STUDIES ON PORPHYRIN BIOSYNTHESIS AND ON RELATED TOPICS IN RHODOPSEUDOMONAS SPHEROIDES

STUDIES ON PORPHYRIN BIOSYNTHESIS AND ON RELATED TOPICS IN RHODOPSEUDOMONAS SPHEROIDES

A thesis submitted by

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in partial fulfilment of the requirements
for the Degree of
Doctor of Philosophy
in the
University of London.

July 1961

St. Mary's Hospital Medical School,
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ABSTRACT

All the work has been done with the photosynthetic microorganism Rhodopseudomonas spheroides. On incubation under suitable conditions large amounts of an aminoketone, which has been identified as aminoacetone, accumulate. Although aminoacetone is an analogue of δ-aminolaevulic acid it has no effect on enzyme reactions involving δ-aminolaevulic acid, nor has it any significant effect on porphyrin or bacteriochlorophyll biosynthesis in whole organisms.

Two diphosphopyridine nucleotide enzymes have been found. One converts threonine to aminoacetone, the other dehydrogenates β-hydroxybutyric acid. These enzymes have been partially purified and their properties studied.

An L-alanine-δ-aminolaevulic acid transaminase has been discovered and its properties investigated. It is possible that it plays a part in the control of porphyrin and bacteriochlorophyll biosynthesis.

The effect of various growth conditions which are unfavourable for bacteriochlorophyll biosynthesis - biotin, nicotinic acid and thiamine deficiency, and growth in acetate - has been studied. Under all these conditions the specific activity of δ-aminolaevulic acid synthetase is reduced when compared with the activity in organisms grown under optimal conditions. In some of these conditions δ-aminolaevulic acid dehydrase activity is also reduced, while the activities of the three enzymes mentioned above are unaffected.

Ethionine and threonine, alone among a large number of compounds

tested, inhibit bacteriochlorophyll synthesis and stimulate accumulation of free porphyrins, when added to organisms illuminated in a medium containing α-oxoglutarate and glycine. This effect is specifically reversed by methionine and homocysteine. Results with radioactive methionine suggest that it donates its methyl group directly to form the methyl ester group of bacteriochlorophyll, and this is probably the step blocked by ethionine and threonine.

Haemin has been found to inhibit δ-aminolaevulic acid synthetase, and protoporphyrin, δ-aminolaevulic acid dehydrase, in cell-free extracts.

These results, together with those of other workers, suggest possible mechanisms by which Rhodopseudomonas spheroides controls bacteriochlorophyll biosynthesis.

A C K N O W L E D G E M E N T S

I am greatly indebted to Professor A. Neuberger, F.R.S. for his supervision, for his interest and encouragement throughout, and for his many helpful suggestions, without which this work would not have been accomplished.

I would also like to express my appreciation to Dr. K.D. Gibson for his interest and for the many fruitful discussions we had concerning various aspects of this work.

I am grateful to Dr. J. Lascelles of the Microbiology Unit, Department of Biochemistry, University of Oxford for supplying the culture of Rhodopseudomonas spheroides, and for a most stimulating discussion which we had soon after I started this work, and to Dr. D.F. Elliott of the National Institute for Medical Research, Mill Hill, London, for kindly supplying D-threonine, L-allothreonine and D-allothreonine.

I wish to thank other members of this Department for their interest and co-operation, and for supplying compounds which they had prepared. The origin of these compounds is acknowledged in the text.

Thanks are due to the Carnegie Trust for the Universities of Scotland for a Scholarship during the tenure of which this work was carried out.

CONTENTS

ABSTRACT ... ... (i)

ACKNOWLEDGEMENTS ... ... (iii)

CONTENTS ... ... (iv)

TABLES ... ... (vii)

FIGURES ... ... (x)

INTRODUCTION 1

MATERIALS AND METHODS 14

Chemicals 14
Organisms 15
Growth of organisms 15
Procedure for the study of porphyrin synthesis with suspensions of organisms 16
Preparation of enzyme extracts 17
Estimations 18
Assay of enzyme activities 19

EXPERIMENTAL AND RESULTS 21

ACCUMULATION OF AN AMINOKETONE BY WHOLE ORGANISMS UNDER SUITABLE INCUBATION CONDITIONS; ITS PURIFICATION AND IDENTIFICATION 21

Accumulation of aminoketones 22
Purification of the aminoketones 26
Identification of the aminoketones 28

THREONINE DEHYDROGENASE ACTIVITY IN EXTRACTS 32

Mechanisms of L-threonine dehydrogenase action 35
Partial purification of the L-threonine dehydrogenase 36

Experiments with purified L-threonine dehydrogenase 39
Stoichiometry of L-threonine dehydrogenase reaction 39
Effect of inhibitors on L-threonine dehydrogenase 41
Activity of DPN+ analogues with L-threonine dehydrogenase 45
Michaelis constants of L-threonine dehydrogenase 47

β-HYDROXYBUTYRIC ACID DEHYDROGENASE ACTIVITY IN EXTRACTS 47

Assay system for β-hydroxybutyric acid dehydrogenase 49
Michaelis constants for β-hydroxybutyric acid dehydrogenase 52

δ-AMINOLAEVULIC ACID TRANSAMINASE IN EXTRACTS 54

Identification of the products of the reaction 58
Ability of amino acids other than L-alanine to transaminate with γδ-dioxovaleric acid 60
Transamination between L-, D- and β-alanine and γδ-dioxovaleric acid 61
Possible role of aminoacetone and ALA transaminase in the control of porphyrin synthesis 64

THE ROLE OF BIOTIN IN PORPHYRIN AND BACTERIOCHLOROPHYLL BIOSYNTHESIS 65

Enzyme activities in cell-free extracts of normal and biotin deficient organisms 70
Effect of avidin on ALA synthetase 74
Levels of enzymes in other deficiency conditions 76

THE EFFECT OF ETHIONINE AND THREONINE ON PORPHYRIN AND BACTERIOCHLOROPHYLL SYNTHESIS 78

Ethionine effects 78
Threonine effect on porphyrin and bacteriochlorophyll synthesis in Mixture I 85
Conditions required for ethionine and threonine effects 88
Reversal of ethionine and threonine effects 90

THE ORIGIN OF THE METHYL ESTER GROUP OF BACTERIOCHLOROPHYLL 94

Experimental 95
Results 98

EFFECT OF PORPHYRINS ON ALA SYNTHETASE AND ALA DEHYDRASE IN ENZYME EXTRACTS 104

DISCUSSION 105

Aminoacetone 105
β-Hydroxybutyric acid dehydrogenase 110
L-Alanine-ALA transaminase 113
Role of biotin in porphyrin and bacteriochlorophyll synthesis 115
Effects of haemin and protoporphyrin 120
Origin of the methyl ester group in bacteriochlorophyll 122
Effect of ethionine and threonine on porphyrin and bacteriochlorophyll synthesis 124
Control mechanisms for pigment synthesis 129

SUMMARY 139

REFERENCES 142

TABLES

Table 1.	Identification of aminoketones by periodate oxidation followed by gas partition chromatography of the acid produced	30
Table 2	Requirements for L-threonine dehydrogenase activity	31
Table 3	Partial purification of L-threonine dehydrogenase	37
Table 4	Specificity of threonine dehydrogenase towards the four isomers of α-amino-β-hydroxybutyric acid	38
Table 5	Stoichiometry of L-threonine dehydrogenase reaction	41
Table 6	Effect of inhibitors on L-threonine dehydrogenase activity	42
Table 7	Effect of DPN^+ analogues with L-threonine dehydrogenase	45
Table 8	Effect of inhibitors on β-hydroxybutyric acid dehydrogenase	52
Table 9	ALA transaminase activity in extracts	56
Table 10	Effect of pyruvate, methylglyoxal and α-oxoglutarate on L-alanine-ALA transaminase activity	59
Table 11	Evidence for an alanine racemase in crude enzyme extracts	62
Table 12	Effect of biotin deficiency on porphyrin production	66
Table 13	Biotin requirement for growth and porphyrin and bacteriochlorophyll synthesis by biotin-deficient organisms	67
Table 14	Requirement for biotin of biotin-deficient anaerobically grown organisms	68
Table 15	Effect of biotin deficiency on growth and pigment content of organisms	70

Table 16	Enzyme activities in normal and biotin deficient organisms	69
Table 17	Effect of illumination in Mixture I on ALA synthetase and other enzyme activities	71
Table 18	Properties of nicotinic acid and thiamine deficient organisms	75
Table 19	Comparison of enzyme activities in organisms grown anaerobically in the light in malate and in acetate	77
Table 20	Effect of DL-p-fluorophenylalanine, DL-ethionine and biotin on biotin deficient organisms illuminated in Mixture I	79
Table 21	Effect of DL-ethionine on illumination in different media	80
Table 22	Effect of ethionine on normal and biotin deficient organisms	81
Table 23	Effect of different concentrations of DL-ethionine in Mixture I	84
Table 24	Comparison of the effects of DL-threonine and DL-ethionine in Mixture I	86
Table 25	Effect of replacing $(NH_4)_2HPO_4$ in Mixture I by amino acids on porphyrin synthesis by normal and biotin deficient organisms	87
Table 26	Requirement of metal ions in Mixture I for the ethionine effect	89
Table 27	Reversal of ethionine and threonine effects by methionine and homocysteine	91
Table 28	Effect of Fe^{2+} on porphyrin accumulation in Mixture I in the presence and absence of ethionine and threonine	93
Table 29	Quantities of pigments synthesised during illumination with ^{14}C-methionine	97
Table 30	Radioactivity of purified pigments	99

Table 31 Liquid scintillation counting of fragments of bacteriochlorophyll 101

Table 32 Specific radioactivities of methionine added, and of the pigments formed in its presence 102

FIGURES

Fig. 1	Scheme for porphyrin and bacteriochlorophyll biosynthesis	2
Fig. 2	Structures of the major pigments of Rhodopseudomonas spheroides, and of the phytol group of bacteriochlorophyll	5
Fig. 3	Effect of metal ions on aminoketone accumulation	23
Fig. 4	Effect of Mn^{2+} concentration on aminoketone accumulation	23
Fig. 5	Accumulation of aminoketone anaerobically in the light	25
Fig. 6	L-Threonine dehydrogenase: effect of increasing enzyme concentration	33
Fig. 7	L-Threonine dehydrogenase: effect of time on aminoacetone production	33
Fig. 8	L-Threonine dehydrogenase: pH curve	34
Fig. 9	Effect of sodium bicarbonate on L-threonine dehydrogenase activity	40
Fig. 10	Inhibition of L-threonine dehydrogenase by EDTA	44
Fig. 11a and b	K_M for L-threonine, and for DPN^+ for L-threonine dehydrogenase	46
Fig. 12	β-Hydroxybutyric acid dehydrogenase: effect of increasing the enzyme concentration	48
Fig. 13	β-Hydroxybutyric acid dehydrogenase: pH curve	50
Fig. 14a and b	K_M for β-hydroxybutyric acid, and for DPN^+ for β-hydroxybutyric acid dehydrogenase	53
Fig. 15	L-Alanine-ALA transaminase: ALA production with time	55

Fig. 16	L-Alanine-ALA transaminase: effect of increasing the enzyme concentration	55
Fig. 17	L-Alanine-ALA transaminase: effect of varying the substrate concentrations	57
Fig. 18	L-Alanine-ALA transaminase: pH curve	57
Fig. 19	Effect of α-ketoacids on ALA synthesis	63
Fig. 20	Effect of avidin on ALA synthetase activity	73
Fig. 21a and b	Effect of DL-ethionine on growth, porphyrin, bacteriochlorophyll and carotenoid synthesis	83
Fig. 22	Measurement of radioactivity of bacteriochlorophyll by scintillation counting	100
Fig. 23	Effect of haemin on ALA synthetase activity	103
Fig. 24	Effect of protoporphyrin on ALA dehydrase activity	103

INTRODUCTION

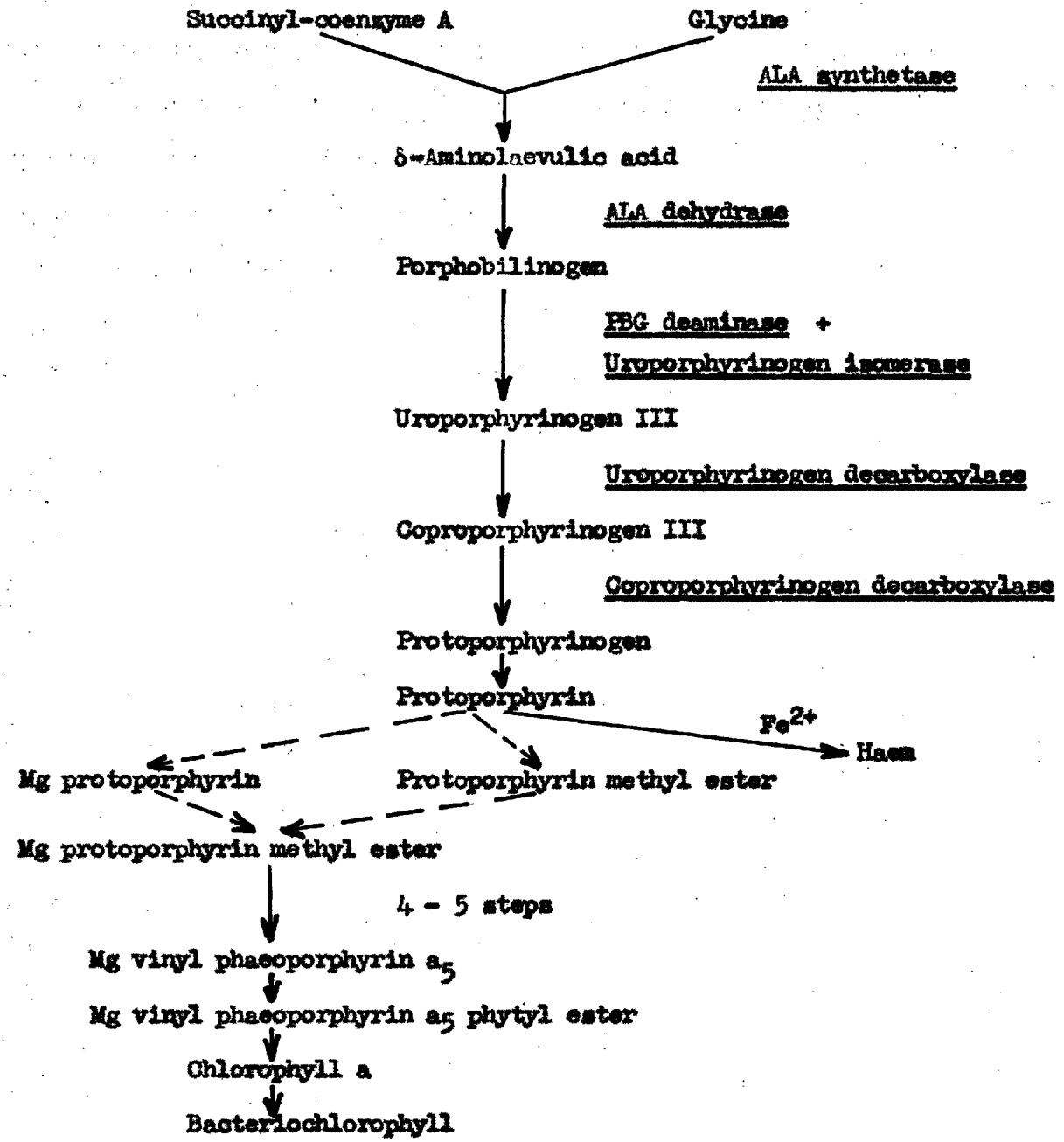
INTRODUCTION

The first clue to the mechanism of porphyrin biosynthesis was obtained by Bloch & Rittenberg (1945) who showed that haemin, isolated from rats fed with deuteriacetate contained significant concentrations of deuterium. This was followed by the reports from Shemin & Rittenberg (1945; 1946a and b) that feeding ^{15}N labelled glycine to man or rats resulted in a relatively high isotope content in the porphyrin moiety of haemoglobin. Further studies by Wittenberg & Shemin (1949, 1950), Shemin & Wittenberg (1951) and Muir & Neuberger (1949, 1950) showed that all the atoms in protoporphyrin, derived from haemoglobin, were supplied by acetate and glycine and strongly suggested that each of the four pyrrole rings in the porphyrin molecule was derived from a common precursor. On the basis of their results Shemin & Wittenberg (1951) postulated that acetate was labelling porphyrin by being converted to a succinyl-coenzyme complex and that this compound plus glycine were the direct precursors. Evidence for this was obtained using ^{14}C-succinate (Shemin & Kumin, 1952).

It was then realised that all the experimental findings could be explained if it was assumed that a succinyl-coenzyme complex condensed with glycine to form δ-aminolaevulic acid (ALA), that two molecules of ALA condensed to form the pyrrole precursor, and that four molecules of pyrrole condensed to form the porphyrin ring. The various porphyrins could then be formed by modification of the side chains on the four pyrrole rings.

δ-Aminolaevulic acid was synthesised, tested in animals, and its role

Fig. 1. Scheme for porphyrin and bacteriochlorophyll biosynthesis



as an intermediate in porphyrin synthesis demonstrated (Shemin & Russell, 1953; Neuberger & Scott, 1953; Dresel & Falk, 1956). ALA was found to be an intermediate for porphyrin biosynthesis in the photosynthetic microorganism Rhodopseudomonas spheroides by Lascelles (1956). Since then work with partially purified fractions of animal and plant tissues and with microorganisms has confirmed these findings and it is probable that the same biosynthetic pathway for porphyrins is operative in all organisms.

The next stage of the research dealt with the elucidation of the enzymic steps in porphyrin synthesis and these are shown in Fig. 1.

In the first step succinyl-coenzyme A condenses with glycine to form ALA. The enzyme responsible for this step, ALA synthetase, was described in chicken erythrocytes (Laver, Neuberger & Udenfriend, 1958; Gibson, Laver & Neuberger, 1958; Brown, 1958), in rabbit reticulocytes (Granick, 1958) and in Rps. spheroides (Gibson, 1958; Kikuchi, Kumar, Talmage & Shemin, 1958). The postulated first product α-amino-β-oxoadipic acid is extremely unstable under physiological conditions, rapidly losing CO_2 to form ALA (Laver, Neuberger & Scott, 1959), consequently its presence has never been demonstrated during the enzymic reaction. The enzyme requires pyridoxal phosphate. It has been purified forty to eighty times and a mechanism for its action proposed (Kikuchi, Kumar & Shemin, 1959).

The conversion of ALA to porphobilinogen (PBG) is catalysed by ALA dehydrase. This enzyme has been obtained in a highly purified state from liver (Gibson, Neuberger & Scott, 1955) and its presence has also been reported in Rps. spheroides (Neuberger, Scott & Shuster, 1956), in

Chlorella (Bogorad & Granick, 1953), in spinach (Bogorad, 1955) and in rabbit reticulocytes (Granick & Mauzerall, 1958).

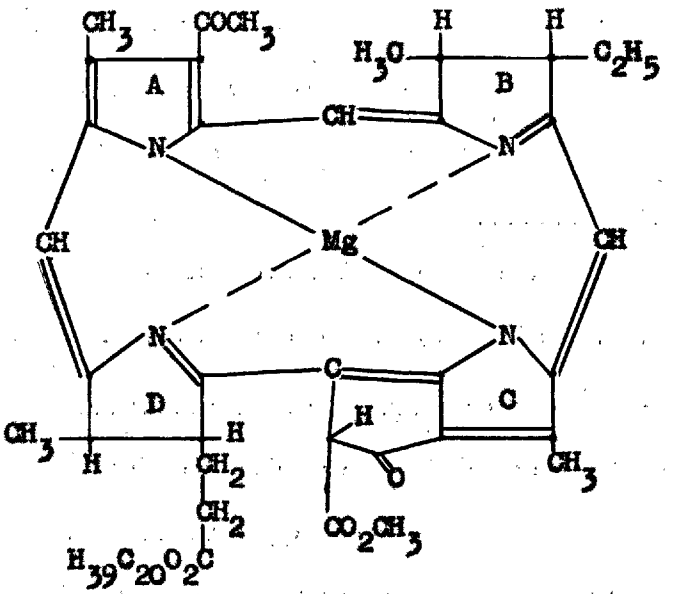
The next step has not been so clearly established. It was thought that porphobilinogen was converted to uroporphyrin, and that the porphyrins were the intermediates in further transformations. However, evidence accumulated that the porphyrins themselves could not serve as substrates (Heath & Hoare, 1959) and it was found that the porphyrinogens, which contain methylene bridges between the pyrroles instead of the methene bridges in the porphyrins were the true intermediates (Granick & Mauzerall, 1958; Bogorad, 1958c; Hoare & Heath, 1958).

Granick & Mauzerall (1958) obtained from rabbit reticulocytes a porphobilinogenase which converted PBG to uroporphyrinogen III. Bogorad (1958a and b) isolated two enzymes from plant sources which are involved in this step. One, PBG deaminase, probably converts PBG to an intermediate which is then acted on by the second, uroporphyrinogen isomerase, to form uroporphyrinogen III. The mechanism of the overall reaction is not known yet, although many hypotheses have been advanced (Bogorad, 1960).

