complete protein. The failure to detect labelled albumin could, however, be the result of very great dilution with the pool of unlabelled albumin present in the system. It is hoped that the results of an investigation now in progress into the nature of the soluble protein labelled in the microsome-cell-sap system will clarify this point.

SUMMARY

1. When a microsome-cell-sap preparation from guinea-pig liver is incubated together with 3-phosphoglycerate, as adenosine triphosphate generator, and Mg^{2+} ions, there is incorporation of [^{14}C]amino acids into the protein of both microsome and cell-sap fractions. There is a delay of about 10 min. before appreciable label appears in the protein of the cell sap.

2. There is no significant incorporation under a variety of conditions of [^{14}C]amino acids into the protein of the cell sap in the absence of microsome material.

3. When microsome material is labelled by incubation with [^{14}C]amino acids for 10 min., then reincubated with fresh unlabelled cell sap, there is an increase in the specific activity of the cell-sap protein when the system is supplemented with 3-phosphoglycerate and Mg^{2+} ions; this is accompanied by an approximately equivalent decrease in the radioactivity of the microsome protein. The evidence obtained suggests that there is transfer of radioactivity from microsome to cell-sap protein.

4. In the absence of 3-phosphoglycerate, there is a marked reduction in the extent of labelling of cell-sap protein, indicating that an adenosine triphosphate-dependent reaction is associated with the transfer of radioactivity from microsome material. Absence of Mg^{2+} ions also reduces the labelling of cell-sap protein. Replacement of 75 %

of the cell sap by 0.25 M-sucrose reduces the transfer of radioactivity from labelled microsomes, despite supplementation with adenosine triphosphate and guanosine triphosphate.

5. Microsome material labelled by incubation with [^{14}C]amino acids for 10 min. is still able to incorporate amino acids into its protein when resuspended in fresh cell sap, although at a somewhat reduced rate.

6. The labelled material present in the cell sap appears to be largely in the form of soluble protein, and the possible nature of this material is discussed.

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REFERENCES

- Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1950). *J. biol. Chem.* **187**, 839.
Campbell, P. N., Greengard, O. & Kernot, B. A. (1958). *Biochem. J.* **68**, 18r.
Craddock, V. M. & Dalgliesh, C. E. (1957). *Biochem. J.* **66**, 250.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Hendler, R. W. (1957). *J. biol. Chem.* **229**, 553.
Hultin, T. (1950). *Exp. Cell Res.* **1**, 376.
Keller, E. B. (1951). *Fed. Proc.* **10**, 206.
Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **221**, 45.
Korner, A. & Tarver, H. (1957). *J. gen. Physiol.* **41**, 219.
Petermann, M. L. & Hamilton, M. G. (1957). *J. biol. Chem.* **224**, 725.
Peters, T. (1957). *J. biol. Chem.* **229**, 659.
Rabinovitz, M. & Olson, M. E. (1956). *Exp. Cell Res.* **10**, 747.
Rabinovitz, M. & Olson, M. E. (1957). *Fed. Proc.* **16**, 235.
Rademaker, W. & Soons, J. B. J. (1957). *Biochim. biophys. Acta*, **24**, 451.
Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.
Simkin, J. L. & Work, T. S. (1957a). *Biochem. J.* **65**, 307.
Simkin, J. L. & Work, T. S. (1957b). *Biochem. J.* **67**, 617.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.

The Relationship of the Tricarboxylic Acid Cycle to the Synthesis of δ -Aminolaevulinic Acid in Avian Erythrocyte Preparations

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Shemin & Russell (1953) showed conclusively by ^{15}N - and ^{14}C -labelling experiments that δ -aminolaevulinic acid incubated with duck blood was incorporated into the haem formed. Confirmation of δ -aminolaevulinic acid as a porphyrin precursor in lysed red-cell systems was forthcoming from Neuberger & Scott (1953) and Dresel & Falk (1953).

It was postulated by Shemin & Kumin (1952) that succinyl-coenzyme A was involved in porphyrin synthesis and, upon the recognition of δ -aminolaevulinic acid as a porphyrin precursor, it was suggested that succinyl-coenzyme A coupled with glycine to form δ -aminolaevulinic acid via the unstable α -amino- β -oxoadipic acid (Shemin & Russell, 1953).

The evidence for the involvement of succinyl-coenzyme A is that an unsymmetrical four-carbon unit closely related to succinate is concerned in porphyrin biosynthesis (Shemin & Kumin, 1952) and that coenzyme A stimulates the synthesis from succinate.

The present work was initiated in an attempt to providemore direct evidence for succinyl-coenzyme A being a precursor of δ -aminolaevulinic acid and at the same time to explore more fully the relationships between the tricarboxylic acid cycle and the initial stages of porphyrin biosynthesis. A system prepared from normal chicken erythrocytes and capable of synthesizing efficiently δ -aminolaevulinic acid *in vitro* has been employed for this purpose.

