered potassium chloride, the activity was greatly reduced. It is likely that enzyme denaturation occurred under these conditions.

δ -Aminolaevulinic acid formation from enzymically-generated succinyl-coenzyme A

It can be seen from Table 7 that freeze-dried particles form a considerable amount of ALA from

Table 7. *Formation of δ -aminolaevulinic acid from glycine and succinate with succinic thiokinase*

The control tube contained freeze-dried particles (30 mg.), phosphate pH 7.4 (0.04M), $MgCl_2$ (2.7 mM), glycine (100 μ moles), potassium succinate (25 μ moles), ATP (10 μ moles), CoA (0.05 μ mole), pyridoxal phosphate (0.2 μ mole), and crude succinic thiokinase from *E. coli* (4.4 mg. of protein) in a volume of 1.85 ml. After incubation for 1 hr. at 37°, the tubes were deproteinized and ALA was estimated as described. Omissions are shown below.

Omissions	ALA formed (μ mole)	Activity as % of control
None	0.064	(100)
Succinate	0.005	8
CoA	0.003	5
ATP	0.002	3
<i>E. coli</i> extract	0.006	9
Freeze-dried particles	0.000	0

glycine and succinyl-CoA when this is generated from succinate, CoA and adenosine triphosphate (ATP) by means of succinic thiokinase from *E. coli*. In order to obtain ALA by this reaction it was necessary to add all the substrates and cofactors and succinic thiokinase; omission of succinate, CoA, ATP, thiokinase or particles reduced ALA synthesis by 90% or more. A similar experiment in which succinyl-CoA was generated from α -oxoglutarate and CoA is described in Table 8. Here use was made of the reaction catalysed by purified α -oxoglutarate dehydrogenase (Sanadi & Littlefield, 1953; Kaufman, Gilvarg, Cori & Ochoa, 1953) in which α -oxoglutarate and CoA form succinyl-CoA and carbon dioxide with concomitant reduction of DPN. Glutamic dehydrogenase and ammonia were

added in order to reoxidize reduced DPN. The table shows that more than 0.1 μ mole of ALA was formed in the presence of all the additions; but, when glycine or pyridoxal phosphate was omitted, ALA synthesis decreased by 70–75 %, and in the absence of added α -oxoglutarate, CoA or DPN very little ALA was formed. In contrast, the omission of α -oxoglutaric dehydrogenase or glutamic dehydrogenase led to a much smaller decrease in ALA formation. The significance of these results is discussed below.

Identification of the product of condensation of succinyl-coenzyme A and glycine

The material formed by freeze-dried particles from glycine and succinyl-CoA behaved as ALA both in the assay with acetylacetone and in the method of Shuster (1956) as modified by Laver *et al.* (1958). Good agreement between the two methods was shown in an experiment in which freeze-dried particles (80 mg.) were incubated for 1 hr. with glycine (100 μ moles), pyridoxal phosphate (0.4 μ -mole), succinyl-CoA (1.2 μ moles), EDTA (0.25 mM) and phosphate (0.03 M), pH 7.4, in a final volume of 3.0 ml. In a control tube from which succinyl-CoA was omitted, 0.2 μ mole of ALA was added to serve as an internal standard. The amount of ALA formed in the experimental tube was estimated as 0.227 μ mole by the modified picric acid method and as 0.174 μ mole when assayed by condensation with acetylacetone. None of the pyrrole formed in this latter reaction could be extracted into ether at pH 8. In this experiment iodoacetamide was not added before assay and the lower figure obtained by the second method may have been due to inter-

ference in the colour reaction by the thiol group of CoA.

Fractionation of freeze-dried particles

Freeze-dried particles (327 mg.) were suspended in water (10 ml.) and homogenized, as described under Methods. When this extract (1 ml.) was incubated for 1.5 hr. with glycine, succinyl-CoA and pyridoxal phosphate under the same conditions as in Table 4, 0.083 μ mole of ALA was formed. The supernatant obtained by centrifuging this extract (1500 g; 20 min.) when incubated under identical conditions formed 0.041 μ mole of ALA and the supernatant obtained by centrifuging the extract at high speed (20 000 g; 15 min.) formed 0.038 μ mole of ALA. It appeared therefore that part of the activity was soluble, but no further fractionation of the soluble material was attempted.