Uroporphyrinogen is converted to coproporphyrinogen by a uroporphyrinogen decarboxylase (Granick & Mauzerall, 1958).

The formation of protoporphyrinogen from coproporphyrinogen is catalysed by a coproporphyrinogen oxidase, partially purified from liver mitochondria by Sano & Granick (1961). Oxygen is the only oxidant used by this enzyme. An enzymic oxidation of protoporphyrinogen to protoporphyrin was also detected in mitochondria by Sano & Granick (1961).

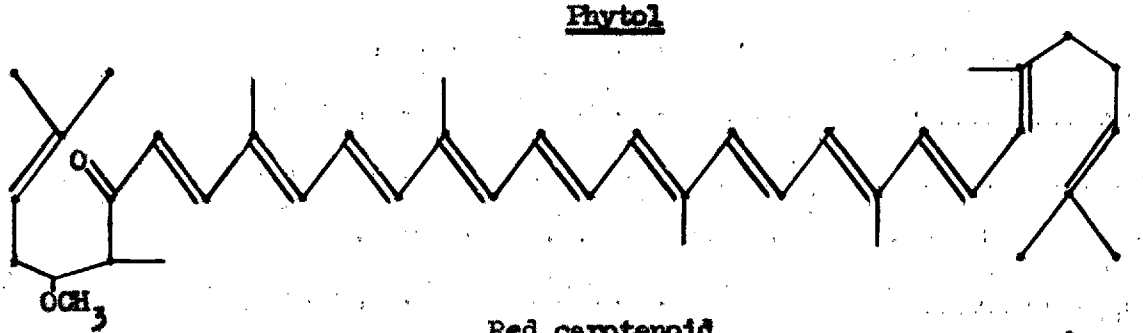
Fig. 2 Structures of the major pigments of Rhodopseudomonas spheroides and of the phytol group of bacteriochlorophyll



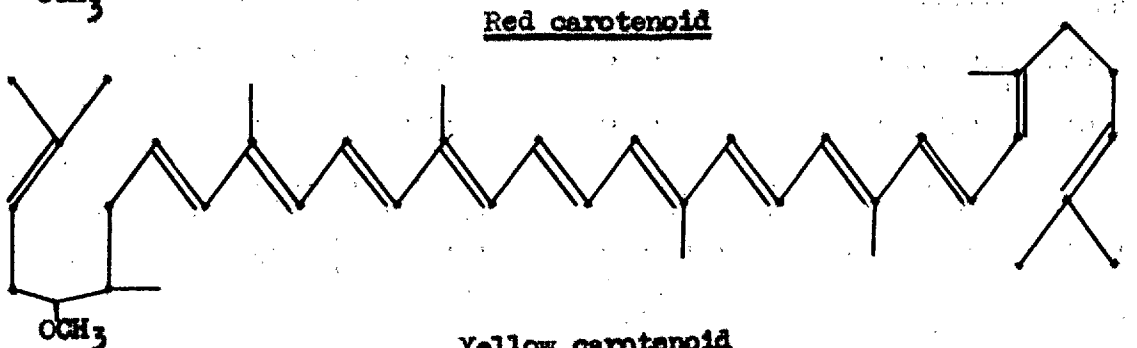
Bacteriochlorophyll



Phytol



Red carotenoid



Yellow carotenoid

Lochhead & Goldberg (1959) observed the enzymic incorporation of iron into protoporphyrin in a number of human and rat tissues, and Labbe & Hubbard (1961) isolated an enzyme in soluble form from rat liver mitochondria which catalyses the incorporation of Fe^{2+} into protoporphyrin to form haem.

The pathway leading to the magnesium porphyrins, chlorophyll and bacteriochlorophyll (whose structure is shown in Fig. 2), which is the one of interest in this study, is not well worked out. None of the enzymic steps are known and the order of the intermediates is that proposed by Granick on the basis of his work with mutants of Chlorella. Granick (1948a and b; 1950) found that protoporphyrin, Mg protoporphyrin and Mg vinyl phaeoporphyrin a_5 accumulated in different mutants of Chlorella which were unable to form chlorophyll. Recently Granick (1960, 1961) identified Mg protoporphyrin monomethyl ester and protoporphyrin monomethyl ester in another mutant of Chlorella, and detected Mg protoporphyrin monomethyl ester in barley seedlings incubated with ALA. The first two steps in bacteriochlorophyll synthesis presumably involve formation of Mg protoporphyrin monomethyl ester, but it is not known in which order the Mg and the methyl group are added to protoporphyrin. The next steps probably involve formation of the isocyclic ring between the propionic acid methyl ester side chain of ring C and the methene carbon between ring C and D, to form Mg vinyl phaeoporphyrin a_5. Four or five enzymic steps may be involved, but no intermediates have ever been detected. The next step appears to be the addition of the phytol group to the propionic acid side chain on ring D to form Mg vinyl phaeoporphyrin a_5 phytyl ester,

which is then converted to chlorophyll a by reduction, and from there to bacteriochlorophyll by oxido-reduction. There is some evidence (Smith, 1960) that chlorophyllide a is phytylated to form chlorophyll a, and Bogorad (1960) has suggested that bacteriochlorophyll is not formed from chlorophyll a but is derived from coproporphyrin instead of from protoporphyrin. Accumulation of compounds when biosynthetic pathways are blocked does not necessarily mean that these compounds are the true intermediates. This has already been shown for porphyrin biosynthesis, where the porphyrinogens are the true intermediates, and for a number of other biosynthetic pathways including purine biosynthesis (Buchanan, 1958). Therefore, too much reliance should not be placed on the pathways proposed above, leading from protoporphyrin to bacteriochlorophyll.

The origin of the methyl ester group in chlorophyll was studied by Green, Altman, Richmond & Salomon (1957) who showed that Chlorella grown in the presence of ^{14}C-formate, incorporated label into the methyl ester group, but not into the rest of the chlorophyll molecule.

The origin of the phytol group is not known. As can be seen in Fig. 2 both phytol and carotenoids (the major carotenoids of Rps. spheroides are shown) are very similar structurally. Other terpenes have been shown by the studies of Bloch, Lynen, Popjak and their co-workers (Lynen & Henning, 1960) to be synthesised from mevalonic acid and it is probable that phytol and carotenoids are also synthesised from mevalonic acid.

Control mechanisms. It is probable that in organisms enzymic reactions do not occur at random rates and numerous observations show that

there are mechanisms whereby organisms can control their metabolic pathways so that they do not waste their resources in making compounds they do not require, or even in making compounds they do require in excess amounts (Pardee, 1959). Alteration of the growth conditions, which changes the requirements of organisms, evokes responses from the control mechanisms, which allow the organisms to make the best use of the new conditions. While the presence of control mechanisms is firmly established, the elucidation of their nature is still in its infancy. One of the main difficulties is to reproduce *in vivo* conditions in experiments *in vitro*, and when results have been obtained to relate them back to conditions in the whole organism.

Since metabolic reactions are enzyme catalysed, the activities of enzymes and their absolute amounts are of very great importance. For instance pH, oxygen tension, availability of substrates and of cofactors, and the presence and absence of inhibitors can affect the activity of enzymes. The absolute amounts of enzymes can also be affected by these factors. For example, some enzymes, such as lysine decarboxylase (Sher & Mallette, 1954), appear to have only a limited stability *in vivo* and must be constantly renewed. *In vitro* many enzymes are unstable in the absence of their cofactor, and this may happen *in vivo*, the presence of cofactor not only controlling the activity of the enzyme but also the synthesis and stability of the apoenzyme (Lichstein, 1956; Burch, Lowry, Padilla & Combs, 1956).

The localisation of enzymes of a pathway in different cell structures may also play a part, the movement of substrates from one enzyme to the

next depending on the permeability of the particle membranes. In addition, compounds which are intermediates in more than one pathway will be metabolised by the pathway favoured by the prevailing conditions.

These control mechanisms, although known to operate, are difficult to evaluate quantitatively, and the mechanisms which appear to be most important are enzyme induction (adaptation) and negative-feedback control.

Enzyme induction, or adaptation, occurs when the organism synthesises a large amount of enzyme in response to the addition of a substrate, or in general, a compound closely related to the substrate, although the enzyme may not be present in detectable amounts in the absence of the substrate. Sequential induction also occurs when the product formed by the first induced enzyme, induces the next enzyme in the pathway, and so on (Cohn & Monod, 1953). While most adaptation studies have been done in micro-organisms, a similar phenomenon has been observed in animals (Knox, Auerbach & Lin, 1956).

Negative feedback control involves inhibition of the action of, or repression of the synthesis of, the early enzymes in a biosynthetic pathwa by excess of the end-product of the pathway. This has already been demonstrated for the biosynthesis of arginine (Vogel, 1957), pyrimidines (Yates & Pardee, 1957), tryptophan (Gibson, Jones & Teltscher, 1956), isoleucine (Umbarger & Brown, 1958), purines (Wyngaarden & Ashton, 1959), histidine (Ames & Garry, 1959) and methionine (Rowbury & Woods, 1961a and b

Various observations have shown that mechanisms do exist for the control of porphyrin pigment synthesis, and some have indicated ways in which they act.

Shemin & Russell (1953) found that in rats administered ALA labelled with ^{14}C in the carbon atom bearing the amino group, the label appeared in respiratory CO_2, urinary formate and in the ureido groups of uric acid. To explain this they proposed that ALA was metabolised by a cyclic pathway, the succinate-glycine cycle, in the process of which one-carbon fragments were released. More recently direct evidence for this pathway was produced by Kowalski, Dancewicz & Szot (1957), Kowalski, Dancewicz, Szot, Lipinski & Rosiek (1959) and Bagdasarian (1959), who detected ALA transaminases in mammalian tissues and in Corynebacterium diphtheriae. The quantitative importance of these reactions is not known but they may compete with the reactions of porphyrin synthesis for ALA.

Lascelles (1956) found that on illumination of Rps. spheroides in a medium containing *a*-oxoglutarate and glycine, porphyrins, mainly copro-porphyrin were excreted into the medium. Organisms previously grown in a medium deficient in biotin, nicotinic acid or thiamine did not form porphyrins under these conditions unless the missing factor was added.

The first careful study on the effect of growth conditions on bacteriochlorophyll and carotenoid production by Rps. spheroides was that of Cohen-Bazire, Sistrom & Stanier (1957) who showed that organisms grown aerobically in the dark in a medium containing malate contained little pigment, while those grown anaerobically in the light in the same medium were highly pigmented. Transferring organisms from the dark to the light resulted in pigment synthesis after a lag phase. Addition of oxygen to organisms growing and forming pigment anaerobically in the light inhibited pigment synthesis immediately, but did not inhibit growth. Removal of

the oxygen then resulted in an immediate synthesis of pigment. Increasing the light intensity had the same effect as addition of oxygen and lowering the light intensity the same effect as removal of oxygen. Organisms grown at a low light intensity were more highly pigmented than ones grown at a high light intensity, the pigment content being approximately inversely proportional to the light intensity. On the basis of this they suggested that the synthesis of bacteriochlorophyll and carotenoid, both of which are affected equally by the various conditions, is controlled by the state of oxidation or reduction of the electron-transport chain.

Lascelles (1959, 1960a) analysed these conditions more closely and found that organisms grown aerobically, at a high oxygen tension, not only contained much less pigment than those grown photosynthetically, but also contained only a quarter of the ALA synthetase and ALA dehydrase. On growing these organisms anaerobically in the light or at a low oxygen tension in the dark, pigment was formed and the specific activities of ALA synthetase and ALA dehydrase increased. This adaptation could be blocked by protein synthesis inhibitors. Addition of oxygen to organisms growing anaerobically in the light not only inhibited pigment synthesis but also inhibited ALA synthetase and ALA dehydrase synthesis, and increasing the light intensity had the same effect. Removal of the oxygen reversed these changes.

Lascelles (1956) reported that addition of iron to Rps. spheroides illuminated in a medium containing α-oxoglutarate and glycine inhibited porphyrin excretion, and more recently (Lascelles, 1960a) she found that haemin and ALA, added to cultures growing in the light, repressed the

synthesis of ALA synthetase and ALA dehydrase.

Kikuchi et al. (1958) reported the presence of a heat-labile inhibitor of ALA synthetase in extracts of aerobically grown Rps. spheroides but not in extracts of anaerobically grown organisms, but Lascelles (1959) could not repeat this finding.

Sano & Granick (1961) postulated that, in liver, porphyrin synthesis is controlled by the permeability, either active or passive, of the mitochondria, since ALA is synthesised in the mitochondria, ALA is converted to coproporphyrinogen by soluble enzymes, and protoporphyrin is formed in the mitochondria.

The organism used in the studies to be reported in this thesis is the photosynthetic microorganism Rps. spheroides, which is a member of the non-sulphur purple bacteria Athiorodaceae. This organism has been a popular choice for work on porphyrin synthesis and on the control of pigment synthesis, mainly due to its ease of growth both aerobically and photosynthetically and to the fact that it contains relatively high concentrations of the enzymes of porphyrin synthesis and of the porphyrin pigments themselves. Rps. spheroides can grow both aerobically in the dark and anaerobically in the light at the expense of a number of simple aliphatic acids or alcohols.

Initially the object of the work was to investigate the role of biotin in the synthesis of ALA, since Lascelles (1956) had indicated that biotin was involved in the production of ALA, but not in its further metabolism to porphyrins. ALA synthetase has not been closely studied previously and since its action involves a decarboxylation and most biotin

enzymes, so far described, catalyse carboxylations, it was thought that biotin might be required for the condensation of succinyl CoA and glycine to form ALA. Although the ALA synthetase activity is reduced in biotin deficiency, this is not a specific effect, as other unfavourable growth conditions also reduce the activity. These results, together with those obtained using avidin, show that the ALA synthetase is not a biotin enzyme. The work has developed into a study of various aspects of porphyrin and bacteriochlorophyll biosynthesis including control mechanisms. Aminoacetone, another α-aminoketone, is produced in relatively large amounts under suitable conditions. A powerful L-alanine-ALA transaminase has been discovered. Ethionine and threonine, alone among a large number of compounds, inhibit bacteriochlorophyll synthesis and greatly stimulate excretion of porphyrins. Some evidence has been obtained that the methyl ester group of bacteriochlorophyll is donated directly by methionine, and that this is the step blocked by ethionine and threonine. Haemin has been found to inhibit ALA synthetase activity in cell-free extracts, and protoporphyrin to inhibit ALA dehydrase activity. These results and those of other workers have been used to discuss possible mechanisms for the control of porphyrin, bacteriochlorophyll and carotenoid synthesis in Rps. spheroides. During the course of the work a threonine dehydrogenase, which converts L-threonine to aminoacetone, and a β-hydroxybutyric acid dehydrogenase have been discovered and some of their properties investigated.

MATERIALS AND METHODS

MATERIALS AND METHODS

Chemicals

Oxidised diphosphopyridine nucleotide (DPN^+), reduced diphosphopyridine nucleotide (DPNH), oxidised triphosphopyridine nucleotide (TPN^+), α-oxoglutaric acid, sodium pyruvate, adenosine triphosphate (ATP) and reduced glutathione (GSH) were obtained from Boehringer & Soehne, Mannheim, Germany; deamino-DPN^+, 3-acetylpyridine-DPN^+ and DL-homocysteine thiolactone from Mann Research Laboratories, Inc., New York; coenzyme A (CoA, 70% pure) and avidin (2.5 units/mg. protein, 1 unit of avidin combines with 1 μg of biotin) from Nutritional Biochemicals Corporation, Cleveland, Ohio; pyridoxal 5-phosphate, D-biotin and DL-ethionine from Roche Products, Ltd., Welwyn Garden City, Herts.; D-ethionine, L-ethionine and DL-homoserine from California Corporation for Biochemical Research, Los Angeles; L-[methyl-^{14}C]methionine and DL-[2-^{14}C]mevalonolactone from the Radiochemical Centre, Amersham, Bucks. D-Threonine, L-allothreonine and D-allothreonine were very kindly supplied by Dr. D.F. Elliott, National Institute for Medical Research, Mill Hill, London, N.W. 7. δ-Aminolaevulic acid (ALA) was prepared by the method of Shemin (1957). Aminoacetone was prepared by catalytic hydrogenation of oximinoacetone, followed by recrystallisation from ethanol-ether. γ-Amino-β-hydroxybutyric acid was prepared by Dr. J.B. Neilands (Neilands, Neuberger & Scott, 1960). Preparations of avidin (4.9 units/mg. protein and 9.6 units/mg. protein) were supplied by Dr. N.M. Green. Ovalbumin and ovomucoid were supplied by Dr. R.D. Marshall. γδ-Dioxovaleric acid was

prepared by Dr. A.T. Carpenter, and methylglyoxal by Dr. J.J. Scott. Dimethyl protoporphyrin and protoporphyrin were prepared by the method of Grinstein (1947), Mg protoporphyrin dimethyl ester and Mg protoporphyrin by the method of Granick (1948b), and haemin by the method of Fischer (1955). Succinyl-coenzyme A (succinyl-CoA) was prepared by the method of Simon & Shemin (1953), acidified to pH 3 with HCl, and kept frozen until required. The values quoted for succinyl-CoA are based on the amount of CoA used, assuming it to be 70% pure. Phosphate buffers were made from KH_2PO_4 and K_2HPO_4. Tris buffers were prepared from 2-amino-2-hydroxymethylpropane-1:3-diol and HCl (Gomori, 1955).

Organisms

The strain of Rhodopseudomonas spheroides was obtained from Dr. Lascelles (National Collection of Industrial Bacteria [N.C.I.B.] no. 8253).

Growth of organisms. The organisms were maintained in stab culture exactly as described by Lascelles (1956).

Anaerobic growth: in all experiments, unless otherwise stated, organisms were grown semi-anaerobically in the light at 32 - 34° for 44 hr. in Medium S of Lascelles (1956). The illumination was supplied, and the temperature maintained by 60 watt bulbs placed at the appropriate distance from the flasks. Cultures were grown in 500 ml. or 1000 ml. lots in conical flasks. Organisms grown anaerobically under conditions of vitamin deficiency were grown exactly as stated above, except that the vitamin concentration in the medium was reduced. The vitamin concentrations for normal growth are biotin 4×10^{-8} M, nicotinic acid

8×10^{-6} M, and thiamine 3×10^{-6} M. Acetate-grown organisms were grown anaerobically in the light as described above except that the malate (.02 M) in Medium S was replaced by acetate (.04 M) plus bicarbonate (.005 M).

Aerobic growth in the dark was in Medium S, exactly as described by Lascelles (1956).

Complete anaerobiosis is not essential (Lascelles, 1959) and in all the work anaerobic actually means semi-anaerobic, the illuminations being done in an atmosphere of air but without shaking.

Harvesting of organisms. At the end of growth the organisms were harvested by centrifugation at 0°, washed with a volume of .05 M phosphate buffer, pH 7.4, equal to half the original culture volume, and suspended in buffer at a concentration of 80 mg. dry wt./ml. This suspension was kept frozen at -20° until required.

Procedure for the study of porphyrin synthesis with suspensions of organisms

Synthesis from α-oxoglutarate and glycine. The washed cells were suspended at a final concentration of 1.0 mg. dry wt./ml. in Mixture I of Lascelles (1956). Incubations were done semi-anaerobically in the light for 18 - 24 hr. at 32 - 34° in 100 x 10 mm. tubes containing 5 ml. of reaction mixture. The heat and light were supplied by 60 watt bulbs placed at the appropriate distance from the tubes. This medium will be referred to as Mixture I, and the above conditions as standard illumination conditions. Any variations will be described at the appropriate place. The composition of Mixture I is α-oxoglutaric acid,

0.01 M; glycine, 0.01 M; fumaric acid, 0.02 M; $(NH_4)_2HPO_4$, 0.003 M; $MgSO_4$, 8×10^{-4} M; $CaCl_2$, 4×10^{-4} M; $MnCl_2$, 5×10^{-6} M; phosphate buffer pH 6.8, .006 M. The pH was adjusted to 6.8 with NaOH.

Synthesis from succinate and glycine. The conditions used were exactly as described above. Mixture I S differs from Mixture I in containing succinic acid (.01 M) instead of α-oxoglutaric acid.

Synthesis from δ-aminolaevulic acid. The washed cells were illuminated in Mixture II of Lascelles (1956) using the same conditions as for illumination in Mixture I. This medium, which will be referred to as Mixture II, contains ALA, 2×10^{-3} M; $MgSO_4$, 8×10^{-4} M; and phosphate buffer pH 6.8, 0.1 M.