During the course of the investigations described here, two communications have appeared (Laver & Neuberger, 1957; Gibson, Laver & Neuberger, 1958) which describe work with systems prepared from the erythrocytes of hens rendered anaemic with phenylhydrazine. The utilization of succinyl-coenzyme A for δ -aminolaevulinic acid synthesis has been demonstrated in these systems.

EXPERIMENTAL

Materials

The blood used was collected from normal adult domestic fowls (*Gallus domesticus*) pooled for marketing. Collection was made into a polythene bottle containing penicillin G, streptomycin and heparin as described by Dresel & Falk (1954).

Citric acid (A.R.), fumaric acid, succinic acid and malic acid were obtained from British Drug Houses Ltd. Fumaric and succinic acids were recrystallized as sodium salts from aqueous ethanol. Malic acid was recrystallized from *iso*-butyl methyl ketone and chloroform (Montgomery & Webb, 1956a). Oxaloacetic acid, α -oxoglutaric acid and DL-*iso*-citric acid lactone were obtained from L. Light and Co. Ltd. The lactone was hydrolysed before use, as described by Krebs & Eggleston (1944). Adenosine triphosphate (ATP) and coenzyme A (CoA) were supplied by the Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. D-*iso*-Citric acid lactone was a gift from Sir Hans Krebs, F.R.S., fluorocitric acid a gift from Sir Rudolph Peters, F.R.S., and α -lipoic acid a gift from Dr J. A. Elvidge. 5- δ -Dimethylbenzimidazole was a gift from Glaxo Ltd. (through the good offices of Dr T. F. Macrae). Parapyruvate (γ -hydroxy- γ -methyl- α -oxoglutaric acid) was a gift from Dr H. Heath.

Methods

Preparation of tissue system. Chick erythrocytes were washed, lysed and restored to isotonicity as described by Dresel & Falk (1956). After centrifuging at about 1800 g for 15 min., the supernatant from the haemolysate was discarded and the precipitate was washed three times by re-suspension in 0.9% NaCl followed each time by recentrifuging. The volume of the washed cell debris was then measured and to each 4 ml. was added 1 ml. of 0.9% KCl. The preparation was stirred well to ensure homogeneity

and stored on crushed ice. All steps in the above procedure were carried out at about 4°. The preparation so obtained contained all the succinic-oxidase activity (as assayed in the Warburg respirometer) of the original haemolysate and synthesized δ -aminolaevulinic acid (ALA) from glycine and succinate without concomitant production of detectable amounts of porphobilinogen or porphyrins.

Incubation. Incubation was conducted in 50 ml. Erlenmeyer flasks at 38° with shaking at about 100 shakes/min. Except where otherwise stated, 2 ml. of preparation was incubated in each flask in the presence of 0.5 ml. of 0.5M-MgCl₂, 0.05 ml. of 5 mM-pyridoxal 5'-phosphate, 1.2 ml. of 0.1M-phosphate buffer (pH 6.8) and 0.9% (w/v) KCl to bring the total volume after the addition of substrates, co-factors, etc. to 5 ml.

Identification and estimation of δ -aminolaevulinic acid. ALA was identified by paper chromatography after prior conversion into 4,2'-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole by the method of Mauzerall & Granick (1956). The pyrrole was extracted with ether and chromatographed alongside, and as a mixed sample with, a specimen of the pyrrole prepared from authentic ALA. Ascending chromatograms were run on Whatman no. 1 paper with ammonia (sp.gr. 0.880)-water-butanol (2:98:100, by vol.). The pyrrole was detected as a pink spot (R_F 0.40) on spraying the paper with the *p*-dimethylaminobenzaldehyde reagent described by Mauzerall & Granick (1956).

For estimation of ALA the reaction mixture (5 ml.) was partially deproteinized by treatment with 0.5 ml. of 10% (w/v) solution of ZnSO₄·7H₂O. After centrifuging, Mauzerall & Granick's (1956) procedure was applied to a measured volume of the supernatant, small quantities of residual protein brought down during heating being removed by recentrifuging.

That the material estimated as ALA was in fact this substance was verified by the chromatographic technique described each time a new substrate or addition was made to the incubate.

Identification of keto acids. Keto acids were identified by paper chromatography of their 2,4-dinitrophenylhydrazones as described by Cavallini, Frontali & Toschi (1949a, b).

Identification of hydroxamates. Hydroxamates were separated and identified by the paper-chromatographic method described by Stadtman & Barker (1950).