Formation of aminoacetone and analogous amino ketones by freeze-dried particles

Shemin (1955) suggested that the condensation of glycine with succinate to form ALA might be only one example of a more general reaction. The ability of acetyl-CoA, propionyl-CoA and glutaryl-CoA to form amino ketones with glycine was therefore investigated. When synthetic acetyl-CoA was incubated with freeze-dried particles, together with glycine and pyridoxal phosphate, a substance was formed which reacted with acetylacetone at pH 4.6 to give a pyrrole that could be extracted into ether at pH 8. It was found that there was no disappearance of synthetic aminoacetone during incubation at a concentration of 0.3 mM with particles for 1 hr. at 38° and pH 7.4, and that it could be assayed by condensation with acetylacetone at pH 4.6 by using exactly the same method as that employed for ALA. The pyrrole resulting from this condensation, having no free carboxyl group, could be completely extracted into ether at pH 8. The amino ketone formed from acetyl-CoA and glycine may therefore be presumed to be aminoacetone. The effect of time of incubation on the formation of aminoacetone is shown in Fig. 3. Freeze-dried particles form much less aminoacetone than they do ALA. The fact that enzymic synthesis continues for a longer period is probably due to the greater stability of acetyl-CoA compared with that of succinyl-CoA.

Propionyl-CoA and glutaryl-CoA were also found to give rise to amino ketones when incubated with glycine, pyridoxal phosphate and freeze-dried particles. Since authentic samples of 6-amino-5-oxohexanoic acid and 1-aminobutan-2-one were not available, the amounts of these substances formed enzymically could not be estimated quantitatively, but appreciably less colour was produced with Ehrlich's reagent than in the case of ALA.

Table 8. *Formation of δ -aminolaevulic acid from glycine and α -oxoglutarate with α -oxoglutaric dehydrogenase*

The control tube contained freeze-dried particles (24.0 mg.), phosphate, pH 7.4 (0.09 M), glutathione (1.5 μ mole), glycine (100 μ moles), pyridoxal phosphate (0.2 μ mole), CoA (0.03 μ mole), potassium α -oxoglutarate (5 μ moles), DPN (0.2 μ mole), α -oxoglutaric dehydrogenase (49 μ g.), glutamic dehydrogenase (100 μ g.), and $(\text{NH}_4)_2\text{SO}_4$ (20 μ moles) in a volume of 1.7 ml. After incubation for 1 hr. at 37°, the tubes were deproteinized and ALA was estimated as described. Omissions are indicated below.

Omissions	ALA formed (μ mole)	Activity as % of control
None	0.115	(100)
Glycine	0.028	24
α -Oxoglutarate	0.012	10
CoA	0.017	15
Pyridoxal phosphate	0.034	30
DPN	0.016	14
α -Oxoglutaric dehydrogenase	0.075	65
Glutamic dehydrogenase	0.082	71

*Formation of aminoacetone from
pyruvate by whole particles*

Whole particles (0.6 ml.) were incubated for 1.5 hr. with pyruvate (3 mM), glycine (0.06 M), pyridoxal phosphate (0.2 μ mole), CoA (0.05 μ mole), $MgCl_2$ (3 mM) and phosphate, pH 7.4 (65 mM), in a final volume of 1.7 ml. Under these conditions 0.029 μ mole of aminoacetone was formed. When pyruvate was replaced by 3 mM- α -oxoglutarate, 0.257 μ mole of ALA was formed under identical conditions.