Preparation of enzyme extracts

Two methods for disrupting the organisms have been used. Suspensions containing about 40 mg. dry wt./ml. were disrupted ultrasonically (Mullard Ultrasonic Generator, type E 7590A, Mullard Ltd., London, W.C. 1) for 8 min. at 25 kc/sec. Alternatively, suspensions, 80 mg. dry wt./ml. were disrupted in the Hughes Press (Hughes, 1951) and then diluted with two volumes of buffer. The resulting suspensions were centrifuged for 90 min. at 105,000 g. in the Spinco Model L Ultracentrifuge, and the clear supernatants removed. These crude enzyme extracts, which are clear yellow in colour, were put in the deep freeze, either immediately or after dialysis against .05 M phosphate pH 7.4 at 4° for 17 hr., and kept until required for assay.

Most of the experiments to be described were performed with enzyme

extracts prepared by the Hughes Press method, and this can be assumed to be the method used unless otherwise stated.

Estimations

All spectrophotometric estimations were performed on the Unicam spectrophotometers, models SP 500 or SP 600.

Dry weight of organisms. The extinction of suspensions was measured at 680 mμ and the dry weight calculated from a calibration curve. The calibration curve was constructed by drying suspensions of organisms in water to constant weight at 100°, after having measured the extinction of the suspensions at 680 mμ. Absorption due to the photopigments is minimal at this wavelength (Cohen-Bazire et al., 1957).

Carotenoids and bacteriochlorophyll. These pigments were extracted from the organisms with methanol or acetone-methanol (7:2 v/v) and measured spectrophotometrically by the method of Cohen-Bazire et al. (1957). In some of the early experiments bacteriochlorophyll was estimated by the method of Van Niel & Arnold (1938). This method was found to be unsatisfactory and all further estimations were done by the method of Cohen-Bazire et al. (1957).

Porphyrins. These were estimated spectrophotometrically in the supernatant after removal of organisms by centrifugation, exactly as described by Lascelles (1956).

Protein. The concentration of protein in extracts was measured by the biuret reaction (Layne, 1957), using crystalline bovine plasma albumin as standard.

Protein and nucleic acid. When estimations of both these components in extracts were required, the optical densities at 260 mμ and 280 mμ were measured. The concentration of each component was calculated from the extinction coefficients for enolase and nucleic acid given by Warburg & Christian (1941).

δ-Aminolaevulic acid. The amount of ALA was estimated colorimetrically after condensation with acetylacetone by the method of Mauzerall & Granick (1956). The colour was measured 15 min. after addition of the Ehrlich's reagent.

Aminoacetone. This compound was estimated by the same method as for ALA except that the colour was measured immediately after addition of the Ehrlich's reagent.

Authentic ALA and aminoacetone were used to construct standard curves.

Assay of enzymic activities

δ-Aminolaevulic acid synthetase. The reaction mixture contained crude enzyme extract, 0.05 - 0.3 mg. protein (0.25 - 1.5 mg. if the extract was prepared by ultrasonic disintegration of organisms); phosphate buffer pH 7.4, 37.5 μmoles; glycine, 12.5 μmoles; succinyl-CoA, 0.2 μmoles; pyridoxal phosphate, 0.05 μmoles; water to 0.375 ml. Incubation was at 37° for 30 min. The reaction was stopped by addition of 0.175 ml. of 20% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation and washed with 0.45 ml. of 5% trichloroacetic acid. The combined supernatants were estimated for ALA

as described above. The amount of ALA formed was proportional to the amount of extract added over the range stated. Every enzyme extract assayed, was tested at two or three different concentrations within this range to ensure that the results were valid. Formation of porphyrins was not detected under the conditions used.

δ-Aminolaevulic acid dehydrase. This enzyme was assayed by the method of Gibson et al. (1955). The system contained enzyme extract, 1 - 5 mg. of protein (2 - 10 mg. if the extract was prepared by ultrasonic disintegration of organisms); phosphate buffer pH 6.8, 200 μmoles; GSH, 30 μmoles; water to 3.0 ml. - in the main compartment of Thunberg tubes, and ALA, 33 μmoles in 1.0 ml. of water in the side arm. The tubes were evacuated on the water pump and incubated at 37° for 60 min. The contents were mixed and the incubation continued for another 60 - 90 min. Deproteinisation, destruction of SH groups and estimation of porphobilinogen were performed by the method of Gibson et al. (1955). The rate of porphobilinogen production was proportional to the protein added over the range used. Assays of all enzyme extracts were done at two or three different enzyme concentrations within this range.

EXPERIMENTAL AND RESULTS

ACCUMULATION OF AN AMINOKETONE BY WHOLE ORGANISMS UNDER SUITABLE INCUBATION CONDITIONS; ITS PURIFICATION AND IDENTIFICATION

Lascelles (1956) noted that Rps. spheroides incubated aerobically in the dark in Mixture I did not form porphyrins. On repeating this experiment, although no porphyrin could be detected, an aminoketone, which was at first assumed to be ALA, was found. It was noted, however, that the aminoketone behaved like aminoacetone, not like ALA, in the Mauzerall & Granick (1956) estimation method, giving a colour immediately the Ehrlich reagent was added. The pyrroles, formed from aminoacetone ($CH_3COCH_2NH_2$) and the aminoketone on condensation with acetylacetone, could both be extracted from aqueous solution at pH 8 into ether. The pyrrole formed on condensation of ALA ($HOOC.CH_2CH_2COCH_2NH_2$) with acetylacetone, having a carboxyl group, could not be extracted from aqueous solution at pH 8 into ether. This confirms that the aminoketone formed is not ALA, and shows that it may be aminoacetone or some similar aminoketone lacking a carboxyl group.

Incubation of organisms with threonine also gave an aminoketone with similar properties to the one found above.

Elliott (1958, 1959, 1960a) has recently reported that an aminoketone is produced when Staphylococcus aureus is incubated aerobically in a medium containing glycine and glucose, or in one containing threonine. He has characterised the aminoketone from threonine as aminoacetone by the preparation of derivatives, but the one from glycine and glucose is produced in very small quantities and has only been identified as

aminoacetone on the basis of its chromatographic and electrophoretic properties.

The aminoketone formed from threonine was assumed to be aminoacetone and no further work has been done on it. Preliminary partial purification indicated that the aminoketone produced in Mixture I might be different from aminoacetone, so it was decided to purify it and identify it. It was also thought that this aminoketone, being an analogue of ALA, might play some part in the control of porphyrin synthesis by interfering with the synthesis or metabolism of ALA.

Accumulation of aminoketones

Incubation conditions. Organisms grown anaerobically in the light were suspended at 4 mg. dry wt./ml. in Mixture I, or in Mixture I modified in various ways. The suspensions, 5 - 10 ml. in volume, were contained in boiling tubes. The tubes were connected in series and the suspensions vigorously aerated by bubbling air through from a cylinder. The incubations were done at 35°. Samples were removed at various times, 0.06 volumes of 100% (w/v) trichloroacetic acid added and aminoketone estimated colorimetrically in the supernatant after removal of the precipitate by centrifugation. Quantities of aminoketone formed are expressed as aminoacetone.

Results

In Mixture I the buffer concentration is low (.006 M) and it was noted that during incubation the pH rose from 6.8 to 9 or higher. At a

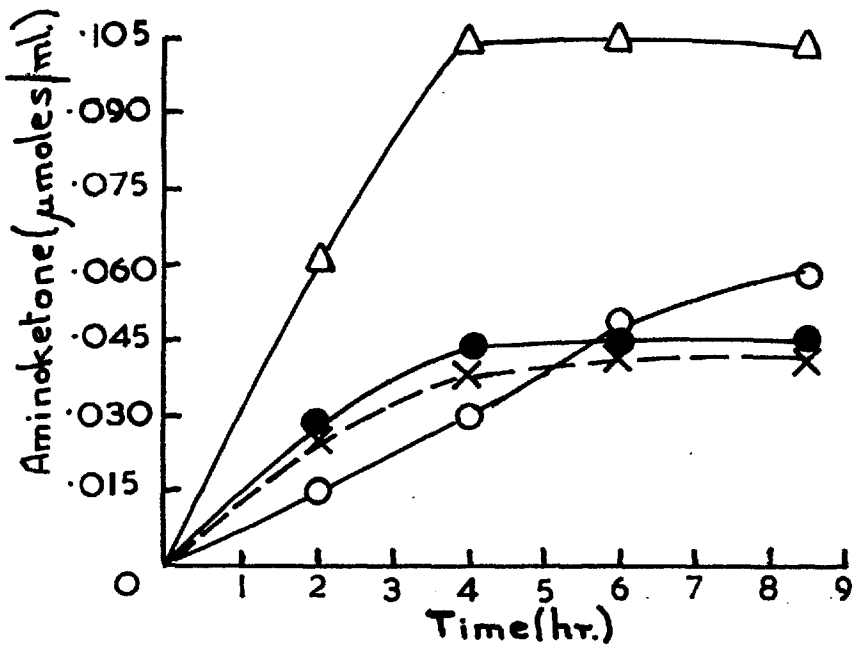


Fig. 3 Effect of metal ions on aminoketone accumulation. Sodium α-oxoglutarate, .02M; sodium fumarate, .04M; glycine, .02M; phosphate buffer pH 6.6, 0.2M; organisms 4 mg. dry wt./ml. Aerated at 35°. Samples removed at times shown and estimated for aminoketone. Metal ions Mg^{2+} 1.6 mM (O——O); Ca^{2+} 0.8 mM (X- - -X); Mn^{2+} 10 μM (Δ——Δ); no metals (●——●).

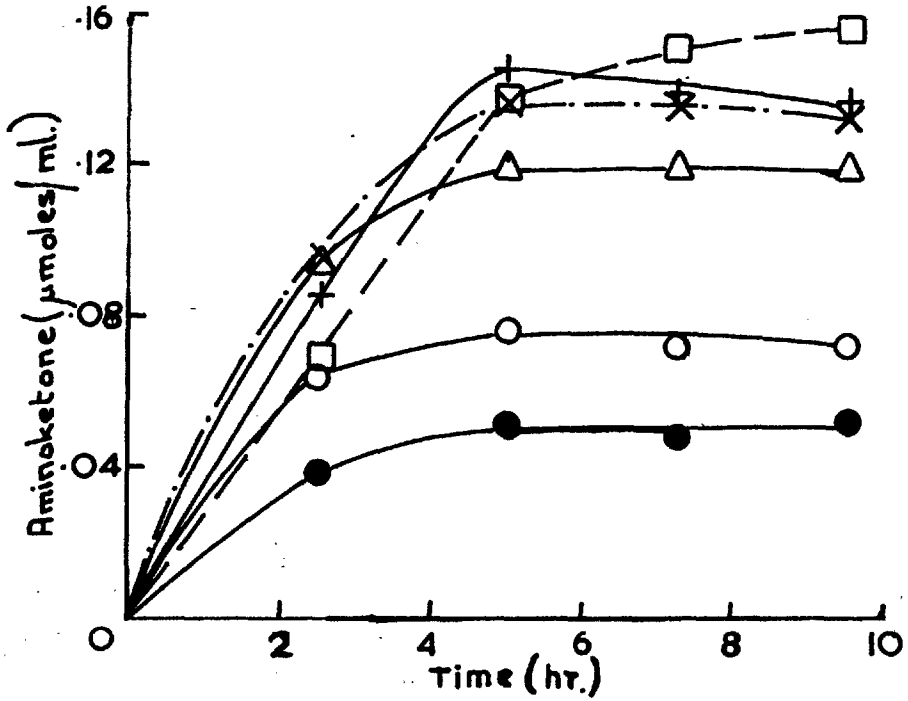


Fig. 4 Effect of Mn^{2+} concentration on aminoketone accumulation. Sodium α-oxoglutarate, .02M; sodium fumarate, .04M; glycine, .02M; phosphate buffer pH 6.6, 0.2 M; organisms, 4 mg. dry wt./ml. Aerated at 35°. Samples removed at times shown and estimated for aminoketone. Mn^{2+} conc.: 10^{-3}M (□- - -□); 5 x 10^{-4}M (+——+); 10^{-4}M (X- · -X); 5 x 10^{-5}M (Δ——Δ); 10^{-5}M (O——O); no Mn^{2+} (●——●).

phosphate buffer concentration of 0.2 M the pH did not rise markedly, and the amount of aminoketone which accumulated in 16 hr. (.063 μmoles/ml.) was six times the amount which could be detected in Mixture I without added buffer (.010 μmoles/ml.). All further experiments have therefore been done in the presence of 0.2 M phosphate buffer pH 6 - 7.

It was found that omission of $(NH_4)_2HPO_4$ from the medium did not affect the yield of aminoketone. The accumulation of aminoketone was found to be linear for 4 hr., then it completely stopped, and the amount of aminoketone detected remained constant over the next 18 hr. α-Oxoglutarate and fumarate, if added after 4 hr., gave a further increase in aminoketone, but adding glycine had no effect.

When the incubation was done in a medium lacking metal ions, the accumulation of aminoketone was low and variable, and it was concluded that one or more of the metals - Mg^{2+}, Ca^{2+}, Mn^{2+} - were required. Mn^{2+} greatly enhanced the yield of aminoketone, Ca^{2+} had no effect, and Mg^{2+} lowered the initial rate of accumulation, but the final yield was slightly greater than that obtained in the absence of Mg^{2+} (Fig. 3). Increasing the concentration of Mn^{2+} in the medium increased the yield of aminoketone (Fig. 4).

The optimum pH for aminoketone accumulation was found to be 6.2. No aminoketone was formed if α-oxoglutarate and fumarate were replaced by acetate.

Accumulation of aminoketone on illumination

When organisms are illuminated in Mixture I under standard

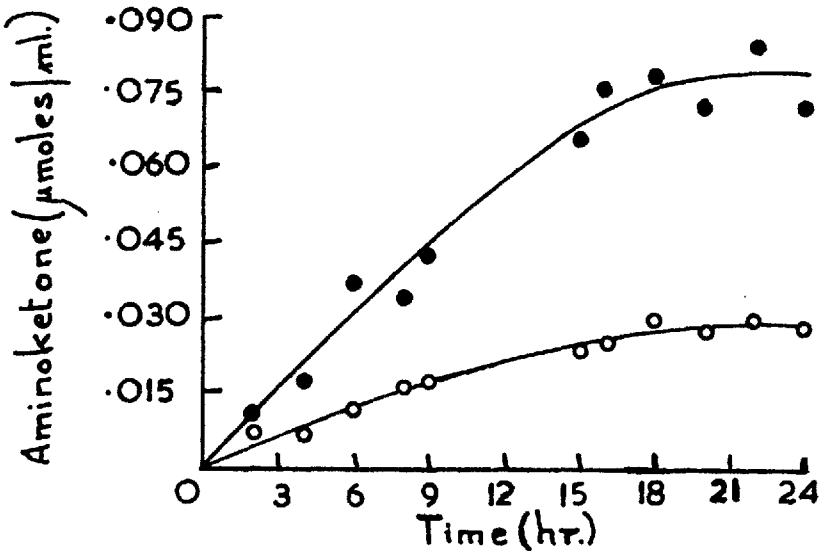


Fig. 5 Accumulation of aminoketone anaerobically in the light

Organisms illuminated under standard conditions in Mixture I (p. 16). Incubation at 32 - 34°. Samples removed at times shown and estimated for aminoketone. Phosphate buffer pH 6.8, .15M (●——●); .05M (○——○).

conditions (p. 16) a small amount of an aminoacetone-like aminoketone accumulates (0.012 μmoles/ml.) during 24 hr. The yield has been greatly improved by increasing the phosphate buffer concentration (Fig. 5). This effect is not specific for potassium phosphate buffer, a similar yield of aminoketone being obtained with 0.15 M sodium phosphate or tris buffers.

Purification of the aminoketones

The aminoketone which accumulates aerobically in the dark ("dark" aminoketone), and the one which accumulates anaerobically in the light ("light" aminoketone) were produced in large amounts, using the optimal conditions worked out above. The conditions used were:

I. "Dark" aminoketone: Sodium α-oxoglutarate, .027 M; sodium fumarate, .054 M; glycine, .027 M; $MnSO_4$, 5×10^{-4} M; phosphate buffer pH 6.1, 0.2 M; organisms, 4 mg. dry wt./ml. Suspensions were aerated at 34° for 8 hr.

II. "Light" aminoketone: Mixture I, plus phosphate buffer pH 6.8, 0.15 M; organisms, 1 mg. dry wt./ml., illuminated under standard conditions at 34° for 21 hr.

At the end of the incubations the pH was adjusted to pH 5 with HCl, the organisms removed by centrifugation, and the supernatants used for purification of the aminoketones. The incubations were done in batches and the supernatants pooled. The purification was started with 173 μmole of "dark" aminoketone and 159 μmoles of "light" aminoketone.

Purification. Identical methods were used for both aminoketones.

Step I. The supernatants, after dilution with water, were passed through

columns of Zeokarb 225 (40 - 80 mesh) in the H^+ form. Solution was passed until the columns were almost saturated, as judged by the colour change. The columns were then washed with water until the effluents were neutral. The aminoketone was eluted with N-HCl followed by 1.5 N-HCl. 10 ml. fractions were collected. All the fractions containing aminoketone were combined and dried. The large residue obtained is mainly KCl. The yields obtained by colorimetric estimation were: "dark" aminoketone, 144 µmoles (84%); "light" aminoketone, 134 µmoles (85%).

Step 2. The aminoketone and amino acids were extracted from the solid residue with acetone containing 5% of 6N-HCl (Smith, 1958). This extraction left most of the salt behind. The extracts were dried and the residue taken up in water. Yields: "dark" aminoketone, 125 µmoles (72%); "light" aminoketone, 130 µmoles (82%).

Step 3. The solution from step 2 was applied to a column of Zeokarb 225 (8% cross-linked, 50 µ particle size) in the H^+ form. The column, 24 x 1 cm. in size, was washed with water until the effluent was neutral. Elution was performed with 0.5 N-HCl, at a flow rate of 5 ml./20 min., and 5 ml. fractions collected. The aminoketones were eluted with approximately 200 ml. of 0.5 N-HCl. Analysis showed a symmetrical peak with very little tailing. The fractions containing aminoketone were pooled, dried and the residue dissolved in water. Yields: "dark" aminoketone, 113 µmoles (65%); "light" aminoketone, 72 µmoles (45%).

Step 4. On paper chromatography in butanol-acetic acid-water (60:15:25 by vol.) the aminoketones from the last step gave a yellow ninhydrin spot

with R_f 0.39 (aminoacetone, R_f 0.39; ALA, R_f 0.29) and a small purple spot with R_f 0.51. The final purification was therefore done by paper chromatography using butanol-acetic acid-water and the aminoketone eluted off the paper with HCl. This method gave a complete separation from the amino acid impurity, but the recovery of aminoketone was low. Yield: "dark" aminoketone, 13.7 μmoles from 50 μmoles; "light" aminoketone, 16.8 μmoles from 32.9 μmoles.

Identification of the aminoketones

The following properties of both "dark" aminoketone and "light" aminoketone are identical with those of aminoacetone.

1. The R_f values are the same as aminoacetone in butanol-acetic acid-water (60:15:25 by vol.).
2. On paper they give yellow spots with ninhydrin, which turn to brown, then to purple on standing. Aminoacetone and ALA show this also.
3. The pyrroles formed from the aminoketones on condensation with acetylacetone have the same R_f values as the pyrrole from aminoacetone and acetylacetone, in butanol-acetic acid-water (63:10:27 by vol.) (R_f 0.87) and in butanol-ammonia (equal volumes of butanol and 1.5 N ammonia) (R_f 0.85) (Mauzerall & Granick, 1956).

Final proof of the identity of the aminoketones was obtained by periodate oxidation and identification of the acid formed by gas partition chromatography.

Shemin, Russell & Abramsky (1955) have reported that periodate oxidation of ALA gives good yields of succinic acid. Aminoacetone on

periodate oxidation should give acetic acid.

Periodate oxidation. Aminoacetone and purified aminoketones were oxidised in 10 ml. of a solution containing 0.1 g. of 50% (w/v) periodic acid and 0.15 g. of $NaHCO_3$. The reaction was allowed to proceed for 16 hr. in the dark at room temperature. The periodate and iodate were precipitated by adding excess 0.2 $\underline{\underline{M}}$-$BaCl_2$. After centrifugation the pH of the supernatants was adjusted to 3 with 3 $\underline{\underline{N}}$-$H_2SO_4$ and the precipitate again centrifuged off. The supernatants were steam-distilled at pH 3 (no HCl distils over at this pH), and distillate collected until no more acid came over. The distillates were kept alkaline, and dried in vacuo.

Gas partition chromatography. This was carried out according to James & Martin (1952). The apparatus was built by Dr. R.D. Marshall, and I am grateful to him for instruction in its use. The column was made from kieselguhr (Celite 545, Johns Manville Co., Ltd.) which was mixed with half its weight of silicone (MS 550, Hopkins and Williams, Ltd.) containing 10% (w/w) stearic acid. The column was 130 cm. long and had an internal diameter of 0.5 cm. and was contained in a steam jacket. Partition was accomplished by passing nitrogen at a pressure of 45 cm. Hg. The acids coming off the column were titrated with 0.02 $\underline{\underline{N}}$-NaOH using 0.001% phenol red as indicator. When the colour of the indicator changed from red to yellow as acid came off the column, drops of NaOH were added from a burette to reverse the colour change, and the time when the drops were added was noted.