RESULTS

Glycine and succinate as substrates

Fig. 1 illustrates the relationship between production of ALA and time of incubation. It shows increasing synthesis which attains a plateau after a period of about 3 hr. Except where otherwise indicated, an incubation period of 3 hr. was used, therefore, in the ensuing experiments. The relatively marked loss of activity by the system on ageing can also be seen in Fig. 1.

The effect of glycine concentration on synthesis of ALA is shown in Fig. 2. The curve does not pass through the origin because of the presence of a small amount of endogenous glycine. Maximal synthesis was obtained with glycine concentrations of the order of 30 mM.

The relationship between succinate concentration and the production of ALA under the standard conditions of incubation and glycine concentration is illustrated in Fig. 3, maximal effects being observed with succinate levels of about 10 mM. The enzyme system was fully saturated when these substrates were present in the concentrations indicated.

Fig. 1. Effect of duration of incubation on ALA synthesis. ●, Fresh preparation; ○, preparation which had been stored on crushed ice for 24 hr. before use. Preparations contained 25 mM-succinate and 30 mM-glycine, and were incubated under the standard conditions described in the text.

Fig. 2. Effect of glycine concentration on formation of ALA. Succinate was present in a concentration of 25 mM. Incubations were conducted under standard conditions.

Fig. 3. Effect of succinate concentration on production of ALA. Glycine was present in a concentration of 30 mM. Incubations were conducted under standard conditions.

Glycine and other tricarboxylic cycle intermediates as substrates

Various intermediates of the tricarboxylic acid cycle at molar concentrations comparable to that found to give maximal synthesis of ALA from succinate (i.e. 10 mM) were examined for capacity to replace succinate in the system. The results of this preliminary screening are shown in Table 1. They indicate vast superiority of citrate and *iso*-citrate over either α -oxoglutarate or succinate as substrate for synthesis of ALA. Fumarate, malate and oxaloacetate had very little activity in this respect. The small but detectable yield of ALA obtained without added substrate is due to traces of endogenous substrate in the preparation (see also Fig. 3).

More detailed examination of citrate, *isocitrate* and α -oxoglutarate, at various concentrations, as alternative substrates to succinate was made and the results are recorded in Fig. 4. It is noted that excess of these substrates is inhibitory.

Table 1. Comparison of various tricarboxylic acid cycle intermediates as substrates for the synthesis of δ -aminolaevulic acid

Incubations were conducted under standard conditions (see under Methods) with 30 mM-glycine. All substrates were examined at a concentration of 25 mM. No change in pH was observed after incubation with any of the substrates.

Substrate	ALA (μ m-moles)
Citrate	181
<i>D</i> -isoCitrate	198
α -Oxoglutarate	93
Succinate	68
Fumarate	6
Malate	10
Oxaloacetate	21
None added	4

Fig. 4. Comparison of citrate, *D*-isocitrate and α -oxo-glutarate with succinate as substrates for synthesis of ALA. ●, Citrate; ○, *D*-isocitrate; ■, α -oxoglutarate; □, succinate. Incubations were conducted under standard conditions in the presence of 30 mM-glycine.

Paper chromatography of the above incubates for identification of keto acids showed the formation of α -oxoglutarate from citrate, *isocitrate* and succinate although with succinate the amount, as judged visually from the fluorescence of the dinitrophenylhydrazones in ultraviolet light, was very small compared with that formed from the two other substrates.

Effect of various co-factors. Pyridoxal 5'-phosphate (50 μ M) was added to the incubates as a matter of routine throughout the present work as a result of the report of Schulman & Richert (1956) that it is involved in the early stages of porphyrin synthesis before the formation of ALA. Omitting it from the system caused a decrease of approximately 40% in ALA synthesis, as can be seen from Table 2.

Other possible co-factors examined for effect on ALA synthesis by the present preparation were CoA, ATP, α -lipoic acid, thiamine pyrophosphate and the ribosides adenosine, guanosine and inosine. The results are shown in Table 2. CoA was found to stimulate ALA synthesis from each of the three tricarboxylic acid cycle intermediates, *isocitrate*, α -oxoglutarate and succinate. ATP, however, exerted a stimulatory influence only when succinate was the substrate and produced slight but reproducible inhibition when either *isocitrate* or

α -oxoglutarate was being utilized for the production of ALA. α -Lipoic acid produced an increase in ALA yield from *isocitrate* or α -oxoglutarate but had little effect on that from succinate. Neither thiamine pyrophosphate nor the ribosides had any significant influence on ALA synthesis from any of the three substrates examined.