DISCUSSION

Activation of succinate and α -oxoglutarate

It was shown in Part 1 (Laver *et al.* 1958) that washed whole particles obtained from the lysates of erythrocytes of chickens, which had been made anaemic, can synthesize ALA from α -oxoglutarate (or succinate) and glycine. No ALA was formed on incubation of the particles with glycine and succinyl-CoA. The present paper deals with the behaviour of preparations obtained from freeze-dried particles. Such preparations are inactive on incubation with α -oxoglutarate, glycine, coenzyme A, magnesium chloride and phosphate (Table 1), but a relatively small amount of ALA is formed on addition of DPN or of DPN and ammonium chloride. When α -oxoglutarate in this system is replaced by succinyl-CoA, a further and very considerable increase in activity is found. Indeed, the amount of ALA formed under these conditions with freeze-dried preparations is only slightly smaller than that obtained with whole particles and α -oxoglutarate. Whole particles were found to be active without addition of CoA and addition of this coenzyme produced on the average an activation of 20 or 45 % only with α -oxoglutarate and succinate respectively. With freeze-dried preparations, on the other hand, there is no ALA formation unless succinyl-CoA is added as such (Fig. 1) or is enzymically produced in the system either by the action of succinic thiokinase of *E. coli* on succinate (Table 7) or by the action of purified α -oxoglutaric dehydrogenase on α -oxoglutarate (Table 8).

It must be presumed therefore that succinyl-CoA is the intermediate in ALA formation in whole particles as well as in freeze-dried preparations. The formation of succinyl-CoA in whole particles is almost certainly due to α -oxoglutaric dehydrogenase. The presence of α -oxoglutaric dehydrogenase in freeze-dried preparations can be inferred from the fact that ALA formation from α -oxoglutarate is markedly stimulated by addition of DPN (Table 1), the known hydrogen-acceptor in this reaction (Sanadi & Littlefield, 1953; Kaufman *et al.* 1953). The further increase of ALA synthesis produced by addition of ammonium ions to this system implies

that freeze-dried preparations also contain glutamic dehydrogenase, which reoxidizes reduced DPN while forming glutamate from α -oxoglutarate and ammonia. The correctness of this view is borne out by the experiments reported in Table 8, in which purified preparations of these two enzymes were added. Omission of only one of these enzymes resulted in a relatively small decrease in ALA formation, indicating that the freeze-dried preparations contain both α -oxoglutaric dehydrogenase and glutamic dehydrogenase. When DPN was left out, there was a great reduction in ALA synthesis, which is to be expected if α -oxoglutaric dehydrogenase is involved.

In the earlier paper (Laver *et al.* 1958) it was reported that with whole particles DPN had a slight, but definite, activating effect in the absence of oxygen. The smallness of this effect and the non-availability of succinyl-CoA to whole particles is most satisfactorily explained by the existence of permeability barriers, which disappear on freeze-drying and on mechanical homogenization (Gibson *et al.* 1958). The fact that ALA formation from α -oxoglutarate in whole particles is not inhibited by azide may also be explained in terms of a permeability barrier. Alternatively, the oxidation of reduced DPN may occur through enzymes other than those of the cytochrome system, such as the oxidases which have been found in *Streptococcus faecalis* (Dolin, 1955).

The path by which succinate is used for ALA synthesis in whole particles is not clearly established. The work reported in Part 1 and the results shown in Table 7 are consistent with the data of Shemin & Kumin (1952), which indicate that the conversion of succinate into succinyl-CoA in normal duck red cells occurs mainly by oxidation through the Krebs cycle. Their figures show that there is in addition a direct conversion of succinate into its CoA derivative. Although the latter reaction may contribute to ALA formation from succinate in whole particles, the results shown in Table 7 indicate that there is little or no thiokinase in freeze-dried preparations.