With the column used and running it at a pressure of 45 cm. Hg and

at 100°, the elution time of the acids was: formic acid, 7 min.; acetic acid, 20 min.; propionic acid, 35 min.; and butyric acid, 65 min.

The residues from the steam distillates were dissolved in 0.3 ml. of water and one drop of 100% H_3PO_4 added to each. The fatty acids were extracted into ether (3 x 5 ml.) and the combined ether extracts dried over anhydrous $MgSO_4$. After filtering, the ether solutions were taken to a small volume with a stream of nitrogen and applied to the end of the column. The column was replaced in the steam jacket, and the chromatography performed. The results are shown in Table 1.

TABLE 1

Identification of aminoketones by periodate oxidation followed by gas partition chromatography of the acid produced

Sample	µmoles	Acid recovered on gas chromatography		Elution time (min.)
		µmoles	%	
Reagent blank	-	0.0	-	-
Aminoacetone	10	4.2	42	20
Aminoacetone	20	11.4	57	20
Aminoacetone	50	26.6	53	20
Aminoacetone	100	49.4	49	20
"Dark" aminoketone	13.7	7.8	57	20
"Light" aminoketone	16.8	11.2	67	20

The columns were run for a total of from 60 - 80 min. but no more acid

TABLE 2

Requirements for L-threonine dehydrogenase activity

Assay: L-threonine, 25 μmoles; pyridoxal phosphate (where added), 0.05 μmoles; DPN^+ or TPN^+, as indicated; enzyme - crude dialysed extract (≡ 1.0 mg. protein); phosphate buffer, pH 7.4, 75 μmoles; water to 0.75 ml. Incubation at 37° for 1 hr. Reaction stopped with trichloroacetic acid. Supernatant, after removal of protein, estimated for aminoacetone.

DPN^+ (μmoles)	TPN^+	Pyridoxal phosphate	Aminoacetone (μmoles)
0.5	-	+	.032
1.0	-	+	.042
-	0.5	+	.004
-	1.0	+	.005
1.0	-	-	.038
-	1.0	-	.005
-	-	+	.002

was eluted. Samples of sodium acetate, on acidification with H_3PO_4, extraction into ether, and gas chromatography also gave a recovery of about 50%.

These results show that both the dark aminoketone and the light aminoketone are aminoacetone.

THREONINE DEHYDROGENASE ACTIVITY IN EXTRACTS

An enzyme has been found in extracts of Rps. spheroides which produces aminoacetone from threonine in the presence of DPN^+, that is a threonine dehydrogenase (Table 2). All the experiments have been performed with extracts prepared from organisms grown anaerobically in the light. Table 2 shows that TPN^+ cannot replace DPN^+ as hydrogen acceptor, and there is no reaction in the absence of the latter. Pyridoxal phosphate does not enhance the activity.

The aminoketone formed in the above reaction has not been rigorously identified, but it is identical with aminoacetone in that the pyrrole formed by condensation with acetylacetone can be extracted into ether at pH 8. The spectrum of the pyrrole-Ehrlich complex formed in the Mauzerall & Granick (1956) colour reaction is also identical with that for aminoacetone. The nature of the reaction also indicates that the aminoketone is aminoacetone.

The production of aminoacetone has been measured in the presence of increasing amounts of enzyme (Fig. 6), showing a linear enzyme-activity curve, and at different times (Fig. 7).

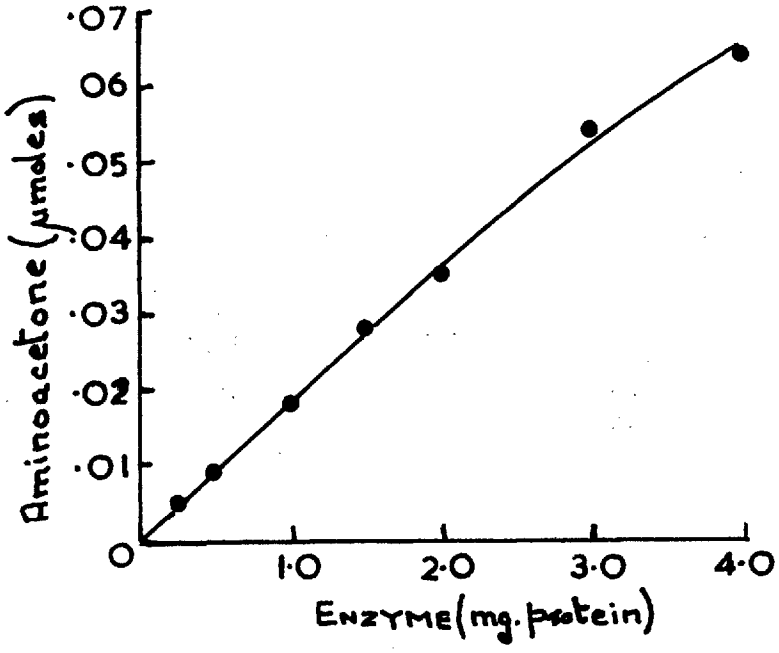


Fig. 6 L-Threonine dehydrogenase: effect of increasing enzyme concentration. L-threonine, 25 µmoles; DPN^+ 0.5 µmoles; phosphate pH 7.4, 75 µmoles; crude dialysed enzyme extract, as stated; water to 0.75 ml. Incubation at 37° for 1 hr. Reaction stopped with trichloroacetic acid, and aminoacetone estimated.

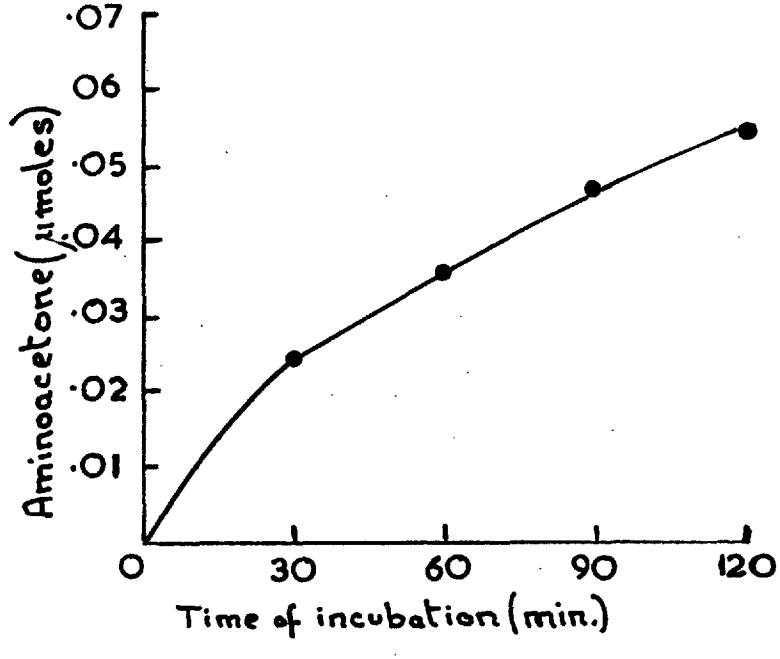


Fig. 7 L-Threonine dehydrogenase: effect of time on aminoacetone production. L-threonine, 25 µmoles; DPN^+ 0.5 µmoles; phosphate, pH 7.4, 75 µmoles; crude dialysed enzyme extract (= 2.0 mg. protein); water to 0.75 ml. Incubation at 37° for times stated. Reaction stopped with trichloroacetic acid and aminoacetone estimated.

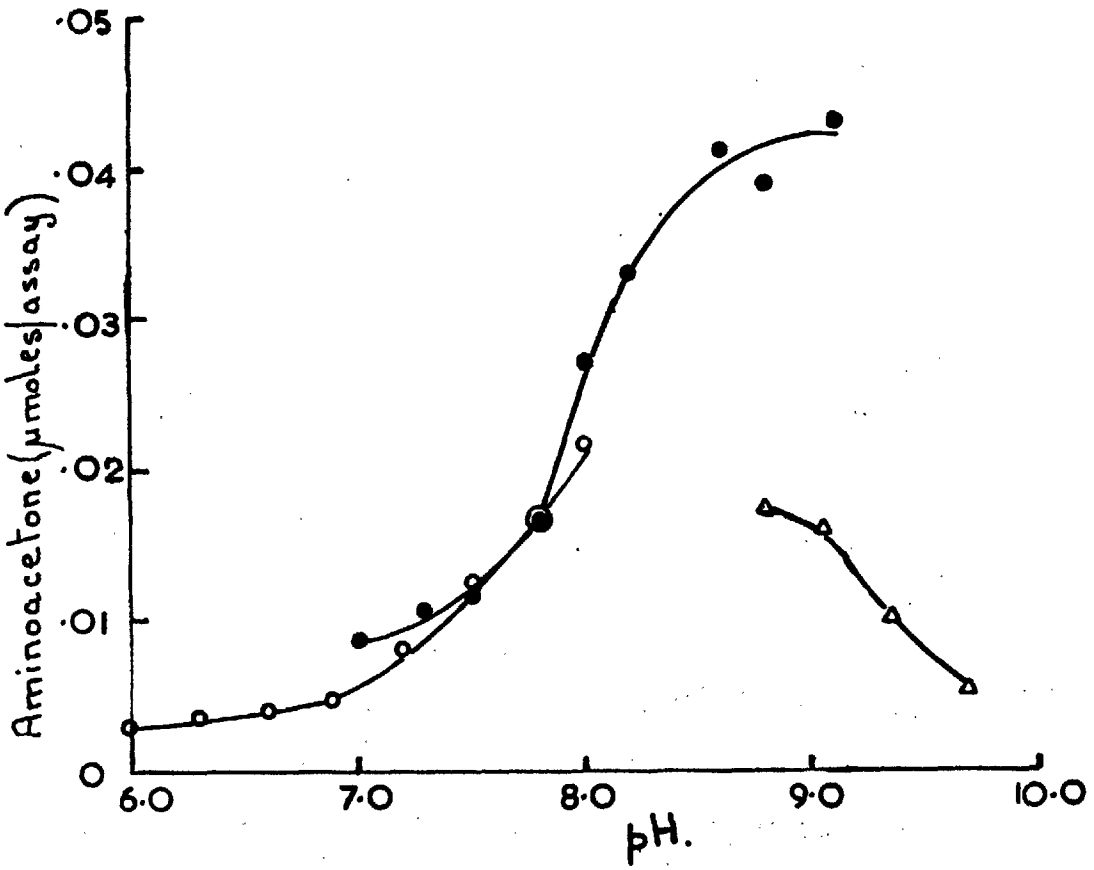


Fig. 8 L-Threonine dehydrogenase: pH curve

L-threonine, 25 μmoles; DPN^+, 0.3 μmoles; buffer, 75 μmoles; crude dialysed enzyme extract (= 1.0 mg. protein); water to 0.75 ml. Incubation 1 hr. at 37°. Reaction stopped with trichloroacetic acid and aminoacetone estimated. Buffer: tris ●——●; phosphate O——O; carbonate-bicarbonate Δ——Δ.

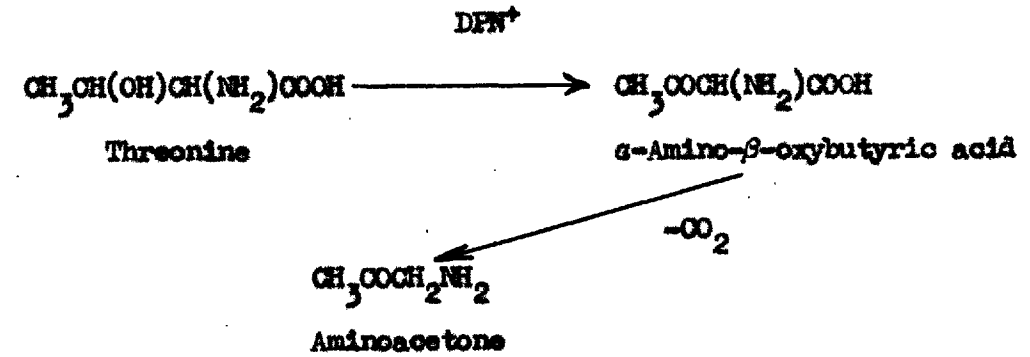
The reaction has been done in tris, phosphate, and carbonate-bicarbonate buffers at various pH values (Fig. 8). The optimum pH in tris is 8.6 - 9.1. Carbonate-bicarbonate buffer inhibits the reaction.

A comparison of threonine dehydrogenase activities using L-threonine and DL-threonine indicates that D-threonine is not acted on and does not inhibit the reaction with the L-isomer (this has been confirmed, see p. 38). The enzyme is therefore an L-threonine dehydrogenase.

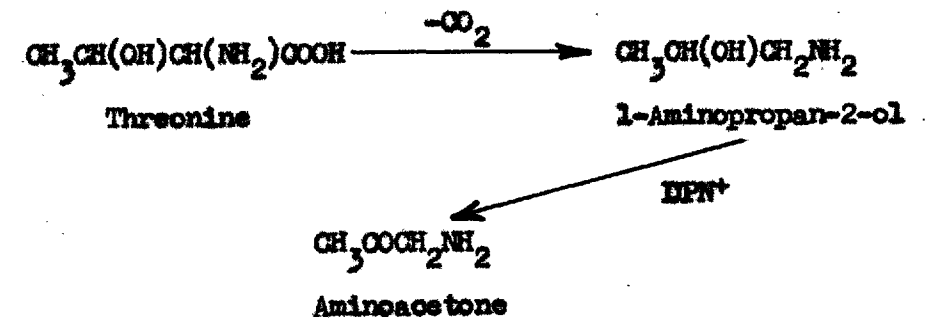
Mechanism of L-threonine dehydrogenase action

There are two possible ways in which L-threonine can be converted to aminoacetone. These have also been considered by Elliott (1960a).

I.



II.



The intermediate in pathway I is extremely unstable, Laver *et al.* (1959) having shown that it has a half-life of much less than 1 min. at

pH 7; and therefore it would decarboxylate spontaneously to give aminoacetone.

If the reaction went by pathway II, D-1-aminopropan-2-ol should be able to replace L-threonine as substrate for aminoacetone production.

DL-1-Aminopropan-2-ol has been tested with the enzyme system, in the presence of DPN^+ and TPN^+ at a range of pH values between 6.0 and 8.8. No aminoacetone could be detected. DL-1-Aminopropan-2-ol did not inhibit aminoacetone production from L-threonine. It is therefore obvious that pathway II does not operate.

Partial purification of the L-threonine dehydrogenase

Protein and nucleic acid were estimated by absorption measurements at 260 mμ and 280 mμ.

Assay of activity in fractions. DL-Threonine, 50 μmoles; DPN^+, 0.2 μmoles; tris, pH 8.8, 75 μmoles; enzyme; water to 0.75 ml.; incubation at 37° for 1 hr. Reaction stopped with trichloroacetic acid and aminoacetone estimated.

All purification steps were carried out at 0 - 4°.

Step 1: $MnCl_2$ treatment. To undialysed crude enzyme extract was added 0.05 volumes of M-$MnCl_2$. After standing for 15 min. the mixture was centrifuged. The supernatant was dialysed overnight against 0.05 M phosphate, pH 7.4. A precipitate which formed was removed by centrifugation.

Step 2. The supernatant was fractionated with solid $(NH_4)_2SO_4$ at 35%, 45%, 60% and 80% saturation. Each precipitate was collected by

centrifugation, dissolved in a volume of buffer equal to 0.22 volumes of the $MnCl_2$ supernatant and dialysed.

Step 3. The solution containing the 45 - 60% $(NH_4)_2SO_4$ precipitate was adjusted to pH 6 and treated with 0.1 volume, 0.1 volume and 0.2 volumes of $Ca_3(PO_4)_2$ gel (13.5 mg./ml.). The supernatant was recovered from one gel treatment before addition of the next batch of gel. To the last supernatant was added 0.6 volumes of gel. The precipitate obtained by centrifugation was eluted once with water and twice with 0.05 M phosphate pH 7.4.

TABLE 3

Partial purification of L-threonine dehydrogenase

Fraction	Specific activity μmoles aminoacetone/ mg./protein/hr.	Purification (based on protein)	Recovery %
Crude extract	.113	1.00	100
$MnCl_2$ supernatant	.126	1.11	65
35% $(NH_4)_2SO_4$ precipitates	-	-	-
45% $(NH_4)_2SO_4$ precipitates	.155	1.37	15
60% $(NH_4)_2SO_4$ precipitates	.224	2.00	40
80% $(NH_4)_2SO_4$ precipitates	.132	1.16	12
1st buffer eluate	.545	4.82	24
2nd buffer eluate	.465	4.11	8

The first buffer eluate, hereafter referred to as "purified enzyme" shows a purification of 4.82 fold with respect to protein and contains very

TABLE 4

Specificity of threonine dehydrogenase towards the four isomers of α-amino-β-hydroxybutyric acid

Assay: α-amino-β-hydroxybutyric acid isomers, as stated; DPN^+, 0.5 μmoles; tris, pH 8.8, 75 μmoles; crude enzyme (≡ 0.96 mg. protein); purified enzyme (≡ .105 mg. protein); water to 0.75 ml. Incubated at 37° for 1 hr. Reaction stopped with trichloroacetic acid and aminoacetone estimated.

Crude enzyme			Purified enzyme		
Substrates	(μmoles)	Aminoacetone (μmoles)	Substrates	(μmoles)	Aminoacetone (μmoles)
L-threonine	5.0	.0332	L-threonine	5.0	.0166
L-threonine	5.0	.0345	L-threonine	10.0	.0316
D-allothreonine	5.0	.0106	D-allothreonine	5.0	.0093
D-allothreonine	5.0	.0106	D-allothreonine	10.0	.0146
D-threonine	5.0	.000	D-threonine	10.0	.000
L-allothreonine	5.0	.000	L-allothreonine	10.0	.000
			L-threonine + D-threonine	5.0 + 5.0	.0166
			L-threonine + L-allothreonine	5.0 + 5.0	.0166
			L-threonine + D-allothreonine	5.0 + 5.0	.0233
			D-threonine + D-allothreonine	5.0 + 5.0	.0084
			D-allothreonine + L-threonine	5.0 + 5.0	.0084

little nucleic acid. This purified enzyme has been used in a number of experiments.

Experiments with purified L-threonine dehydrogenase

A linear increase in the production of aminoacetone with increasing amounts of enzyme was obtained at pH 8.8. The pH curve in tris is very similar to that obtained with crude enzyme (Fig. 8), giving an optimal activity at pH 8.8 - 8.9.

The specificity of crude enzyme and purified enzyme towards the four isomers of α-amino-β-hydroxybutyric acid is described in Table 4. Only L-threonine and D-allothreonine are acted on by both crude and purified enzymes. D-Threonine and L-allothreonine are not acted on, and they do not inhibit the reaction of the other two isomers. D-Allothreonine is acted on at 34% the rate of L-threonine with crude enzyme, and at 50% the rate with purified enzyme.

With the purified enzyme, serine does not inhibit threonine dehydrogenase activity. DL-β-Hydroxybutyric acid appeared to inhibit strongly, but this is due to the presence of an active β-hydroxybutyric acid dehydrogenase which competes for the DPN^+ (see next section).

With crude enzyme γ-amino-β-hydroxybutyric acid is not oxidised to form an aminoketone, nor does it interfere with threonine dehydrogenase activity.