Inhibitors. Fluorocitrate, a known inhibitor of aconitase (Morrison & Peters, 1954), was examined for effect on ALA synthesis from each of the four tricarboxylic acid cycle intermediates, citrate, *isocitrate*, α -oxoglutarate and succinate. The results recorded in Table 3 show, as would be expected if the tricarboxylic acid cycle is the main pathway by which a C_4 unit is provided for ALA synthesis, a marked inhibition of ALA synthesis from citrate but not from the other three substrates.

Another inhibitor examined was parapyruvate (γ -hydroxy- γ -methyl- α -oxoglutaric acid). This substrate was found by Montgomery & Webb (1956*b*) and Montgomery, Fairhurst & Webb (1956) to be an efficient and specific inhibitor of the oxidative decarboxylation of α -oxoglutarate by guinea-pig-heart mitochondria. The results with this material, shown in Table 3, indicate a suppression of ALA formation from citrate, *isocitrate* and α -oxoglutarate but not from succinate.

Table 2. *Effect of possible co-factors on the synthesis of δ -aminolaevulinic acid from isocitrate, α -oxoglutarate or succinate*

Standard incubation procedure was followed with 30 mM-glycine, 25 mM- α -oxoglutarate, 25 mM-succinate and 80 mM-DL-*isocitrate* (experimentally found to be equivalent, in synthesis of δ -aminolaevulinic acid by this preparation, to 25 mM-D-*isocitrate*).

Additions	ALA (μ -moles)		
	<i>iso</i> Citrate	α -Oxoglutarate	Succinate
None	144	76	58
None (pyridoxal 5'-phosphate omitted)	82	43	33
ATP (0.5 mM)	124	48	164
CoA (0.5 mM)	218	114	146
Thiamine pyrophosphate (mM)	126	70	54
α -Lipoic acid (mM)	216	91	60
Adenosine (mM)	145	76	58
Guanosine (mM)	140	72	52
Inosine (mM)	145	72	57

Table 3. *Effect of fluorocitrate and parapyruvate on δ -aminolaevulinic acid formation*

After pre-incubation of inhibitors and preparation for 15 min. under standard conditions plus 30 mM-glycine, substrates were added and the incubation conducted for a further 3 hr. Citrate, α -oxoglutarate and succinate were used in a concentration of 25 mM and DL-*isocitrate* was employed at a concentration equivalent to 25 mM-D-*isocitrate*. Fluorocitrate was 0.23 mM and parapyruvate 4 mM. Percentage inhibitions are shown in parentheses.

Inhibitor	ALA (μ -moles)			
	Citrate	<i>iso</i> Citrate	α -Oxoglutarate	Succinate
None	121	126	55	41
Fluorocitrate	48 (60%)	124 (1%)	55 (0%)	40 (2%)
Parapyruvate	16 (87%)	15 (88%)	12 (78%)	45 (0%)

Antagonistic effect of succinate on the utilization of citrate and isocitrate

In an experiment designed to test the effect of simultaneous addition of more than one substrate to the system, it was observed that ALA formation from succinate and isocitrate together was considerably less than that obtained in a control incubate containing isocitrate alone.

More detailed examination of this effect indicated that increasing concentrations of succinate progressively depressed ALA synthesis until a level was attained comparable with the maximal yield obtained with succinate alone. Further, it was found that the inhibition could be reversed by increasing the isocitrate concentration although, as can be seen from Fig. 4, this substrate was already at its optimum concentration for ALA synthesis. The results of a typical experiment are depicted in Fig. 5.

This inhibitory effect of succinate on ALA formation could also be demonstrated when either citrate or α-oxoglutarate was the second substrate. In the latter case, however, the inhibition was potentiated and not reversed by increasing the concentration of the second substrate (α-oxoglutarate). This is shown in Fig. 6.

Fig. 5. Succinate inhibition of synthesis of ALA from isocitrate and its reversal by increasing concentrations of isocitrate. DL-isocitrate, prepared by hydrolysis of the lactone, was present initially at its optimum concentration for ALA synthesis (equivalent to 30 mM-D-isocitrate). At the point indicated by the arrow increasing amounts of the DL-isocitrate preparation were added. Incubations were conducted under standard conditions in the presence of 30 mM-glycine.

The depression of ALA synthesis from citrate by addition of succinate could not be alleviated by CoA or α-lipoic acid (Table 4). ATP, however, did relieve the inhibition to a slight extent.

Nature of the inhibitory effect of succinate. In order to elucidate the mode of action of succinate as an inhibitor of ALA synthesis from citrate, isocitrate or α-oxoglutarate, other tricarboxylic acid cycle intermediates were examined for similar effects. The primary product of succinate oxidation in the tricarboxylic acid cycle is fumarate, and at equimolar concentration it was found to be even

Fig. 6. Succinate inhibition of synthesis of ALA from α-oxoglutarate and its potentiation by increasing amounts of α-oxoglutarate. Incubations were conducted under standard conditions in the presence of 30 mM-glycine.