Role of pyridoxal phosphate

The evidence that pyridoxal phosphate is a cofactor in the synthesis of ALA was briefly discussed in Part 1. As with whole particles, the decrease in ALA formation which occurred when pyridoxal phosphate was not added to the test system varied from one freeze-dried preparation to another; but there was always a decrease of at least 30 % and usually of 40–50 % (see Table 2). In one case the decrease was 70 % (Table 8). Similar results were reported for ALA formation by homogenized preparations (Gibson *et al.* 1958). The results reported in Table 6 show that added pyrid-

oxal phosphate is bound in such a way that it is not removed by dialysis and that it is the bound pyridoxal phosphate that acts catalytically in ALA formation. The finding that freeze-dried preparations are active without addition of pyridoxal phosphate, and that this activity is not decreased by dialysis against buffered potassium chloride solution, indicates that these preparations contain bound pyridoxal phosphate.

The conclusion that enzyme-bound pyridoxal phosphate is a cofactor in ALA synthesis will also explain results obtained with penicillamine, cysteine and cyanide. There is no evidence from the present work that the inhibitory effect of penicillamine is due to its combination with pyridoxal phosphate; but this is likely on chemical grounds, as du Vigneaud, Kuchinskas & Horvath (1957) have shown that these compounds rapidly form a thiazolidine compound under physiological conditions. These authors have also shown that liver transaminases, which contain pyridoxal phosphate as prosthetic group, are strongly inhibited by L-penicillamine. The enzyme system which forms ALA from succinyl-CoA and glycine has approximately the same sensitivity to L-penicillamine as have the transaminases studied by du Vigneaud *et al.* (1957). D-Penicillamine is a much less effective inhibitor. The steric specificity of this inhibition is consistent with the conclusion drawn above that the catalytically active pyridoxal phosphate is bound to protein. The effects shown by L- and D-cysteine closely resemble those shown by the two isomers of penicillamine. The fact that glutathione inhibits only slightly accords with the suggestion of du Vigneaud *et al.* (1957) that the effect of penicillamine is caused by thiazolidine formation with pyridoxal phosphate.

The formation of ALA from succinyl-CoA and glycine by freeze-dried preparations is inhibited by cyanide to an even greater extent than the synthesis by whole particles from α -oxoglutarate and glycine. It follows that cyanide inhibits the condensation reaction. Furthermore, this inhibition is unlikely to be due to some reaction with succinyl-CoA, since an inhibition of 80% was observed under conditions in which the molar concentration of succinyl-CoA was about five times that of cyanide (Table 4). It is therefore concluded that cyanide inhibits by reaction with pyridoxal phosphate. Enzymes which contain pyridoxal phosphate are inhibited to a greater or lesser degree by cyanide. Of all the bacterial amino acid decarboxylases studied by Taylor & Gale (1945) only glutamic and lysine decarboxylases approach the ALA-forming enzyme in sensitivity to cyanide. Diaminopimelic decarboxylase, which almost certainly contains pyridoxal phosphate (Dewey, Hoare & Work, 1954), is considerably less sensitive

to cyanide, as are liver transaminases (Cohen, 1939; Vyshepan, 1940) and kynureninase (Jakoby & Bonner, 1953). The finding that inhibition by cyanide is not reversed by an excess of pyridoxal phosphate is consistent with the assumption that cyanide has a much greater affinity for enzyme-bound than for free pyridoxal phosphate. It is generally assumed (see, for example, Gale, 1946) that cyanide inhibits pyridoxal phosphate enzymes through cyanohydrin formation, but the facts just discussed suggest that the active centre of the enzyme contains a metal in addition to pyridoxal phosphate (Metzler, Ikawa & Snell, 1954) and that cyanide reacts with this complex. However, it should be pointed out that no inhibition, but a slight activation, was observed on addition of EDTA or 8-hydroxyquinoline and no effect was obtained with azide and $\alpha\alpha'$ -dipyridyl (Table 4).