Stoichiometry of L-threonine dehydrogenase reaction

The reaction mechanism was assumed to be as follows:

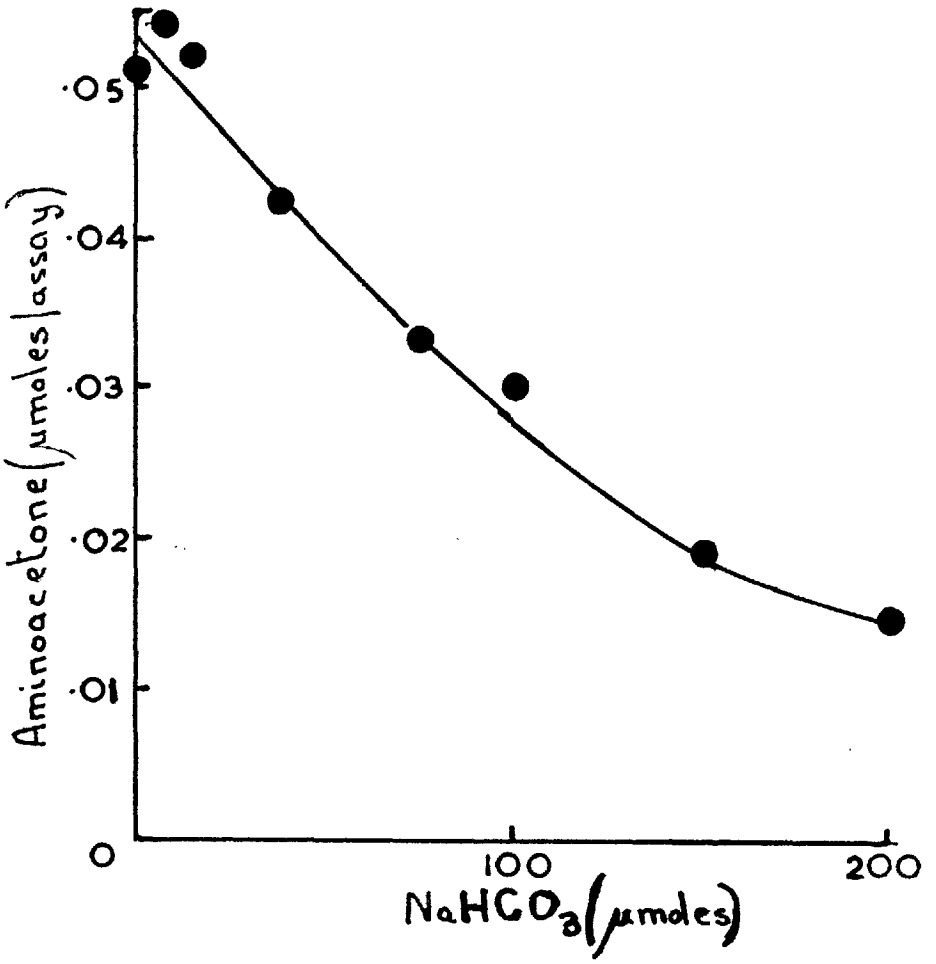


Fig. 9 Effect of sodium bicarbonate on L-threonine dehydrogenase activity

L-threonine, 25 μmoles; DPN^+, 0.5 μmoles; tris, pH 8.8, 75 μmoles; $NaHCO_3$ (pH 8.9) as stated; crude enzyme extract (≡ 0.45 mg. protein); water to 0.75 ml. Incubation 1 hr. at 37°. Reaction stopped with trichloroacetic acid and aminoacetone estimated.

$$\text{Threonine} + DPN^{+} \longrightarrow \text{Aminoacetone} + DPNH + H^{+} + CO_2$$

This has been confirmed by finding that the ratio of aminoacetone formed to DPNH formed is approximately unity (Table 5).

TABLE 5

Stoichiometry of L-threonine dehydrogenase reaction

Assay: DL-threonine, 200 μmoles; DPN^{+}, 20 μmoles; tris pH 8.8, 300 μmoles; purified enzyme (≡ .21 mg. protein) and water to 3.0 ml. were incubated at 37° in the heated cell-holder of the Unicam SP 500. The formation of DPNH was followed at 340 mμ (1 μmole DPNH/3.0 ml. ≡ 2.07 o.d. at 340 mμ with 1 cm. light path). The reaction was stopped after 90 min. and the aminoacetone estimated.

DPNH (μmoles)	Aminoacetone (μmoles)
.070	.084

When determining the pH optimum of crude enzyme (Fig. 8) it was noted that a low enzyme activity was obtained in carbonate-bicarbonate buffer as compared with tris buffer. This has been extended by finding that $NaHCO_3$ inhibits threonine dehydrogenase activity (Fig. 9)

Effect of inhibitors on L-threonine dehydrogenase

A range of known enzyme inhibitors has been tried for an effect on threonine dehydrogenase activity (Table 6). The controls were performed in each case to allow for non-enzymic destruction of aminoacetone.

TABLE 6

Effect of inhibitors on L-threonine dehydrogenase activity

Enzyme assay: DL-threonine, 50 μmoles; DPN^+, 0.3 μmoles; tris pH 8.8, 75 μmoles; enzyme (crude extract ≡ .45 mg. protein; $MnCl_2$ supernatant ≡ .26 mg. protein); inhibitor as indicated; water to 0.75 ml. Incubated at 37° for 1 hr. Reaction stopped with trichloroacetic acid and aminoacetone estimated. Controls: aminoacetone, 0.052 μmoles; tris pH 8.8, 75 μmoles; inhibitor and water to 0.75 ml. were mixed. Trichloroacetic acid was added immediately - non-incubated control, or after incubation at 37° for 30 min. - incubated control, and aminoacetone was estimated. Results are expressed as percentage of aminoacetone in the tubes without added inhibitor. The non-incubated control gave the same amount of aminoacetone as the incubated control, in the absence of inhibitor.

Added compound	(M)	Enzyme activity in crude extract	Added compound	Enzyme activity in $MnCl_2$ supernatant	Controls	
			(M)		Non-incubated	Incubated
$HgSO_4$	10^{-3}	5	10^{-3}	10	95	10
$CuSO_4$	10^{-3}	13	10^{-3}	6	100	24
$AgNO_3$	2×10^{-3}	5	2×10^{-3}	12	107	99
$ZnSO_4$	10^{-3}	92	10^{-3}	77	100	98
$PbAc_2$	10^{-3}	63	10^{-3}	54	105	103
$FeSO_4$	10^{-3}	100	10^{-3}	125	105	106
$FeCl_3$	10^{-3}	98	10^{-3}	125	111	112
$MgSO_4$	10^{-3}	121	10^{-3}	119	103	101
$MnSO_4$	10^{-3}	59	10^{-3}	62	81	61
$CaCl_2$	10^{-3}	126	10^{-3}	142	105	103
Thiosemicarbazide	2×10^{-3}	98	4×10^{-3}	116	111	105
p-Chloromercuri-benzoate	5×10^{-4}	14	5×10^{-4}	16	104	100
NaCN	10^{-4}	61	2×10^{-4}	50	99	95
L-Penicillamine	2×10^{-3}	88	2×10^{-3}	112	114	105
D-Penicillamine	2×10^{-3}	90	2×10^{-3}	119	115	107
L-Cysteine	2×10^{-3}	96	2×10^{-3}	125	115	107
Iodoacetamide	2×10^{-3}	80	5.4×10^{-3}	74	101	120
Sodium azide	2×10^{-4}	108	2×10^{-4}	112	114	105
$\alpha\alpha'$-Dipyridyl	2×10^{-3}	100	2×10^{-3}	97	101	98
8-Hydroxyquino-line	2×10^{-3}	78	5.4×10^{-3}	65	103	100
EDTA	10^{-3}	18	10^{-3}	17	106	100

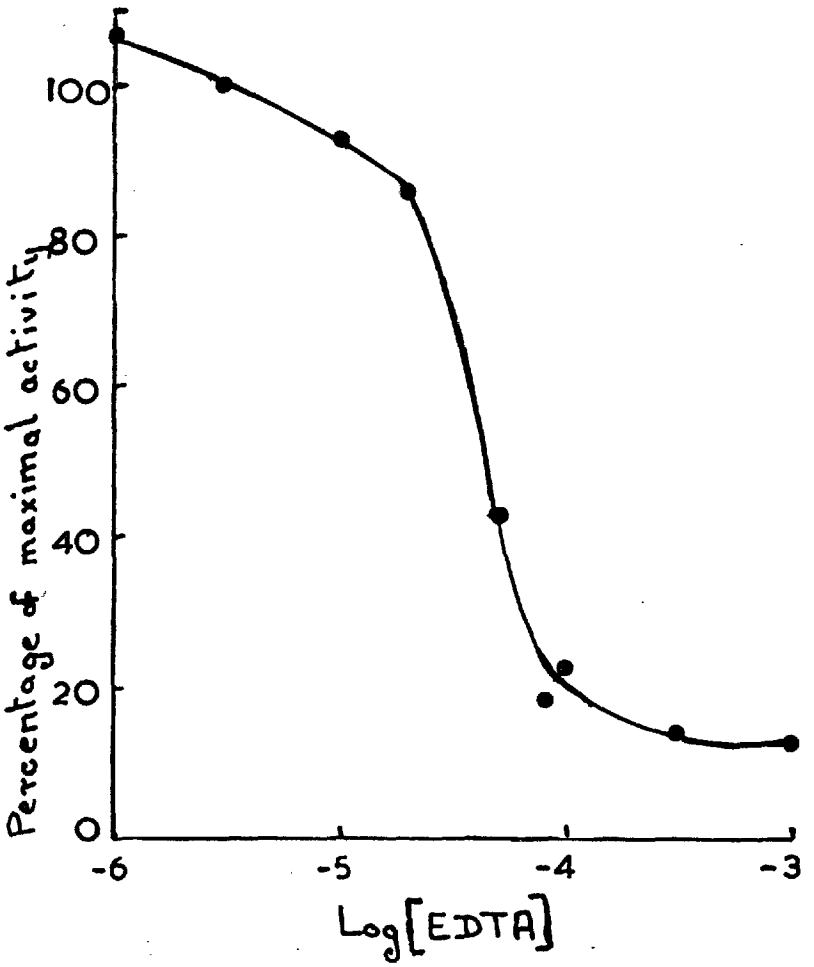


Fig. 10 Inhibition of L-threonine dehydrogenase by EDTA

DL-threonine, 50 μmoles; DPN^+, 0.3 μmoles; tris pH 8.8, 75 μmoles; crude enzyme extract (= 0.45 mg. protein); water to 0.75 ml. Incubation at 37° for 1 hr. Reaction stopped with trichloroacetic acid and aminoacetone estimated.

None of the compounds tested has any appreciable effect on the colorimetric estimation of aminoacetone, but aminoacetone disappears when incubated at 37° in the presence of $HgSO_4$, $CuSO_4$ or $MnSO_4$.

The inhibition due to ethylenediaminetetraacetic acid (EDTA) has been investigated further (Fig. 10); 50% inhibition occurs at 5.6×10^{-5} M EDTA.

Activity of DPN$^+$ analogues with L-threonine dehydrogenase

Deamino-DPN$^+$ cannot replace DPN$^+$ for threonine dehydrogenase activity and 3-acetylpyridine-DPN$^+$ has only 40% the activity of DPN$^+$. Neither analogue inhibits threonine dehydrogenase activity with DPN$^+$ (Table 7).

TABLE 7

Activity of DPN$^+$ analogues with L-threonine dehydrogenase

Assay: DL-threonine, 50 µmoles; DPN$^+$ or analogue, 0.2 µmoles; tris pH 8.8, 75 µmoles. Crude enzyme extract (4.5 mg. protein/ml.), and water to 0.75 ml. Incubation at 37° for 45 min. Reaction stopped with trichloroacetic acid and aminoacetone estimated.

Enzyme	Aminoacetone (µmoles)				
(ml./assay)	DPN$^+$	Deamino-DPN$^+$	3 Acetyl pyridine DPN$^+$	DPN$^+$ + deamino-DPN$^+$	DPN$^+$ + 3 acetyl pyridine-DPN$^+$
0.04	.0087	.000	.0030	-	-
0.08	.0170	.000	.0070	.0190	.0200
0.12	.0250	.000	.0100	-	-

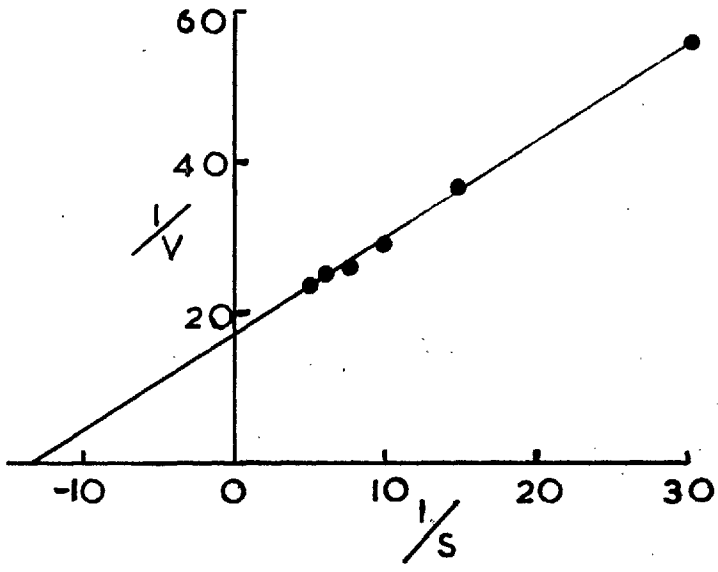


Fig. 11a K_M for L-threonine for L-threonine dehydrogenase. L-threonine, as stated (S = moles/l.); DPN^+, 0.3 µmoles; tris pH 8.8, 75 µmoles; crude enzyme extract (= 0.27 mg. protein); water to 0.75 ml. Incubation at 37° for 30 min. Reaction stopped with trichloroacetic acid and aminoacetone estimated. V (initial reaction velocity) expressed as µmoles aminoacetone/30 min.

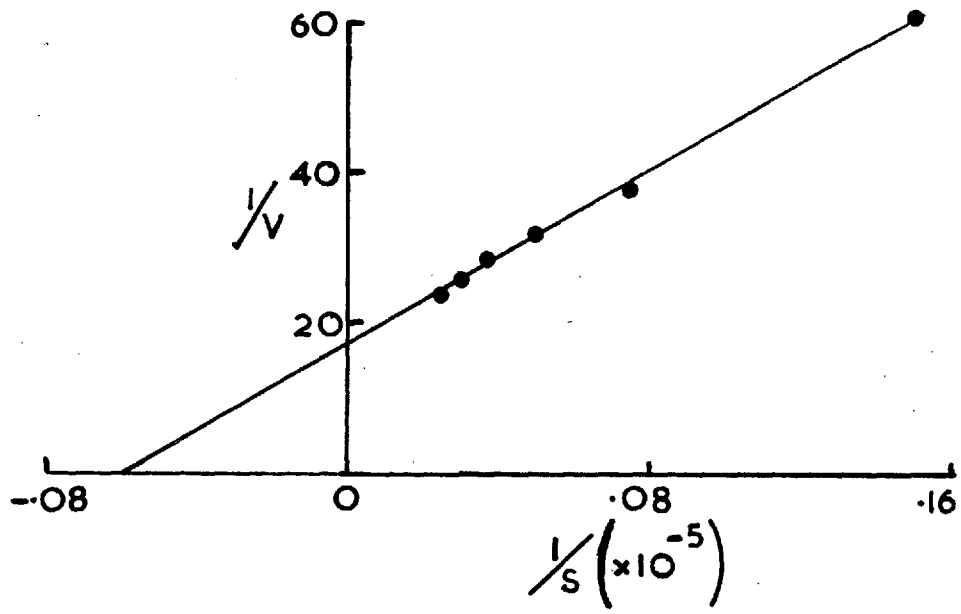


Fig. 11b K_M for DPN^+ for L-threonine dehydrogenase. DPN^+ as stated (S = moles/l.); L-threonine, 150 µmoles; tris pH 8.8, 75 µmoles; crude enzyme extract (= 0.27 mg. protein); water to 0.75 ml. Incubation at 37° for 30 min. Reaction stopped with trichloroacetic acid and aminoacetone estimated. V (initial reaction velocity) expressed as µmoles aminoacetone/30 min.

Michaelis constants of L-threonine dehydrogenase

The K_M values for L-threonine and DPN^+ for threonine dehydrogenase have been determined. For L-threonine the assays were done in the presence of enough DPN^+ to saturate the enzyme and for DPN^+ in the presence of enough L-threonine to saturate the enzyme. The results, plotted as 1/V against 1/S after Lineweaver & Burk (1934) (Fig. 11a and b) give:

L-threonine $K_M = 7.4 \times 10^{-2}$ M at 37°

DPN^+ $K_M = 1.7 \times 10^{-4}$ M at 37°.

Extracts from organisms grown under a variety of conditions have been assayed for a range of enzyme activities (see later). One of these enzymes is L-threonine dehydrogenase. The assay conditions used in all the experiments are: DL-threonine, 50 µmoles; DPN^+, 0.3 µmoles; tris pH 8.8, 75 µmoles; crude enzyme extract; water to 0.75 ml. Incubation at 37° for 45 or 60 min. Reaction stopped with trichloroacetic acid and aminoacetone estimated. The activity of the enzyme is expressed as µmoles aminoacetone/mg. protein/hr.

One result which is relevant to this section is the finding that the threonine dehydrogenase activity is greater in extracts from biotin deficient organisms than from normal organisms. This has been shown to be an increase in L-threonine dehydrogenase activity and not the development of activity towards D-threonine.

β-HYDROXYBUTYRIC ACID DEHYDROGENASE ACTIVITY IN EXTRACTS

It was found that β-hydroxybutyric acid strongly inhibited threonine

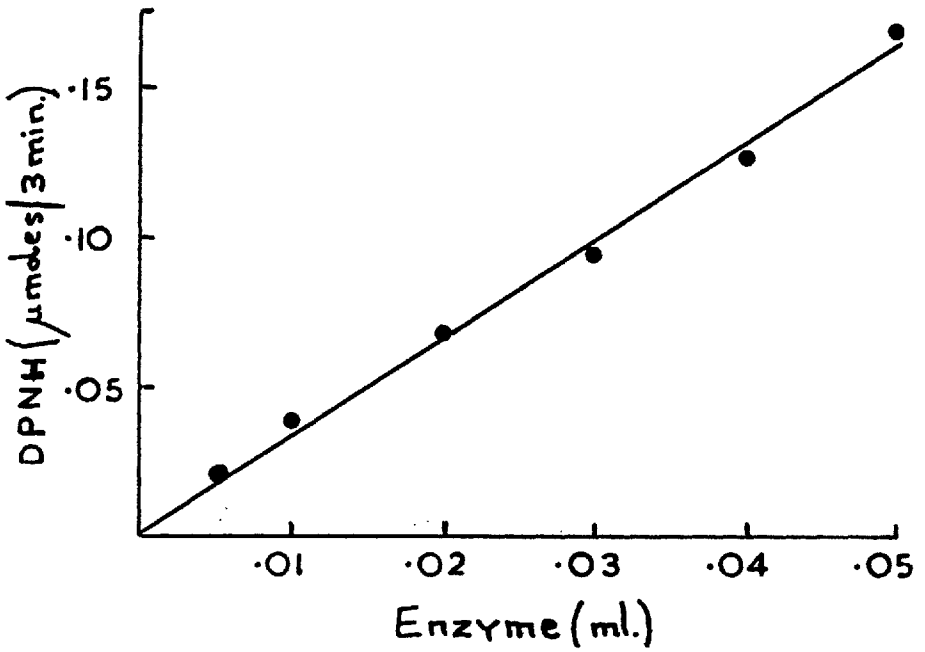


Fig. 12 β-Hydroxybutyric acid dehydrogenase: effect of increasing the enzyme concentration

DL-β-hydroxybutyric Na^+ salt, 10 μmoles; DPN^+, 1 μmole; tris pH 8.8, 300 μmoles; crude enzyme extract (4.5 mg. protein/ml.) added as stated; water to 3.0 ml. Incubation at 37° in heated cell holder of the spectrophotometer. Production of DPNH followed at 340 mμ. Readings every 30 sec. for 3 min.

dehydrogenase activity (p. 39). At first this was thought to be due to the similarity in the structures of β-hydroxybutyric acid and threonine (α-amino-β-hydroxybutyric acid). Further investigation showed that the apparent inhibition was due to the extremely rapid reduction of DPN^+ by the crude enzyme extract and by the partially purified threonine dehydrogenase in the presence of β-hydroxybutyric acid.

Some of the properties of this β-hydroxybutyric acid dehydrogenase have been studied, since it was thought that the same enzyme might be responsible for both β-hydroxybutyric and threonine dehydrogenase activities. All the experiments have been performed with extracts prepared from organisms grown anaerobically in the light.

Assay system for β-hydroxybutyric acid dehydrogenase

DL-β-Hydroxybutyric acid Na^+ salt, 10 μmoles; DPN^+, 1 μmole; tris pH 8.8, 300 μmoles; enzyme; and water to 3.0 ml., were incubated at 37° in the heated cell-holder of the spectrophotometer. The production of DPNH was measured by following the increase in optical density at 340 mμ.

Using crude enzyme extract the production of DPNH was linear for 3 min. and the amount of DPNH formed was proportional to the enzyme added (Fig. 12).

This assay method is therefore a valid measure of the enzyme activity and has been used, exactly as stated above, in all the experiments to be described. The specific activity is expressed as μmoles DPNH/mg. protein/min.