Table 4. Inhibition by succinate of synthesis of δ-aminolaevulic acid from citrate in presence of co-factors

Incubations were carried out under standard conditions with glycine and citrate, both at a concentration of 30 mM. Succinate was used at 10 mM. CoA and ATP were employed at concentrations of 0.5 mM, α-lipoic acid at 10 mM and thiamine pyrophosphate (TPP) at 10 mM.

Additions	ALA (μm-moles)	Inhibition (%)
None	149	66
Succinate	50	
CoA	311	65
Succinate + CoA	109	
ATP	140	40
Succinate + ATP	84	
α-Lipoic acid + TPP	215	62
Succinate + α-lipoic acid + TPP	82	

more efficient than succinate in depressing ALA formation. Malonate (10 mM) was found to alleviate the inhibition by succinate to a significant extent. The results are recorded in Table 5.

The observations were interpreted as indicating that succinate exerted its depressant effect on the synthesis of ALA only after prior conversion into fumarate. Consequently, other tricarboxylic acid cycle intermediates lying after fumarate in the oxidative direction of the cycle were also investigated. The results, shown in Table 6, show that

Table 5. *Comparison of fumarate and succinate as inhibitors of the synthesis of δ -aminolaevulinic acid from citrate and the effect of malonate on the inhibitions*

Incubations were carried out under standard conditions with glycine and citrate both at a concentration of 30 mM. Succinate, fumarate and malonate were used at a level of 10 mM.

Additions	ALA (μ m-moles)	Inhibition (%)
None	94	—
Succinate	38	60
Fumarate	19	80
Succinate + malonate	54	42
Malonate	66	30

Table 6. *Comparison of malate, oxaloacetate and acetate with succinate and fumarate as inhibitors of the formation of δ -aminolaevulinic acid from citrate*

All incubates contained 30 mM-glycine and 25 mM-citrate. Incubation was under standard conditions. All additions were made at a level of 25 mM.

Additions	ALA (μ m-moles)	Inhibition (%)
None	195	—
Succinate	80	59
Fumarate	4	98
Malate	26	87
Oxaloacetate	24	88
Acetate	198	0

malate and oxaloacetate also depress ALA formation from citrate more efficiently than does succinate, although not to as low a level as does fumarate.

Formation of succinyl-coenzyme A and synthesis of δ -aminolaevulinic acid

By using similar incubation conditions to those described in the Methods section, but omitting glycine and pyridoxal phosphate, acyl-CoA derivatives were trapped with hydroxylamine and estimated by the method of Lipmann & Tuttle (1945). The formation of ALA was determined in parallel incubations by using the complete system. The results are shown in Table 7. Paper-chromatographic checks on the hydroxamates formed, by the method of Stadtman & Barker (1950), showed that the only detectable product from these incubates was succinohydroxamic acid.

The results show that α -oxoglutarate is a much better substrate for succinyl-CoA formation than was either citrate or succinate, as was to be expected if succinyl-CoA was produced directly from α -oxoglutarate in the tricarboxylic acid cycle. However, α -oxoglutarate is a less efficient substrate than is citrate (Tables 1 and 7) for the synthesis of ALA; the reasons for this are discussed below. Table 7 also indicates that fumarate must inhibit the synthesis of ALA from citrate at some step before the formation of succinyl-CoA.

Effect of 5:6-dimethylbenzimidazole. Abbott & Dodson (1954) showed that 5:6-dimethylbenzimidazole inhibited the incorporation of [15 N]glycine into haem by chicken-erythrocyte preparations and that this could be traced to an inhibition at a level before the formation of ALA in porphyrin synthesis (Abbott, Dodson & Auvil, 1956).

5:6-Dimethylbenzimidazole was examined in the present system at a concentration (7.5 mM) which had been previously found to give maximal inhibition. The results, illustrated in Table 7, show that 5:6-dimethylbenzimidazole produces an apparent

Table 7. *Comparison of the formation of succinyl-coenzyme A and δ -aminolaevulinic acid under various conditions*

Preparations for ALA assay were incubated under the standard conditions with 30 mM-glycine. Citrate, α -oxoglutarate and succinate were 30 mM, fumarate 10 mM and 5:6-dimethylbenzimidazole 7.5 mM. Incubations for hydroxamate estimation were similar, but pyridoxal phosphate was omitted and hydroxylamine (40 mM, pH 7.0) replaced glycine.