Mechanism of the reaction

No direct evidence has been obtained in the present work concerning the activation of the α -carbon atom of glycine, and the possibility that glycyl-CoA, formed by transfer from succinyl-CoA, is the reactive intermediate, cannot at this stage be ruled out. However, the simplest explanation, consistent with the evidence presented, is that the reactive form of glycine is a Schiff's base formed between this amino acid and pyridoxal phosphate bound to the enzyme and that this complex reacts with succinyl-CoA. A considerable amount of work has been done on the Schiff's bases formed under physiological conditions between pyridoxal or pyridoxal phosphate and various amino acids, including glycine (e.g. Blakley, 1955; Matsuo, 1957; Christensen, 1958). The classical studies of Erlenmeyer & Früstück (1895) and of Erlenmeyer (1899) showed that formation of a C-C bond involving the α -carbon atom of glycine is greatly facilitated by prior condensation between the amino group and an aldehyde. More recently it has been shown by Metzler, Longenecker & Snell (1953, 1954) that pyridoxal and a metal catalyse the non-enzymic condensation of formaldehyde and glycine. The reversible enzymic transformation of glycine into serine is also dependent on the presence of pyridoxal phosphate (Blakley, 1954, 1955; Heunneken, Hatefi & Kay, 1957; see also Kisliuk & Sakami, 1955) and so is the enzymic conversion of glycine and acetaldehyde into threonine (Braunshtein & Vilenkina, 1953) and allothreonine (Karasek & Greenberg, 1957). It is now postulated that pyridoxal phosphate fulfils a similar function in the acyl addition involved in ALA synthesis as it does in the aldol condensations discussed above.

The primary product of the condensation has not been identified. It has been suggested (Shemin & Russell, 1953; Neuberger & Scott, 1953) that

α -amino- β -oxoadipate is an intermediate in ALA formation, but the ease with which this compound loses carbon dioxide spontaneously after chemical synthesis has so far prevented its isolation (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work). There is therefore no information as to whether it is enzymically decarboxylated. Even if it should be possible to show such enzymic action, it could not be concluded that α -amino- β -oxoadipate is an intermediate. Although highly purified isocitric dehydrogenase catalyses the decarboxylation of oxalosuccinate (Moyle & Dixon, 1956; Siebert, Carsiotis & Plaut, 1957) it has been shown by means of isotopes that this compound is not an intermediate in the overall oxidation of isocitrate to oxoglutarate (Siebert *et al.* 1957). Similarly, oxaloacetate is not intermediate in the synthesis of malate from pyruvate and carbon dioxide by the 'malate' enzyme (Veiga Salles, Harary, Banfi & Ochoa, 1950; Harary, Korey & Ochoa, 1953).

Freeze-dried preparations have been found to form substances reacting as amino ketones when succinyl-CoA is replaced by other acyl-CoA derivatives. The reaction with acetyl-CoA is much slower than with succinyl-CoA (Fig. 3) and it is probable that propionyl-CoA and glutaryl-CoA react even more sluggishly. It is not known whether all these reactions are catalysed by the same enzyme. It is possible that the reaction with acetyl-CoA has some physiological significance, as suggested by Nemeth, Russell & Shemin (1957), since some aminoacetone is formed on incubation of whole particles with glycine, pyruvate, CoA and pyridoxal phosphate.

Inhibition with thiol reagents

The synthesis of ALA from oxoglutarate and glycine by whole particles was markedly inhibited by iodoacetamide and *p*-chloromercuribenzoate (Laver *et al.* 1958). This is probably due to interaction with coenzyme A. With freeze-dried particles and succinyl-CoA, iodoacetamide is without effect, but *p*-chloromercuribenzoate still inhibits (Table 4), almost certainly by direct combination with the enzyme (Table 5). This inhibition may not be caused by interaction with thiol groups, as it has been shown that inhibition of catalase by organic mercurials is due to causes other than blocking of thiol groups (Sohler, Seibert, Kreke & Cook, 1952). On the other hand, betaine aldehyde dehydrogenase, which is known to be a thiol enzyme, is strongly inhibited by *p*-chloromercuribenzoate, but is much less sensitive to iodoacetamide and iodoacetate (Rothschild & Barron, 1954). The question whether the enzyme responsible for the condensation of succinyl-CoA and glycine has an essential thiol group must therefore be left open.