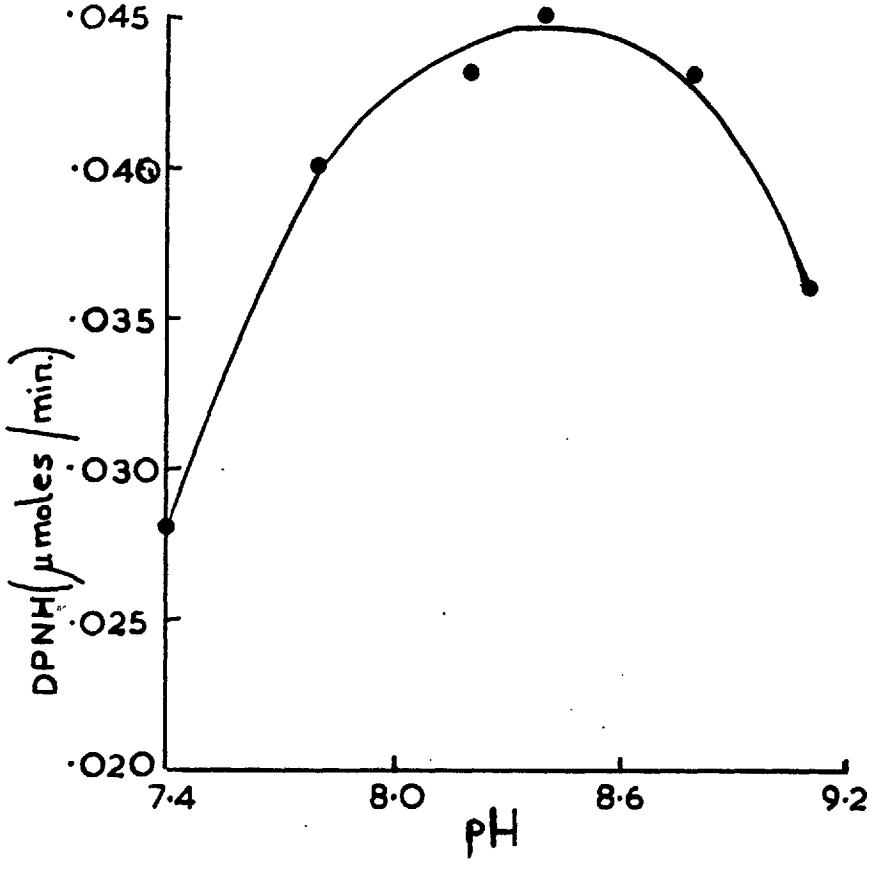


Fig. 13 β-Hydroxybutyric acid dehydrogenase: pH curve

Standard assay conditions, as described on p. 49 and in Fig. 12. Tris buffer pH as stated. Crude dialysed enzyme extract (≡ .18 mg. protein).

Partial purification of the enzyme was carried out exactly as described for threonine dehydrogenase (p. 36). A comparison of activities, purifications, and yields of both threonine and β-hydroxybutyric acid dehydrogenases showed that no separation of enzymic activities had been obtained.

For β-hydroxybutyric acid dehydrogenase TPN^+ cannot replace DPN^+, and TPN^+ does not interfere with the reduction of DPN^+ to DPNH. The pH optimum for the enzyme has been found to be 8.4 - 8.6 (Fig. 13). Sodium bicarbonate inhibits β-hydroxybutyric acid dehydrogenase. The presence of 0.2 M-$NaHCO_3$ in the standard assay reduces the activity to 39% of the control (cf. Fig. 9 where a similar concentration of $NaHCO_3$ inhibits the threonine dehydrogenase activity to 37% of the control).

The effect of some known enzyme inhibitors on β-hydroxybutyric acid dehydrogenase has been determined (Table 8).

The addition of 300 μmoles of DL-threonine to the standard assay for β-hydroxybutyric acid dehydrogenase does not inhibit the rate of DPNH production. γ-Amino-β-hydroxybutyric acid does not reduce DPN^+ in the presence of crude enzyme extract, but when 20 μmoles are added to the standard assay system for β-hydroxybutyric acid dehydrogenase a 30% inhibition of DPNH production is observed.

The reversibility of the β-hydroxybutyric acid dehydrogenase has been shown by observing a rapid DPNH oxidation when the sodium salt of acetoacetic acid and DPNH are incubated with crude enzyme extract.

Using the standard conditions (p. 49) it was found that with this enzyme deamino-DPN^+ was not reduced and 3-acetylpyridine-DPN^+ was reduced

at 8% the rate of DPN^+. Neither analogue inhibited DPN^+ reduction.

TABLE 8

Effect of inhibitors on β-hydroxybutyric acid dehydrogenase

Ehzyme assay: standard conditions on p. 49 . Crude enzyme extract (= 0.135 mg. protein).

Inhibitor	Concentration (M)	Activity µmoles DPNH/min.	% Control
None	-	.033	100
None	-	.034	
p-Chloromercuri-benzoate	5×10^{-4}	.000	0
NaCN	2×10^{-4}	.029	86
Iodoacetamide	4×10^{-3}	.033	100
EDTA	10^{-3}	.019	53
$HgSO_4$	10^{-3}	.000	0
$MgSO_4$	10^{-3}	.031	91
$CaCl_2$	10^{-3}	.039	107

Michaelis constants for β-hydroxybutyric acid dehydrogenase

The K_M values for β-hydroxybutyric acid and DPN^+ for β-hydroxybutyric acid dehydrogenase have been determined. Each estimation was made at a saturating concentration of the other component. Fig. 14a and b show the plots of 1/V against 1/S. Presumably only one isomer of β-hydroxybutyric acid is attacked and the other does not inhibit.

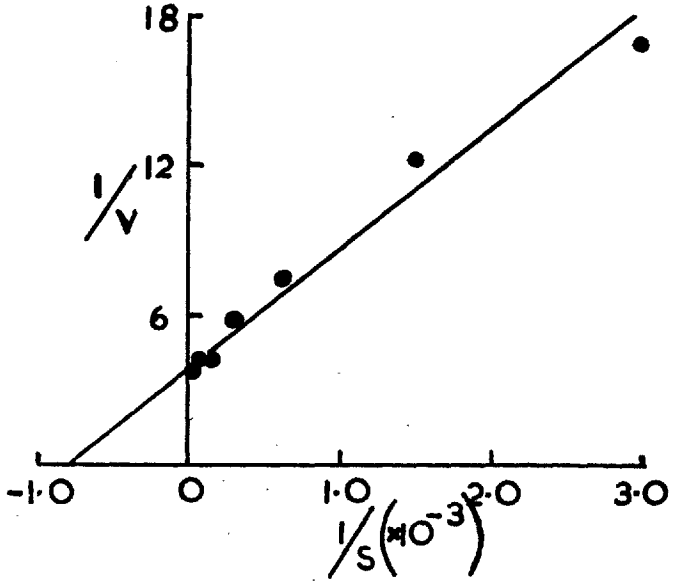


Fig. 14a K_M for β-hydroxybutyric acid for β-hydroxybutyric acid dehydrogenase. DL-β-hydroxybutyric acid Na^+ salt, as stated (S = moles/l.). DPN^+, 1.0 µmoles; tris pH 8.8, 300 µmoles; crude enzyme extract (= .093 mg. protein); water to 3.0 ml. Incubation at 37° for 3 min. V (initial velocity) expressed as change in optical density in 3 min. at 340 mµ.

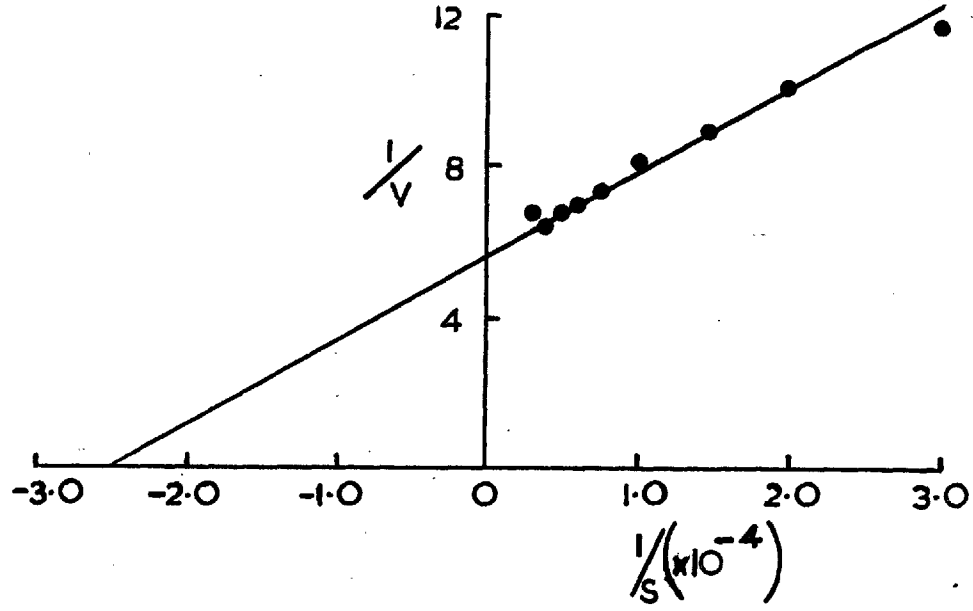


Fig. 14b K_M for DPN^+ for β-hydroxybutyric acid dehydrogenase. DPN^+, as stated (S = moles/l.); DL-β-hydroxybutyric acid Na^+ salt, 20 µmoles; tris pH 8.8, 300 µmoles; crude enzyme extract (= .093 mg. protein); water to 3.0 ml. Incubation at 37° for 3 min. V (initial velocity) expressed as change in optical density in 3 min. at 340 mµ.

As will be discussed later, the isomer attacked is most probably the D-isomer.

For Dβ- hydroxybutyric acid $K_M = 6.25 \times 10^{-4}$ M at 37°

For DPN$^+$ $K_M = 4.0 \times 10^{-5}$ M at 37°.

δ-AMINOLAEVULIC ACID TRANSAMINASE IN EXTRACTS

δ-Aminolaevulic acid transaminases have been reported previously in mammalian tissues by Kowalski et al. (1957) and Kowalski et al. (1959) and in Corynebacterium diphtheriae by Bagdasarian (1957). It was decided to look for an ALA transaminase in extracts of Rps. spheroides since it was realised that such an enzyme could control porphyrin formation by competing with ALA dehydrase for ALA. In the reports mentioned above, transamination was assayed by incubating ALA plus an α-ketoacid in the presence of enzyme and detecting any amino acid formed by chromatography. This is a relatively tedious procedure and only semi-quantitative. Quantitative studies can be made if the reaction is performed in the reverse direction, that is from γδ-dioxovaleric acid and an amino acid, since there is available a very sensitive and specific colorimetric estimation for ALA. In this way an ALA transaminase has been found in crude enzyme extracts of Rps. spheroides (Table 9). All the experiments have been performed with extracts from organisms grown anaerobically in the light.

Methylglyoxal which on transamination with an amino acid ought to give aminoacetone did not do so when an experiment identical with that

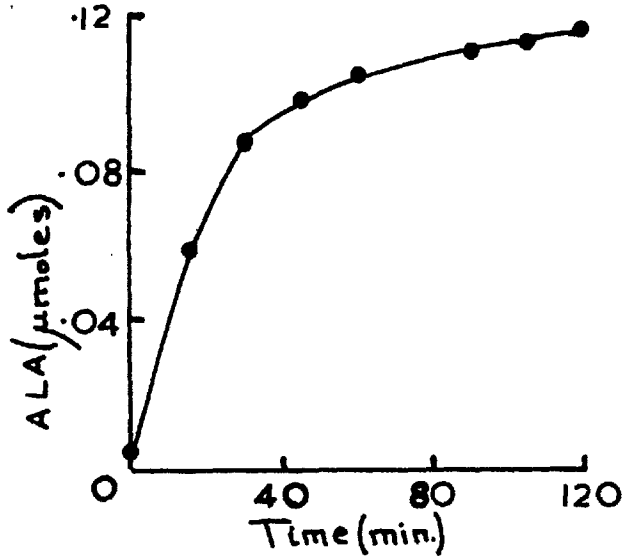


Fig. 15 L-Alanine-ALA transaminase: ALA production with time

L-alanine, 20 μmoles; γδ-dioxovaleric acid, 5 μmoles; pyridoxal phosphate, 0.20 μmole; phosphate pH 7.4, 75 μmoles; crude enzyme extract (≡ 0.41 mg. protein); water to 0.75 ml. Incubation at 37° for times shown. Reaction stopped with trichloroacetic acid and ALA estimated.

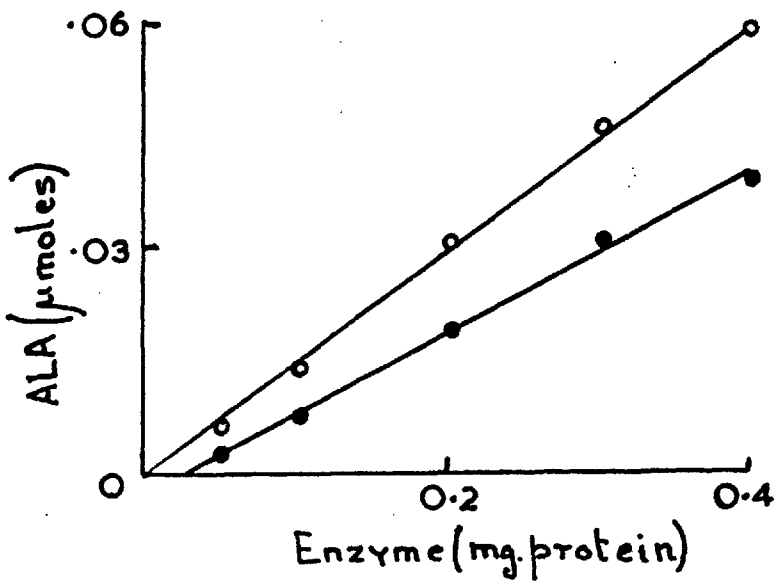


Fig. 16 L-Alanine-ALA transaminase: effect of increasing the enzyme concentration

L-alanine, 20 μmoles; γδ-dioxovaleric acid, 5 μmoles; phosphate pH 7.4, 75 μmoles; crude enzyme extract as stated; water to 0.75 ml. Incubation at 37° for 10 min. (●——●) and for 20 min. (○——○). Reaction stopped with trichloroacetic acid and ALA estimated.

described in Table 9 was performed with methylglyoxal instead of γδ-dioxovaleric acid.

TABLE 9

ALA transaminase activity in extracts

Assay: amino acids, 20 μmoles; γδ-dioxovaleric acid, 10 μmoles; phosphate buffer pH 7.4 or 8.1, 75 μmoles; enzyme - crude extract undialysed (≡ 0.51 mg. protein); water to 0.75 ml. Incubated at 37° for 1 hr. Reaction stopped with trichloroacetic acid and ALA estimated.

Amino acid	pH	ALA μmoles/assay
L-Glutamic acid	7.4	.0104
L-Glutamic acid	8.1	.0096
L-Aspartic acid	7.4	.000
L-Aspartic acid	8.1	.000
L-Alanine	7.4	.076
L-Alanine	8.1	.052

The production of ALA as a function of time has been studied (Fig. 15). A boiled enzyme control gives no ALA, and absence of pyridoxal phosphate does not decrease the activity.

The amount of ALA produced increases linearly with increasing amounts of enzyme (Fig. 16).

Fig. 17 shows that concentrations of γδ-dioxovaleric acid greater

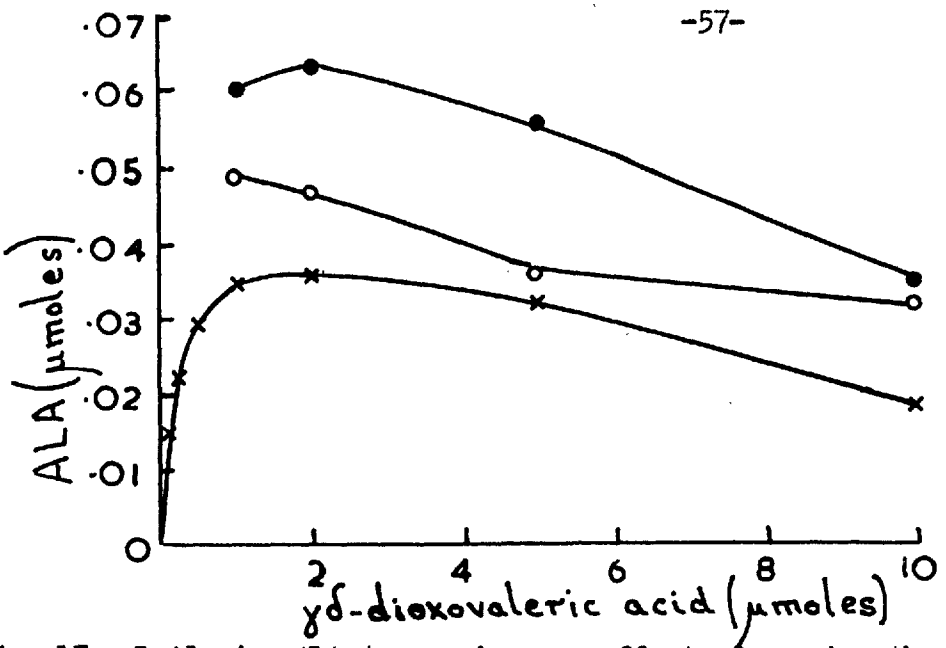


Fig. 17 L-Alanine-ALA transaminase: effect of varying the substrate concentrations

L-alanine and γδ-dioxovaleric acid as stated. Phosphate pH 7.4, 75 µmoles; crude enzyme extract (= .41 mg. protein); water to 0.75 ml. Incubated at 37° for 15 min. Reaction stopped with trichloroacetic acid and ALA estimated. Concentration of L-alanine: 20 µmoles (●——●); 10 µmoles (○——○); 4 µmoles (×——×).

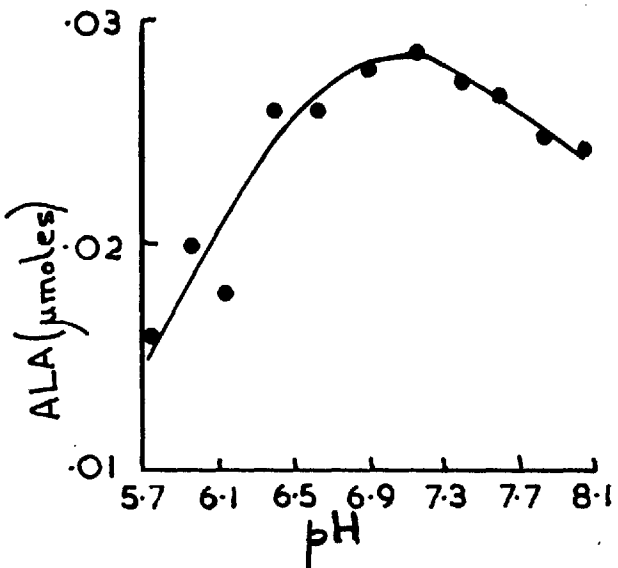


Fig. 18 L-Alanine-ALA transaminase: pH curve

L-alanine, 10 µmoles; γδ-dioxovaleric acid, 1 µmole; Phosphate pH as stated, 75 µmoles; crude enzyme extract (= 0.4 mg. protein); water to 0.75 ml. Incubated at 37° for 20 min. Reaction stopped with trichloroacetic acid and ALA estimated.

than 2 μmoles/assay inhibit ALA production. Increasing the concentration of L-alanine is not inhibitory. In most of the experiments to be described on the L-alanine-ALA transaminase, L-alanine has been used at 10 μmole/assay, and γδ-dioxovaleric acid at 1 μmole/assay.

The pH optimum of the L-alanine-ALA transaminase is 7.0 - 7.2 (Fig.18

Identification of products of the reaction

The product of transamination between γδ -dioxovaleric acid and L-alanine has been assumed to be ALA on the basis of the colour test of Mauzerall & Granick (1956) - the spectrum of the coloured product being identical with that of genuine ALA.

A large scale incubation was done with γδ-dioxovaleric acid and L-alanine, with and without enzyme. After removal of protein and salt the residue was chromatographed in butanol-acetic acid-water (60:15:25 by vol.) and electrophoresis on paper at 2500 v. also performed. On treatment with ninhydrin a yellow spot corresponding to ALA was found. In the control, not containing enzyme, a faint yellow spot was also found. Similarly alanine has been detected as the product when ALA and pyruvate are incubated with the enzyme. No alanine is detected in the absence of enzyme.

Attempts to measure the reverse reaction quantitatively by estimating disappearance of ALA have not been successful. The amounts of ALA disappearing were too low to measure accurately. This result may imply that the equilibrium of the reaction favours ALA production.

TABLE 10

Effect of pyruvate, methylglyoxal and α-oxoglutarate on L-alanine-ALA transaminase activity

Assay: L-alanine, 10 μmoles; γδ-dioxovaleric acid, 1 μmole; phosphate pH 7.15, 75 μmoles; additions as stated; enzyme - crude extract (≡ 0.21 mg. protein) H_2O to 0.75 ml. Incubated at 37° for 1 hr. Reaction stopped with trichloroacetic acid and ALA estimated.

Concentration of inhibitor (μmoles)	ALA (μmoles)		
	Methylglyoxal	Pyruvate	α-Oxoglutarate
-	.0288	.0288	.0288
1.0	.0086	.0060	.0228
2.0	.0047	.0023	.0232
5.0	.0000	.0000	.0206

Ability of amino acids other than L-alanine to transaminate with γδ-dioxovaleric acid

The assay system was: amino acid, 10 μmoles; γδ-dioxovaleric acid, 1 μmole; phosphate pH 7.15, 75 μmoles; crude enzyme extract (≡ .54 mg. protein) and water to 0.75 ml. Incubation was at 37° for 1 hr. The amino acids gave the following amounts of ALA expressed as percentage of the amount formed from L-alanine: D-alanine, 74%; β-alanine, 21%; L-aspartic acid, L-glutamic acid, L-ethionine, L-methionine, L-phenylalanine, and L-threonine - all 7 - 10%. When added at a concentration of 10 μmoles to a system forming ALA in the presence of 10 μmoles of L-alanine the only amino acid which inhibits is L-glutamic acid, the activity being 72% of the control.