Substrate	Additions	Succino- hydroxamic acid (μ m-moles)	ALA (μ m-moles)
Citrate	—	410	134
Citrate	Fumarate	230	6
Citrate	5:6-Dimethylbenzimidazole	550	27
Citrate	α -Oxoglutarate	610	99
α -Oxoglutarate	—	650	60
Succinate	—	270	45
Succinate	5:6-Dimethylbenzimidazole	540	10

increase in succinyl-CoA formation and yet inhibits ALA synthesis by about 80 %. The implications of this are discussed below.

Table 8 shows the result of increasing the glycine and pyridoxal 5'-phosphate concentrations in systems in which ALA synthesis from citrate was partly inhibited by 5:6-dimethylbenzimidazole. The inhibition was apparently relieved to some extent by increasing the glycine concentration, but not by increasing the amount of pyridoxal 5'-phosphate present.

DISCUSSION

For the purposes of the present investigation, an enzyme system was desired which would synthesize ALA in measurable quantity but in which synthesis would not proceed further. In addition, it was considered highly desirable that such a system should originate from normal animals, since intoxication with drugs such as phenylhydrazine could conceivably impair or disorganize the tricarboxylic acid cycle (cf. Sprinz & Waldschmidt-Leitz, 1953; Geber & Rostorfer, 1954) the relation of which to ALA synthesis it was desired to investigate. A suitable system was found in the fowl-erythrocyte preparation described here.

Examination of various tricarboxylic acid cycle intermediates (Table 1, Fig. 1) showed that the following descending order of efficiency as substrates for ALA synthesis exists in this system: *isocitrate*, citrate, α -oxoglutarate, succinate, oxaloacetate, malate, fumarate. Citrate is apparently utilized for ALA synthesis after prior conversion into *isocitrate*, as was shown (Table 3) by the use of the specific inhibitor of aconitase, namely fluoro-citrate.

*iso*Citrate appears to be converted into α -oxoglutarate before the formation of ALA, as has been indicated by: (i) paper-chromatographic identification of keto acids formed; (ii) the inhibition of the synthesis of ALA from these two substrates by

parapyruvate, a specific inhibitor of the oxidative decarboxylation of α -oxoglutaric acid (Table 3); and (iii) the stimulation of the formation of ALA from these substrates by α -lipoic acid, an essential component of the α -oxoglutaric oxidative decarboxylation system (Table 2). These interconversions are all consistent with the presence of an active tricarboxylic acid cycle in the present system.

Confirmation of the involvement of CoA in the synthesis of ALA was obtained by the large stimulatory capacity of this co-factor on all substrates examined. ATP stimulated the formation of ALA from succinate, but not from *isocitrate* or α -oxoglutarate, with which, on the contrary, it produced slight but reproducible inhibitions (Table 2). These observations are in agreement with the direct formation of succinyl-CoA from succinate (requiring ATP) as well as from α -oxoglutarate (not requiring ATP) according to the scheme (cf. Shemin & Kumin, 1952; Wriston, Lack & Shemin, 1955; Kaufman, Gilvarg, Cori & Ochoa, 1953):

Production of succinyl-CoA from citrate, *iso*-citrate or α -oxoglutarate would, however, generate ATP without requiring it at any stage; hence it seems that a relatively large exogenous pool of ATP might depress the formation of succinyl-CoA from these substrates. An analogous situation has been reported by Sanadi & Littlefield (1951) in the inhibition by added ATP of the succinylation of sulphanilamide, when using a coupled α -oxoglutaric 'dehydrogenase' and pigeon-liver acylating system.

The inhibition by succinate of the synthesis of ALA from citrate or *isocitrate* brought the ALA level down to that obtained by using succinate alone (Figs. 4, 5). This confirms the existence of two pathways for succinyl-CoA formation: (i) from α -oxoglutarate, and (ii) from succinate directly; and it is compatible with the scheme shown above.

The inhibitory effect of succinate has been shown (Tables 5, 6) to be attributable not to succinate but to the formation of fumarate, malate or oxaloacetate. The yields of ALA from citrate in the presence of each of these acids appear to be influenced by two complimentary factors: the inhibitory effect and the partial utilization of these inhibitory acids as substrates (compare Tables 1 and 6). This explains why ATP appears to alleviate the succinate inhibition to some degree, for it has already been shown that this co-factor stimulates the utilization of succinate for the synthesis of ALA (Table 4).

Table 8. *Effect of increasing the glycine and pyridoxal 5'-phosphate concentrations on the inhibition of synthesis of 8-aminolaevulinic acid by 5:6-dimethylbenzimidazole*

Incubations were under standard conditions. 5:6-Dimethylbenzimidazole (DMB) was used at a concentration of 7.5 mM. Citrate (30 mM) was used throughout as substrate.