SUMMARY

1. Preparations of freeze-dried particles obtained from erythrocytes of anaemic chickens synthesize δ -aminolaevulinic acid from succinyl-coenzyme A and glycine. This is true, whether succinyl-coenzyme A is chemically synthesized or generated enzymically from succinate or α -oxoglutarate.

2. Such preparations form little or no δ -aminolaevulinic acid from α -oxoglutarate, unless diphosphopyridine nucleotide is added. It is concluded from this and other evidence that the synthesis of δ -aminolaevulinic acid from α -oxoglutarate in whole particles, described by Laver *et al.* (1958), involves the formation of succinyl-coenzyme A by the action of α -oxoglutaric dehydrogenase.

3. The addition of pyridoxal phosphate stimulates the synthesis of δ -aminolaevulinic acid 1.5–3-fold. Evidence was obtained that pyridoxal phosphate is firmly bound to the enzyme. No other cofactor appears to be involved in the condensation of succinyl-coenzyme A and glycine.

4. *p*-Chloromercuribenzoate, cyanide, L-penicillamine and L-cysteine inhibit the reaction strongly. D-Penicillamine, D-cysteine and glutathione inhibit slightly; iodoacetamide, sodium azide, $\alpha\alpha'$ -dipyridyl and carbon monoxide have no effect, while 8-hydroxyquinoline and ethylenediamine-tetra-acetate activate slightly. Inhibition by cyanide is unchanged in the presence of a tenfold excess of pyridoxal phosphate.

5. Freeze-dried particles form aminoacetone on incubation with glycine, acetyl-coenzyme A and pyridoxal phosphate, and substances reacting as aminoketones when acetyl-coenzyme A is replaced by propionyl- and glutaryl-coenzyme A. Whole particles form aminoacetone from pyruvate and glycine. These reactions are slower than the synthesis of δ -aminolaevulinic acid.

6. It is suggested that the α -carbon of glycine is activated, before condensing with succinyl-coenzyme A, by the formation of a Schiff's base between the amino group of glycine and enzyme-bound pyridoxal phosphate. The inhibition of the synthesis of δ -aminolaevulinic acid by cyanide, penicillamine and cysteine is explained on this basis.

Grateful acknowledgement is made to the Rockefeller Foundation, New York, for the gift of a Spinco Ultracentrifuge and Roche Products Ltd., Welwyn Garden City, for supplying us with pyridoxal phosphate.

REFERENCES

- Blakley, R. L. (1954). *Nature, Lond.*, **174**, 652.
- Blakley, R. L. (1955). *Biochem. J.* **61**, 315.
- Braunshtein, A. E. & Vilenkina, G. Ya. (1953). *Uspekhi Sovremennoi Biol.* **36**, 275.