Pyruvate and methylglyoxal strongly inhibit the L-alanine-ALA transaminase at low concentration, while α-oxoglutarate has little effect (Table 10). Pyruvate is a product of the reaction, and methylglyoxal is an analogue of γδ-dioxovaleric acid, and as described previously excess γδ-dioxovaleric acid also inhibits (Fig. 17). Solutions of methylglyoxal, on standing, are oxidised to pyruvic acid. The methylglyoxal solution used in the above experiment was titrated against sodium hydroxide using phenolphthalein as indicator and found to contain 0.026 μmoles of pyruvic acid/μmole of methylglyoxal. This shows that the inhibition found for methylglyoxal is genuine and not due to the pyruvic acid present. Aminoacetone at a concentration of 0.6 μmoles in the above assay system does not inhibit ALA production. The method of estimating ALA in the presence of aminoacetone is to form the pyrroles

with acetylacetone as in the method of Mauzerall & Granick (1956), then adjust the pH to 8 and extract out the neutral pyrrole formed from aminoacetone with ether. The pyrrole from ALA stays in the aqueous phase and can now be estimated in the usual way.

Crude enzyme extract prepared in tris buffer instead of in phosphate gave a low L-alanine-ALA transaminase activity. The activity was increased by dialysis against phosphate buffer. 10 μmoles of tris buffer pH 7.4, when added to the standard assay system described in Table 10, inhibited the reaction, only 34% of the ALA found in the control being formed.

Transamination between L-, D-, and β-alanine and γδ-dioxovaleric acid

L-, D-, and β-Alanine are the only three amino acids tested which transaminate with γδ-dioxovaleric acid at an appreciable rate in the presence of crude enzyme extract. It was of interest to see whether more than one enzyme was involved.

Partial purification of the crude enzyme extract, first with $MnCl_2$ to remove nucleic acid, then by $(NH_4)_2SO_4$ fractionation at 40%, 60%, 80% and 100% saturation showed no separation of the activities towards L-alanine and β-alanine. The activities and recoveries in each fraction were identical. However, much of the activity towards D-alanine was lost on purification. The results in Table 11 strongly indicate the presence of an alanine racemase in the crude enzyme extract. They show that D-alanine, on incubation with crude enzyme extract, is converted to a compound which forms ALA on incubation with γδ-dioxovaleric acid in

the presence of partially purified enzyme. D-Alanine itself gives very little ALA on incubation with γδ-dioxovaleric acid in the presence of partially purified enzyme.

TABLE II

Evidence for an alanine racemase in crude enzyme extracts

Assay: D-alanine or L-alanine, 10 μmoles; pyridoxal phosphate, 0.1 μmole; phosphate pH 7.4, 75 μmoles; crude enzyme extract (= 0.23 mg. protein); and water to 0.55 ml. were pre-incubated at 37° for the times stated. Mixtures were boiled for 3 min. and the protein precipitates removed by centrifugation. To each supernatant was added γδ-dioxovaleric acid, 1 μmole, and 0.1 ml. of the combined 40 - 60% and 60 - 80% $(NH_4)SO_4$ fractions (preparation described in text). Incubation at 37° for 30 min. Reaction stopped with trichloroacetic acid and ALA estimated.

Substrate	Time of pre-incubation (min.)	ALA (μmoles)
D-Alanine	30	.0112
L-Alanine	30	.0181
D-Alanine	0	.0029
L-Alanine	0	.0189

L-Alanine-ALA transaminase activity has been assayed in crude enzyme extracts from organisms grown under different conditions. The results will be described in the appropriate section. The standard assay system used was L-alanine, 10 μmoles; γδ-dioxovaleric acid, 1 μmole; phosphate buffer pH 7.4, 75 μmoles; crude enzyme extract and water to 0.75 ml.;

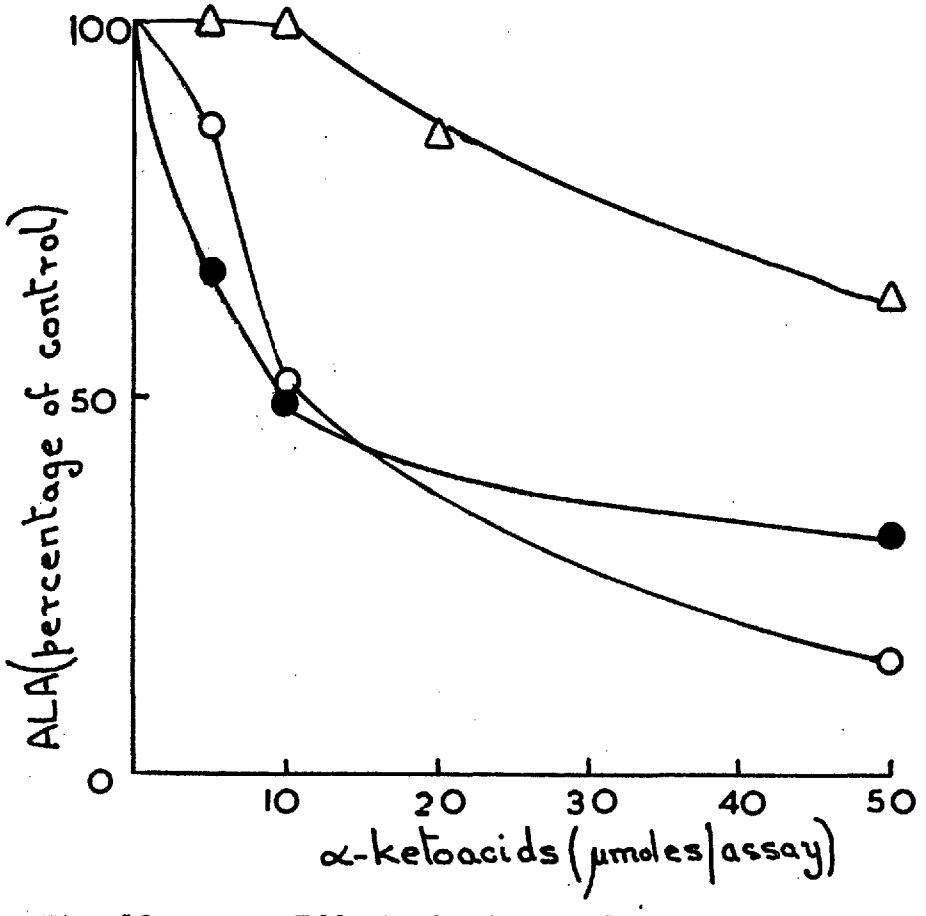


Fig. 19 Effect of *a*-ketoacids on ALA synthesis

Glycine, 25 μmoles; sodium succinate, 25 μmoles; CoA, 0.05 μmoles; ATP, 4 μmoles; pyridoxal phosphate, 0.1 μmoles; $MgSO_4$, 0.5 μmoles; GSH, 2.5 μmoles. Phosphate pH 7.4, 75 μmoles; crude enzyme extract (= .53 mg. protein); pyruvate, *a*-oxoglutarate, oxaloacetate (all adjusted to pH 7 approx. with NaOH) as stated. Total volume 0.75 ml. Incubations at 37° for 45 min. and 90 min. Reaction stopped with trichloroacetic acid and ALA estimated. Results expressed as percentage of controls and average of results at 45 min. and 90 min. plotted.

.Pyruvate ●——●; *a*-oxoglutarate O——O; oxaloacetate Δ——Δ.

incubated at 37° for 15 min. Reaction stopped with trichloroacetic acid and ALA estimated. Specific activity is expressed as μmoles ALA/mg. protein/15 min.

Possible role of aminoacetone and ALA transaminase in the control of porphyrin synthesis

If L-alanine-ALA transaminase is active enough to control the amount of ALA available to form porphyrins in whole organisms, then on addition of pyruvate to a system forming ALA in the presence of crude enzyme extract, some of the ALA ought to be removed by transamination. The results of such an experiment are shown in Fig. 19. The rate of synthesis of ALA was not linear over the 90 min. but the percentage inhibition by the α-ketoacids was approximately the same at both 45 and 90 min. and the average value is shown. The inhibition by pyruvate is what would be expected if ALA were being removed by transamination, but as reported on p. 60 . there is only a weak L-glutamic acid-ALA transaminase activity in crude enzyme extracts. The addition of L-alanine (20 μmoles) does not inhibit ALA production, and when added with pyruvate does not reverse the inhibition caused by pyruvate.

Aminoacetone (1 μmole) added to the system for ALA synthesis under the conditions described in Fig. 19 does not inhibit the reaction.

As found with the enzyme from liver (Gibson et al., 1955) and the one from chicken erythrocytes (Granick, 1958), aminoacetone is not a substrate for ALA dehydrase and at a concentration of 40 μmoles in the standard assay system (described on p. 20) does not inhibit porphobilinogen production.

THE ROLE OF BIOTIN IN PORPHYRIN AND BACTERIOCHLOROPHYLL BIOSYNTHESIS

Lascelles (1956) reported that when Rps. spheroides, after growth, were illuminated in Mixture I, containing α-oxoglutarate and glycine (composition of Mixture I is given on p. 17) free porphyrin accumulated in the medium. This porphyrin was mainly coproporphyrin, but contained some uroporphyrin. If the organisms were grown in a medium deficient in nicotinic acid, thiamine, or biotin (the only three essential vitamins for the growth of Rps. spheroides) no porphyrin was formed on illumination in Mixture I unless the deficient factor was added. Deficient organisms formed porphyrin when illuminated in a medium containing ALA (Mixture II) at the same rate as normal organisms in the absence of added vitamin. These results indicated that the production of ALA, not its utilisation, was being blocked in vitamin deficiency. Lascelles (1956) thought that deficiency of nicotinic acid and thiamine could block the conversion of α-oxoglutarate to succinyl-CoA, but offered no explanation for the role of biotin. It was thought that biotin might be involved in the actual condensation of succinyl-CoA and glycine to form ALA. Since relatively little is known about the properties of the enzyme involved in this condensation, that is ALA synthetase, it was decided to investigate the role which biotin played in porphyrin biosynthesis.

Lascelles' (1956) findings with biotin deficient organisms have been confirmed and extended (Table 12).

Formation of porphyrin by biotin deficient organisms from succinate

TABLE 12

Effect of biotin deficiency on porphyrin production

Organisms which had been grown aerobically in the dark in the presence of 5.4×10^{-9} M biotin were harvested and illuminated, in the media stated, under standard conditions for 24 hr. Biotin where added is 4×10^{-8} M

Medium	Biotin	Porphyrin (μmoles/ml.)
Mixture I	-	13.5
Mixture I	+	50.3
Mixture IS	-	6.6
Mixture IS	+	21.6
Mixture II	-	33.5
Mixture II	+	37.3

and glycine (Mixture IS) also requires the addition of biotin. Since succinate and α-oxoglutarate are converted to succinyl-CoA by two different enzymes, neither of which is a biotin enzyme, this result would indicate the probability that the step which requires biotin is the ALA synthesis step.

When normal organisms are illuminated in Mixture I, growth and bacteriochlorophyll synthesis occur. Biotin was required for growth and bacteriochlorophyll synthesis when biotin deficient organisms were illuminated in Mixture I or Mixture IS (Table 13).

TABLE 13

Biotin requirement for growth and porphyrin and bacteriochlorophyll synthesis by biotin deficient organisms

Aerobically grown biotin deficient organisms illuminated, in the medium stated, under standard conditions for 24 hr. Biotin, 4×10^{-8} M added, where stated. Bacteriochlorophyll estimated by the method of Van Niel & Arnold (1938).

Medium	Biotin (4×10^{-8} M)	Dry weight of organisms (mg./ml.)	Porphyrin (μmoles/ml.)	Bacteriochlorophyll (μmoles/ml.)
Mixture I	-	1.43	1.6	0.88
Mixture I	+	2.64	4.9	7.9
Mixture IS	-	1.08	1.2	0.76
Mixture IS	+	2.38	3.7	4.6
Non-incubated control		1.16	1.6	0.0

No growth or bacteriochlorophyll synthesis occurs on illumination in Mixture II of normal or biotin deficient organisms.

Aerobically grown organisms contain very little bacteriochlorophyll, and Lascelles (1959) reported that adaptation, involving protein synthesis, was required before aerobically grown organisms formed pigment on illumination. Since biotin might be involved in this adaptation all further work has been done with normal and biotin deficient organisms grown anaerobically in the light. It was found that addition of biotin to biotin deficient light-grown organisms is required before growth and porphyrin and bacteriochlorophyll synthesis occur (Table 14).

TABLE 14

Requirement for biotin of biotin deficient anaerobically grown organisms

Normal or biotin deficient organisms grown anaerobically in the light with the biotin concentration stated, illuminated in Mixture I under standard conditions for 23 hr. Biotin added where stated.

Biotin in growth medium	Added biotin (8×10^{-8} M)	Dry weight of organisms (mg./ml.)	Porphyrin (μmoles/ml.)	Bacteriochlorophyll (μmoles/ml.
4×10^{-8} M	-	2.21	13.1	41.2
4×10^{-8} M	+	2.21	9.3	42.6
2×10^{-9} M	-	1.59	3.8	25.4
2×10^{-9} M	+	2.25	9.6	42.0
8×10^{-10} M	-	1.10	0.9	17.2
8×10^{-10} M	+	2.26	7.1	35.3
4×10^{-8} M non-illuminated controls	-	0.99	0.0	25.9
2×10^{-9} M non-illuminated controls	-	0.99	0.0	25.9
8×10^{-10} M non-illuminated controls	-	0.99	0.0	19.2

Reducing the biotin content of the growth medium reduces the yield of organisms obtained. In addition it reduces the pigmentation of the organisms (Table 15).

However, organisms whose growth and pigmentation are not reduced may still be biotin deficient in that they need biotin to be added before they grow, or form porphyrin and bacteriochlorophyll when illuminated in Mixture I. The organisms used in the experiment reported in Table 14 all gave the same growth yield and as can be seen in Table 14

TABLE 16

Enzyme activities in normal and biotin deficient cell extracts

All cultures grown anaerobically in the light; biotin concentration in growth medium as stated. Assays for ALA synthetase p. 19 ; ALA dehydrase p. 20 ; threonine dehydrogenase p. 47 ; β-hydroxybutyric acid dehydrogenase p. 49 ; L-alanine-ALA transaminase p. 63 . Crude enzyme extracts used in all assays. Experiment I was performed with extracts prepared by ultrasonic disruption, the others with Hughes Press extracts.

Exp.	Biotin in growth medium	Enzyme activities				
		μmoles product/mg. protein/hr.			μmoles DPNH/mg. protein/min.	μmoles ALA/mg. protein/15 min.
		ALA synthetase	ALA dehydrase	Threonine dehydrog.	β-OH-butyric dehydrog.	Alanine-ALA transaminase
I	4×10^{-8} M	.072 (100%)	.028 (100%)	-	-	-
I	8×10^{-10} M	.048 (67%)	.028 (100%)	-	-	-
I	2×10^{-10} M	.041 (57%)	.028 (100%)	-	-	-
II	4×10^{-8} M	.288 (100%)	.053 (100%)	-	-	-
II	8×10^{-10} M	.194 (67%)	.051 (96%)	-	-	-
II	4×10^{-10} M	.150 (53%)	.047 (89%)	-	-	-
III	4×10^{-8} M	.172 (100%)	.118 (100%)	.093 (100%)	.142 (100%)	-
III	8×10^{-10} M	.088 (51%)	.099 (84%)	.119 (128%)	.162 (114%)	-
III	2×10^{-10} M	.072 (42%)	.075 (67%)	.171 (184%)	.225 (158%)	-
IV	4×10^{-8} M	.265 (100%)	-	.097 (100%)	.171 (100%)	.126 (100%)
IV	6×10^{-10} M	.121 (46%)	-	.098 (101%)	.211 (122%)	.140 (111%)
V	4×10^{-8} M	-	-	-	-	.094 (100%)
V	8×10^{-10} M	-	-	-	-	.091 (97%)

the organisms grown with 2×10^{-9} M biotin have the same bacteriochlorophyll content as normal organisms.

TABLE 15

Effect of biotin deficiency on growth and pigment content of organisms

The organisms were grown anaerobically in the light in Medium S with the concentrations of biotin stated. Estimations as described on p. 18. The results are the average of two experiments.

Biotin	Dry weight of organisms	Bacteriochlorophyll	Carotenoids	
	mg./ml. of culture	μmoles/mg. dry wt.	μg./mg. dry wt.	
			Yellow	Red.
4×10^{-8} M	1.50	23.9	3.09	1.99
8×10^{-10} M	1.04	16.7	1.63	1.28
4×10^{-10} M	0.80	13.7	1.14	1.16

Enzyme activities in cell-free extracts of normal and biotin deficient organisms

To investigate the problem further, enzyme extracts were made from normal organisms and organisms grown under identical conditions except that the biotin in the growth medium was reduced. These extracts were assayed for ALA synthetase, ALA dehydrase, threonine dehydrogenase, β-hydroxybutyric acid dehydrogenase and L-alanine-ALA transaminase (Table 16).

ALA synthetase is the only enzyme whose specific activity is consistently reduced in biotin deficiency. ALA dehydrase is sometimes

TABLE 17

Effect of illumination in Mixture I on ALA synthetase and other enzyme activities

Organisms, previously grown anaerobically in the light in the presence of different biotin concentrations, were illuminated at 2 mg. dry wt./ml. in Mixture I at 34° for the times stated, with or without added biotin. Organisms harvested and enzyme extracts made. Assays of enzyme activities in crude extracts as described in Table 16.

Exp.	Biotin in growth medium	Biotin added to illumination (4×10^{-8} M)	Time of illumination (hr.)	Enzyme activities: μmoles product/mg. protein/hr.		Enzyme activities: μmole DPNH/mg. protein/min.
				ALA synthetase	Threonine dehydrog.	β-OH-butyric dehydrog.
I	4×10^{-8} M	-	0	.326	.117	.165
I	4×10^{-8} M	-	20	.102	.072	.114
I	6×10^{-10} M	-	0	.210	.153	.206
I	6×10^{-10} M	-	20	.130	.197	.289
I	6×10^{-10} M	+	20	.112	.081	.093
II	4×10^{-8} M	-	0	.242	.082	
II	4×10^{-8} M	-	20	.054	.098	
II	6×10^{-10} M	-	0	.214	.128	
II	6×10^{-10} M	-	20	.118	.170	
II	6×10^{-10} M	+	20	.061	.117	

reduced in extreme deficiency. Both threonine and β-hydroxybutyric acid dehydrogenase are increased significantly in biotin deficiency, and L-alanine-ALA transaminase activity is not affected. Extracts prepared by ultrasonic disruption give lower activities for ALA synthetase and ALA dehydrase than those prepared by the Hughes Press. The reason for the differences in absolute activities of ALA synthetase in different experiments is not known, but the constancy of the ratios in extracts from normal and biotin deficient organisms indicates that the reductions in biotin deficiency are significant.

The ALA synthetase activity in extracts from biotin deficient organisms could not be increased in vitro by addition of biotin, biotin plus ATP, or boiled extracts of normal organisms.

All attempts to purify ALA synthetase failed. If purification had been obtained the presence or absence of biotin as a component of the enzyme could have been determined.

As described above, illumination of biotin deficient organisms in Mixture I plus biotin resulted in growth, and porphyrin and bacteriochlorophyll synthesis. If the synthesis of pigments is being stopped by the deficiency of ALA synthetase, the response to biotin should be accompanied by an increase in ALA synthetase activity. In the experiments described in Table 17 no increase in ALA synthetase activity can be demonstrated when biotin deficient organisms are illuminated in Mixture I in the presence of biotin.