Additions	ALA (μ m-moles)
Glycine (30 mM)	84
Glycine (30 mM) + DMB	4
Glycine (30 mM) + DMB + pyridoxal 5'-phosphate to a final concentration of 0.5 mM	3
Glycine (0.1 mM)	78
Glycine (0.1 mM) + DMB	24

If allowance is made for utilization of these dicarboxylic acids as substrates, it appears, by comparing Tables 1 and 6, that oxaloacetate is probably as effective as fumarate in inhibiting the formation of ALA from citrate. Further, as the tricarboxylic acid cycle is very active in the oxidative direction in this preparation, it would seem most likely that, of fumarate, malate and oxaloacetate, the last is the real inhibitor and that the other two acids mediate their effects only after oxidation to oxaloacetate.

The inhibitory effect of these dicarboxylic acids on the synthesis of ALA from citrate appears to be due to a suppression of the oxidative decarboxylation of α -oxoglutaric acid. Evidence for this is: (i) utilization of α -oxoglutarate for the formation of ALA is also inhibited by these acids (Fig. 6); (ii) the formation of succinyl-CoA from citrate is inhibited in the system in their presence (Table 7), i.e. the inhibition is between α -oxoglutarate and formation of succinyl-CoA. In speculating on purely structural grounds, it might be suggested that oxaloacetate competes in some way with α -oxoglutarate in the oxidative decarboxylation system.

The inhibition does not appear to be relieved by CoA or α -lipoic acid. It can, however, be completely reversed by increasing the citrate concentration (Fig. 5) and hence, by inference, the endogenous α -oxoglutarate concentration. However, this is not the same as adding α -oxoglutarate exogenously, as can be seen from Fig. 6; this is further discussed below.

In the case of succinate-inhibition of the synthesis of ALA from added α -oxoglutarate, the inhibition cannot be relieved by increasing the substrate concentration (Fig. 6). The reason for this can be seen in Table 7, for, although α -oxoglutarate is the most efficient of the substrates examined for the production of succinyl-CoA, it is much less efficient than either citrate or *isocitrate* as a substrate for ALA synthesis. This means that α -oxoglutarate is itself inhibiting the condensation of succinyl-CoA and glycine to form ALA.

The last-mentioned observation affords an explanation for some other apparently anomalous results. First, it explains the marked inhibition of the synthesis of ALA when excess of citrate, *isocitrate* or α -oxoglutarate is used (Fig. 4). Secondly, it shows why the production of ALA from α -oxoglutarate is less than that from citrate; for in the latter case α -oxoglutarate would be generated enzymically in a steady trickle and never attain significantly inhibitory concentrations, as when α -oxoglutarate is supplied exogenously.

5:6-Dimethylbenzimidazole appears, from Table 7, also to be exerting its inhibitory effect on the synthesis of ALA at the condensation step between

succinyl-CoA and glycine. The reasons for this are not obvious, but the fact that the inhibition can be partly relieved by increasing the glycine concentration (Table 8) suggests that 5:6-dimethylbenzimidazole might act in suppressing activation of glycine by pyridoxal phosphate for the condensation. This postulation would explain the apparent increase in succinyl-CoA formation in the presence of 5:6-dimethylbenzimidazole, for without inhibition some succinyl-CoA would be drawn off to form ALA by combination with the small but significant amount of endogenous glycine in the preparation (see Fig. 1), with consequent decrease in the amount of succinyl-CoA detectable as hydroxamate.

Because of the observation that not all succinyl-CoA produced is trapped as hydroxamate, no attempt has been made to calculate the efficiency of the conversion of succinyl-CoA into ALA from the figures in Table 7.

The demonstration, in the present system, of antagonisms and inhibitions in the synthesis of ALA by normal tissue metabolites poses the interesting question whether these factors play a role in co-ordinating cellular metabolism, particularly in the biogenesis of ALA and its further utilization ('succinate-glycine cycle', porphyrin synthesis, etc.). In the present experiments these antagonisms and inhibitions result in drastic suppression of the synthesis of ALA, but, with more nearly physiological concentrations such as exist *in vivo*, they may well represent mechanisms by which the synthesis is normally regulated.

Further work upon this problem is in progress, with particular reference to the mechanism of inhibition by 5:6-dimethylbenzimidazole.

SUMMARY

1. A preparation of lysed normal chick erythrocytes has been described which actively synthesized δ -aminolaevulinic acid from glycine in the presence of various tricarboxylic acid cycle intermediates, but which does not produce porphobilinogen or porphyrins.

2. The tricarboxylic acid cycle intermediates fall into the following decreasing order of effectiveness in δ -aminolaevulinic acid synthesis: *isocitrate*, citrate, α -oxoglutarate, succinate, oxaloacetate, malate and fumarate.