- Christensen, H. N. (1958). *J. Amer. chem. Soc.* **80**, 99.
- Cohen, P. P. (1939). *Biochem. J.* **33**, 1478.
- Dewey, D. L., Hoare, D. S. & Work, E. (1954). *Biochem. J.* **58**, 523.
- Dolin, M. I. (1955). *Arch. Biochem. Biophys.* **55**, 415.
- Dresel, E. I. B. & Falk, J. E. (1953). *Nature, Lond.*, **172**, 1185.
- du Vigneaud, V., Kuchinskas, E. J. & Horvath, A. (1957). *Arch. Biochem. Biophys.* **69**, 130.
- Erlenmeyer, E. (1899). *Liebigs Ann.* **307**, 70.
- Erlenmeyer, E. & Früstück, E. (1895). *Liebigs Ann.* **284**, 36.
- Gale, E. F. (1946). *Advanc. Enzymol.* **6**, 1.
- Gergely, J., Hele, P. & Ramakrishnan, C. V. (1952). *J. biol. Chem.* **198**, 323.
- Gibson, K. D., Laver, W. G. & Neuberger, A. (1958). *Biochem. J.* **68**, 17r.
- Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Harary, I., Korey, S. R. & Ochoa, S. (1953). *J. biol. Chem.* **203**, 595.
- Heunneken, F. M., Hatefi, Y. & Kay, L. D. (1957). *J. biol. Chem.* **224**, 435.
- Jakoby, W. B. & Bonner, D. M. (1953). *J. biol. Chem.* **205**, 699.
- Karasek, M. A. & Greenberg, D. M. (1957). *J. biol. Chem.* **227**, 191.
- Kaufman, S. (1955). In *Methods in Enzymology*, vol. 1, p. 714. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Kaufman, S., Gilvarg, C., Cori, O. & Ochoa, S. (1953). *J. biol. Chem.* **203**, 869.
- Kisliuk, R. L. & Sakami, W. (1955). *J. biol. Chem.* **214**, 47.
- Korkes, S. (1955). In *Methods in Enzymology*, vol. 1, p. 490. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Lascelles, J. (1957). *Biochem. J.* **66**, 65.
- Laver, W. G., Neuberger, A. & Udenfriend, S. (1958). *Biochem. J.* **70**, 4.
- Matsuo, Y. (1957). *J. Amer. chem. Soc.* **79**, 2011.
- Mauzerall, D. & Granick, S. (1956). *J. biol. Chem.* **219**, 435.
- Metzler, D. E., Ikawa, M. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 648.
- Metzler, D. E., Longenecker, J. B. & Snell, E. E. (1953). *J. Amer. chem. Soc.* **75**, 2786.
- Metzler, D. E., Longenecker, J. B. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 639.
- Moyle, J. & Dixon, M. (1956). *Biochem. J.* **63**, 548.
- Nemeth, A. M., Russell, C. S. & Shemin, D. (1957). *J. biol. Chem.* **229**, 415.
- Neuberger, A. & Scott, J. J. (1953). *Nature, Lond.*, **172**, 1093.
- Rothschild, H. A. & Barron, E. S. G. (1954). *J. biol. Chem.* **209**, 511.
- Sanadi, D. R. & Littlefield, J. W. (1953). *J. biol. Chem.* **201**, 103.
- Sanadi, D. R., Littlefield, J. W. & Bock, R. M. (1952). *J. biol. Chem.* **197**, 851.
- 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. & Kumin, S. (1952). *J. biol. Chem.* **198**, 827.
- Shemin, D. & Russell, C. S. (1953). *J. Amer. chem. Soc.* **75**, 4873.
- Shemin, D. & Wittenberg, J. (1951). *J. biol. Chem.* **192**, 315.
- Shuster, L. (1956). *Biochem. J.* **64**, 101.
- Siebert, G., Carsiotis, M. & Plaut, G. W. E. (1957). *J. biol. Chem.* **226**, 977.
- Simon, E. J. & Shemin, D. (1953). *J. Amer. chem. Soc.* **75**, 2520.
- Sohler, M. R., Seibert, M. A., Kreke, C. W. & Cook, E. S. (1952). *J. biol. Chem.* **198**, 281.
- Taylor, E. S. & Gale, E. F. (1945). *Biochem. J.* **39**, 52.
- Veiga Salles, J. B., Harary, I., Banfi, R. F. & Ochoa, S. (1950). *Nature, Lond.*, **165**, 675.
- Vyshepan, E. D. (1940). *Biokhimiya*, **5**, 271.

Studies on the Proteins of Fish Skeletal Muscle

5. MOLECULAR WEIGHT AND SHAPE OF COD FIBRILLAR PROTEINS†

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(Received 3 March 1958)

It was shown in a previous paper in this series (Connell, 1958) that unaggregated myosin is present in extracts of cod skeletal muscle, and that attempts to isolate the monomeric protein from the extracts by employing the usual method of precipitating by dilution failed because it aggregated spontaneously. It has now been confirmed that the

tendency of cod myosin to aggregate is much greater than that of the rabbit protein. Measurements of the molecular weight and shape of unaggregated cod myosin have been undertaken therefore to decide whether some feature of these parameters could explain the unusual behaviour of the fish protein.

Although many attempts to prepare unaggregated cod myosin from extracts containing it have

† Part 4: Connell (1958).