3. Pyridoxal 5'-phosphate and coenzyme A stimulate the synthesis from all substrates examined. Adenosine triphosphate stimulates the production of δ -aminolaevulinic acid from succinate but slightly inhibits that from citrate, *isocitrate* and α -oxoglutarate. α -Lipoic acid enhances synthesis from citrate, *isocitrate* and α -oxoglutarate, but not from succinate.

4. Use of the inhibitors fluorocitrate and parapyruvate shows that the interconversion of substrates before the formation of δ -aminolaevulinic acid is consistent with an active tricarboxylic acid cycle.

5. Succinate inhibits the synthesis of δ -aminolaevulinic acid by suppressing the formation of succinyl-coenzyme A from citrate, isocitrate or α -oxoglutarate after itself being first converted into fumarate, malate or oxaloacetate. The inhibition is reversed by increasing concentrations of citrate or isocitrate, but is potentiated by α -oxoglutarate.

6. α -Oxoglutarate added exogenously inhibits the condensation of glycine and succinyl-coenzyme A in the formation of δ -aminolaevulinic acid.

7. 5:6-Dimethylbenzimidazole also inhibits the condensation of glycine and succinyl-coenzyme A. The inhibition here is partly relieved by increasing concentrations of glycine, but not by increasing concentrations of pyridoxal 5'-phosphate.

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REFERENCES

- Abbott, L. D. & Dodson, M. J. (1954). *J. biol. Chem.* **211**, 845.
- Abbott, L. D., Dodson, M. J. & Auvil, D. K. (1956). *Fed. Proc.* **15**, 208.
- Cavallini, D., Frontali, N. & Toschi, G. (1949*a*). *Nature, Lond.*, **163**, 568.
- Cavallini, D., Frontali, N. & Toschi, G. (1949*b*). *Nature, Lond.*, **164**, 792.
- 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). *Biochem. J.* **63**, 80.
- Geber, W. F. & Rostorfer, H. (1954). *Fed. Proc.* **13**, 52.
- Gibson, K. D., Laver, W. G. & Neuberger, A. (1958). *Biochem. J.* **68**, 17*P*.
- Kaufman, S., Gilvarg, C., Cori, O. & Ochoa, S. (1953). *J. biol. Chem.* **203**, 869.
- Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
- Laver, W. G. & Neuberger, A. (1957). *Biochem. J.* **67**, 22*P*.
- Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
- Mauzerall, D. & Granick, S. (1956). *J. biol. Chem.* **219**, 435.
- Montgomery, C. M., Fairhurst, A. S. & Webb, J. L. (1956). *J. biol. Chem.* **221**, 369.
- Montgomery, C. M. & Webb, J. L. (1956*a*). *J. biol. Chem.* **221**, 347.
- Montgomery, C. M. & Webb, J. L. (1956*b*). *J. biol. Chem.* **221**, 359.
- Morrison, J. F. & Peters, R. A. (1954). *Biochem. J.* **58**, 473.
- Neuberger, A. & Scott, J. J. (1953). *Nature, Lond.*, **172**, 1093.
- Sanadi, D. R. & Littlefield, J. W. (1951). *J. biol. Chem.* **193**, 683.
- Schulman, M. P. & Richert, D. A. (1956). *Fed. Proc.* **15**, 349.
- Shemin, D. & Kumin, S. (1952). *J. biol. Chem.* **198**, 827.
- Shemin, D. & Russell, C. S. (1953). *J. Amer. chem. Soc.* **75**, 4873.
- Sprinz, H. & Waldschmidt-Leitz, E. (1953). *Hoppe-Seyl. Z.* **293**, 16.
- Stadtman, E. R. & Barker, H. A. (1950). *J. biol. Chem.* **184**, 769.
- Wriston, J. C., Lack, L. & Shemin, D. (1955). *J. biol. Chem.* **215**, 603.

Bound Amino Acids in Protein-free Extracts of Italian Ryegrass

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Protein-free diffusates from ryegrass were studied by Synge (1951), who showed that, whilst the greater part of the nitrogen is present as free amino acids, a substantial proportion (usually about 5% of the nitrogen of the diffusates) could be segregated in the form of chemically bound amino acids which could be set free by acid hydrolysis. These fractions of diffusate, obtained by electrical transport in a diaphragm cell, contained also much carbohydrate; their nitrogen content was not

usually much more than 0.1% on dry matter. In the present paper we describe subsequent experiments aimed at characterizing more closely the forms in which bound amino acids occur in diffusates from grass. We have worked chiefly with Italian ryegrass (*Lolium multiflorum* Lam.), but have not found any essential differences from results previously obtained with perennial ryegrass (*L. perenne* L.). A few isolated experiments with diffusates from such diverse leaves as those of clover, raspberry, nettle, cabbage and potato showed that by the diaphragm-cell procedure

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