The level of ALA synthetase in normal organisms falls on illumination as does that in biotin deficient organisms. The results

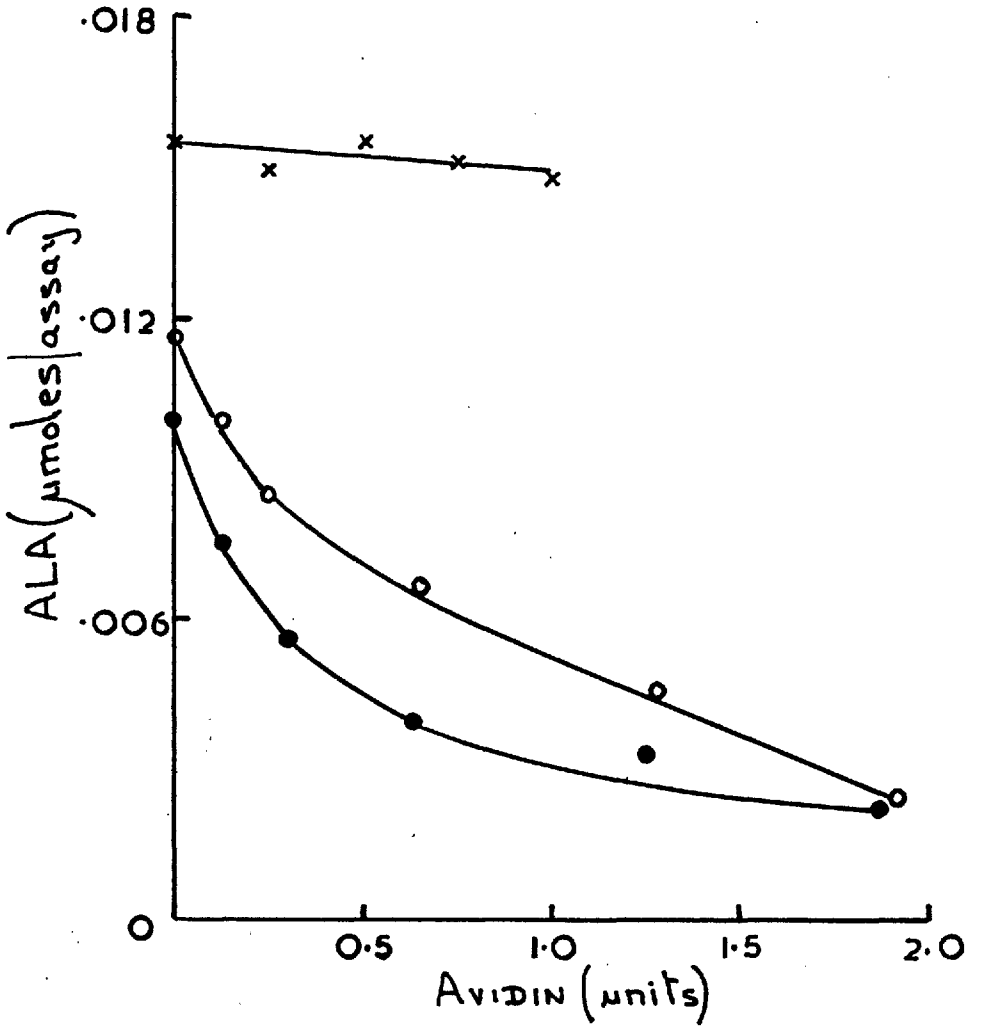


Fig. 20 Effect of avidin on ALA synthetase activity

Assay from succinyl-CoA and glycine, exactly as described on p. 19. Crude enzyme extract containing approx. 0.3 mg. protein used.

●——● avidin 2.5 units/mg. protein

○——○ avidin 4.9 units/mg. protein

×——× avidin 9.6 units/mg. protein

show that the response to biotin does not appear to involve an increase in the activity of ALA synthetase. In biotin deficient organisms illuminated in the absence of biotin the threonine and β-hydroxybutyric acid dehydrogenase activities increase; on addition of biotin these activities fall.

Effect of avidin on ALA synthetase

A commercial preparation of avidin (2.5 units/mg. protein), was tested for its ability to inhibit ALA synthetase activity in crude enzyme extracts. A marked inhibition was obtained (Fig. 20). The effect appeared to be specific. Other egg-white proteins - ovalbumin, ovomucoid and lysozyme - did not inhibit when used at the same concentrations as avidin. The avidin did not inhibit threonine dehydrogenase, nor did it interfere with the colorimetric estimation of ALA. Avidin, preincubated with excess biotin, under a variety of conditions, still inhibited the enzyme. This was surprising since the avidin-biotin complex does not inhibit other biotin containing enzymes under conditions where avidin itself inhibits. Avidin inhibition could not be reversed by having excess succinyl-CoA, or excess pyridoxal phosphate present in the assay system.

The contradiction was resolved by using purer avidin, 4.9 units/mg. protein and 9.6 units/mg. protein. The sample containing 4.9 units/mg. still inhibited (Fig. 20) but the quantity required for 50% inhibition was twice that of the commercial avidin, and avidin, 9.6 units/mg. did not inhibit ALA synthetase activity. This avidin, containing 9.6 units/m of protein, is approximately 80% pure. This estimate is based on the

TABLE 18

Properties of nicotinic acid and thiamine deficient organisms

All cultures grown anaerobically in the light as on p. 15. Levels of vitamins as stated.

Vitamin	Conc. in growth medium	Dry weight of organisms mg./ml. of culture	Bacteriochlorophyll µmoles/mg. dry wt.	Enzyme activities µmoles product/mg. protein/hr.		µmoles DPNH/mg. protein/min.
				ALA synthetase	Threonine dehydrog.	β-OH-butyric dehydrog.
Thiamine	3×10^{-6} M	1.39	19.8	.328	.071	.127
Thiamine	10^{-8} M	0.85	17.3	.224	.090	.136
Thiamine	5×10^{-9} M	0.76	13.4	.140	.088	.132
Thiamine	2.5×10^{-9} M	0.61	-	.156	.076	.135
Nicotinic acid	8×10^{-6} M	1.31	21.8	.348	.072	.118
Nicotinic acid	4.5×10^{-7} M	0.89	18.4	.144	.053	.091
Nicotinic acid	1.5×10^{-7} M	0.39	-	.180	.048	.091
Nicotinic acid	5×10^{-8} M	0.21	-	.052	.072	.074

purest preparation of avidin which Dr. Green has obtained, which binds 3 molecules of biotin per molecule of protein, and which he judges, on the evidence available, to be pure avidin.

These results indicate that it is some other egg-white protein, not avidin itself which is inhibiting ALA synthetase, and that ALA synthetase is not a biotin enzyme.

Levels of enzymes in other deficiency conditions

Since ALA synthetase does not appear to be a biotin enzyme it was decided to see if ALA synthetase activity is reduced in other growth conditions, which are unfavourable for bacteriochlorophyll synthesis. Organisms grown aerobically in the dark contain little pigment and the activities of ALA synthetase and ALA dehydrase are only a quarter of those in light grown organisms (Lascelles, 1959). Organisms grown anaerobically in the light under conditions of nicotinic acid or thiamine deficiency or where malate in the growth medium is replaced by acetate do not grow well and their bacteriochlorophyll content is low. Organisms grown under these conditions have been tested for various enzymes (Tables 18 and 19). Acetate grown organisms are very poorly pigmented, being more closely related to organisms grown aerobically in malate medium than to ones grown anaerobically in malate medium.

Under all these conditions which are unfavourable for bacteriochlorophyll production ALA synthetase activity is significantly reduced. ALA dehydrase activity is reduced in acetate grown organisms, but it has not been tested in nicotinic acid or thiamine deficiency. The other

enzymes tested are not affected. The ALA synthetase activity appears to be related to the ability of the organisms to produce bacteriochlorophyll, and the reduction is not specific for biotin deficiency.

TABLE 19

Comparison of enzyme activities in organisms grown anaerobically in the light in malate and in acetate

Organisms grown anaerobically in the light exactly as described on p. 15 and 16.

Substrate	Enzyme activities				
	µmoles product/mg. protein/hr.			µmoles DPNH/ mg./min.	µmoles ALA/mg./ 15 min.
	ALA synthetase	ALA dehydrase	Threonine dehydrog.	βOH-butyric dehydrog.	ALA transaminase
Malate	.326	.099	.089	.188	.103
Acetate	.035	.020	.087	.160	.104

The other major pigments of these organisms, the carotenoids, are closely related to bacteriochlorophyll in that conditions which reduce the concentration of one pigment, reduce that of the other (cf. Table 15). These pigments are known to be contained in the chromatophores, the intracellular photosynthetic particles. It was thought that some of the unfavourable growth conditions might be inhibiting chromatophore synthesis and so indirectly inhibiting pigment synthesis. Photosynthetic particles were therefore isolated from normal and biotin deficient organisms by the method of Newton & Newton (1957) and various components estimated by the methods reported by Newton & Newton (1957). Protein,

bacteriochlorophyll, carotenoids, carbohydrate, total haemin, total phosphate, lipid phosphate and total inorganic iron were estimated. Expressing the results per mg. of protein, the only ones which were reduced in biotin deficiency were bacteriochlorophyll and carotenoid. Thus biotin deficiency does not appear to be inhibiting chromatophore synthesis by inhibiting the synthesis of one of the components other than the pigments.

The phytol group in the bacteriochlorophyll molecule is very similar structurally to the carotenoid molecule (Fig. 2) and both are probably synthesised in vivo from mevalonic acid. If mevalonic acid synthesis were blocked under deficiency conditions bacteriochlorophyll and carotenoid synthesis would be inhibited. One of the two known biosynthetic pathways leading to mevalonic acid, that from leucine (the details of which have been worked out by Bachhawat, Robinson & Coon, 1956) is known to require biotin for the carboxylation of β-methyl crotonyl-CoA to β-methyl glutaconyl-CoA (Lynen, Knappe, Lorch, Jütting & Ringelmann, 1959).

Addition of mevalonic acid to biotin deficient organisms did not relieve the deficiency. However, ^{14}C-mevalonic acid was not incorporated into bacteriochlorophyll or carotenoids, possibly because the cells are impermeable to mevalonic acid.

THE EFFECT OF ETHIONINE AND THREONINE ON PORPHYRIN AND BACTERIOCHLOROPHYLL SYNTHESIS

In early experiments protein synthesis inhibitors were tried to

see if they could inhibit the response to biotin. The compounds used were DL-p-fluorophenylalanine, 8-azaguanine, 5-bromouracil and DL-ethionin The first three were partially successful in stopping growth and porphyrin and bacteriochlorophyll synthesis when added with biotin to biotin deficient organisms illuminated in Mixture I. In the presence of DL-ethionine, although no growth or bacteriochlorophyll synthesis occurred, a large amount of free porphyrin accumulated in the medium. This effect occurred with ethionine even in the absence of added biotin (Table 20).

TABLE 20

Effect of DL-p-fluorophenylalanine, DL-ethionine and biotin on biotin deficient organisms illuminated in Mixture I

Aerobically grown biotin deficient organisms were illuminated under standard conditions in Mixture I for 26 hr. DL-p-Fluorophenylalanine and DL-ethionine 10^{-2}M and biotin 4×10^{-8}M added where stated.

Additions	Dry weight of organisms mg./ml.	Porphyrin µmoles/ml.	Bacteriochlorophyll µmoles/ml.
None	1.08	2.8	10.8
p-Fluorophenylalanine	0.60	3.9	7.8
Ethionine	1.26	30.5	37.6
Biotin	3.24	4.9	38.6
p-Fluorophenylalanine + biotin	0.54	4.4	10.5
Ethionine + biotin	1.26	29.0	6.6
Non-incubated control	0.96	1.1	4.9

DL-Ethionine gave the same effect when added to biotin deficient organisms illuminated in Mixture I, Mixture IS or Mixture II (Table 21).

TABLE 21

Effect of DL-ethionine on illumination in different media

Aerobic grown biotin deficient organisms illuminated under standard conditions in the media stated for 22 hr. DL-Ethionine 10^{-2}M; biotin 4×10^{-8}M where added.

Medium	Additions	Dry weight of organisms mg./ml.	Porphyrin µmoles/ml.	Bacteriochlorophyll µmoles/ml
Mixture I	None	1.00	1.3	4.1
Mixture I	Biotin	2.73	33.8	12.9
Mixture I	Ethionine	1.20	21.4	3.6
Mixture IS	None	0.87	3.6	3.7
Mixture IS	Biotin	1.86	28.2	8.1
Mixture IS	Ethionine	1.17	37.2	3.6
Mixture II	None	0.87	11.8	2.3
Mixture II	Biotin	0.85	10.7	1.2
Mixture II	Ethionine	0.93	38.1	2.9
Non-incubated control		0.93	1.5	1.8

The effect of ethionine in the ALA medium was found to be different from the effect of ethionine in Mixture I and in Mixture IS. As will be discussed later the effect in Mixture I is specific for ethionine, while in Mixture II other compounds related to ethionine - methionine,

TABLE 22

Effect of ethionine on normal and biotin deficient organisms

Anaerobically grown organisms, grown in the presence of the amounts of biotin stated, illuminated under standard conditions in Mixture I for 20 hr. DL-Ethionine 5×10^{-3} M where added.

Biotin in growth medium	Additions	Dry weight of organisms (mg./ml.)	Porphyrin (μmoles/ml.)	Bacteriochlorophyll (μmoles/ml.)
4×10^{-8} M	None	2.22	13.2	17.6
4×10^{-8} M	Ethionine	1.51	57.6	6.4
2×10^{-9} M	None	0.84	4.8	8.0
2×10^{-9} M	Ethionine	1.50	53.3	6.7
8×10^{-10} M	None	0.72	3.6	4.7
8×10^{-10} M	Ethionine	1.26	32.7	4.0
4×10^{-10} M	None	0.63	1.8	3.6
4×10^{-10} M	Ethionine	0.51	6.3	2.1
4×10^{-8} M	Non-incubated controls	0.93	0.0	12.2
2×10^{-9} M		0.87	0.0	11.7
8×10^{-10} M		0.93	0.0	8.0
4×10^{-10} M		0.87	0.0	3.3

homocysteine, cysteine and glutathione give an increase in free porphyrin. All further work has therefore been done on the ethionine effect in Mixture I.

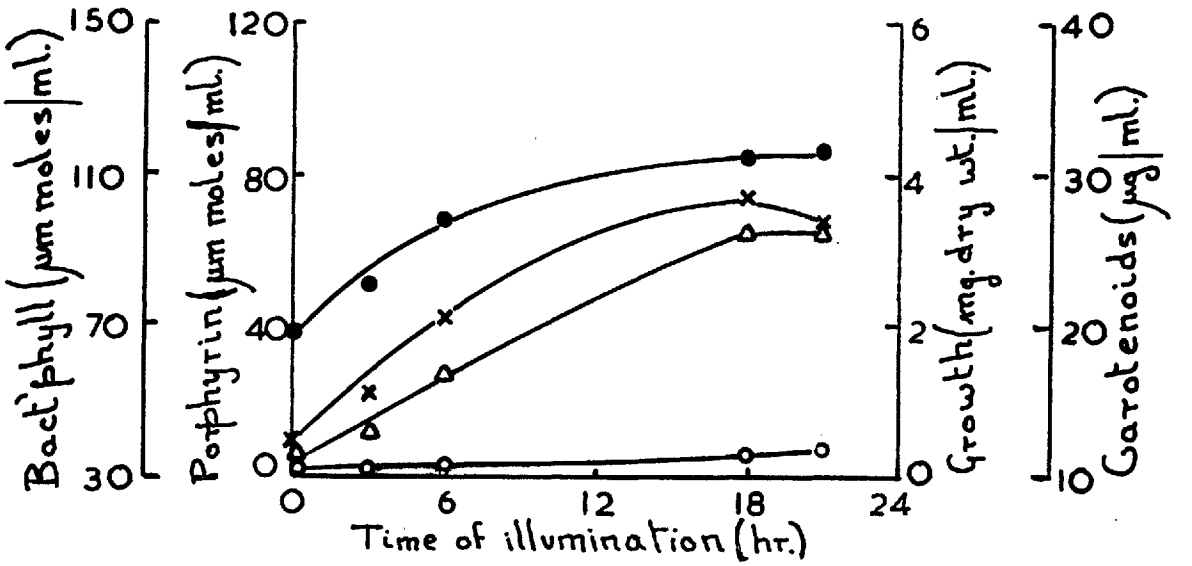
The absorption spectrum of the porphyrins accumulating in the presence of ethionine is identical with that of porphyrins accumulating in its absence over the range 355 - 610 mμ.

At this point the use of aerobically grown organisms was discontinued for the reasons stated on p. 67 and all further work has been done with organisms grown anaerobically in the light.

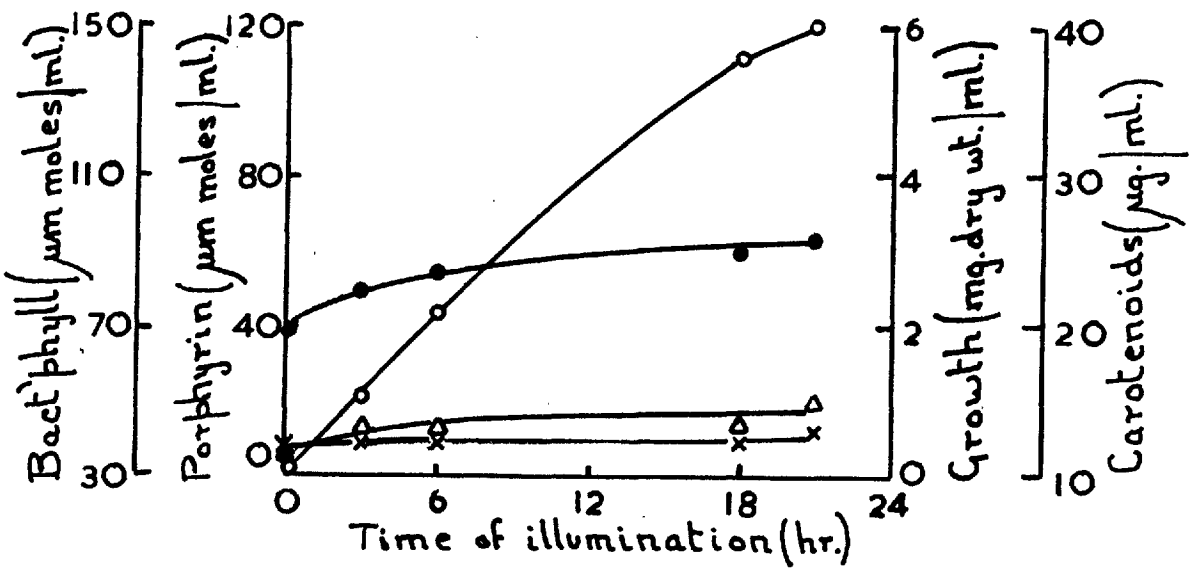
The effect of ethionine on anaerobically grown organisms is the same as that described for aerobically grown organisms. This can be seen in Table 22. The effect of ethionine in promoting accumulation of porphyrin decreases the greater the degree of biotin deficiency of the organisms.

Lascelles (1956) showed that nicotinic acid and thiamine deficient organisms were similar to biotin deficient ones in not forming porphyrin in Mixture I unless the deficient factor was added. DL-Ethionine has also been shown to give porphyrin synthesis, but no growth or bacteriochlorophyll synthesis with nicotinic acid and thiamine deficient organisms in the absence of added vitamin.

As can be seen in Figs. 21a and b, DL-ethionine, when added to normal organisms illuminated in Mixture I, completely stops bacteriochlorophyll and carotenoid synthesis and only partially stops growth. The amount of free porphyrin accumulating in the presence of ethionine is greater by a factor of two than the sum of porphyrin and bacterio-



A



B

Fig. 21a and b Effect of DL-ethionine on growth, porphyrin bacteriochlorophyll and carotenoids

Organisms, previously grown anaerobically in the light, illuminated at 2 mg. dry wt./ml. in Mixture I. Fig. 21a no addition; Fig. 21b plus DL-ethionine 5×10^{-3}M. Samples removed at various times and estimated for growth (●——●), porphyrin (O——O), bacteriochlorophyll (X——X), and carotenoids (Δ——Δ) by the methods stated on p. 18.

chlorophyll formed in its absence. This is a reproducible feature of ethionine action, having been noted in other experiments not described here. In many experiments the difference is three or four fold instead of the twofold difference described in Figs. 21a and b. Ethionine seems to be stimulating porphyrin excretion, the porphyrin formed being more than that expected if all that was happening was a block in bacteriochlorophyll synthesis. This apparent stimulation is also evident from the effect of ethionine on vitamin deficient organisms, which do not form any porphyrin or bacteriochlorophyll in the absence of ethionine.

DL-Ethionine stimulates porphyrin synthesis at a concentration of 10^{-4}M (Table 23). In this experiment the amount of porphyrin formed in the presence of ethionine is also greater than the sum of porphyrin and bacteriochlorophyll formed in its absence.

TABLE 23

Effect on different concentrations of DL-ethionine in Mixture I

Anaerobically grown organisms were illuminated in Mixture I for 20 hr. under standard conditions. DL-Ethionine added as stated.

DL-Ethionine	Porphyrin (μmoles/ml.)	Bacteriochlorophyll (μmoles/ml.)
5×10^{-3}M	67.8	12.3
10^{-3}M	95.0	15.8
5×10^{-4}M	80.0	16.3
10^{-4}M	19.0	23.8
-	5.7	26.7
Non-illuminated control	0.0	13.0

At low concentrations of ethionine, although porphyrin accumulation is still stimulated, some bacteriochlorophyll synthesis occurs.

The effect of L-, D- and DL-ethionine have been tested. All three preparations give porphyrin accumulation. The ratios of activity L:DL:D is 1.5:1.2:1.0.

Threonine effect on porphyrin and bacteriochlorophyll synthesis in Mixture I

A number of compounds related and unrelated to ethionine were tested for an ethionine-like effect on porphyrin and bacteriochlorophyll when added to organisms illuminated in Mixture I under standard illumination conditions. All the compounds were tested at a concentration of between 10^{-2}M and 10^{-3}M. The compounds tried were DL-methionine, DL-homocystine, DL-homocysteine thiolactone, L-cysteine, reduced glutathione, and L and D-penicillamine, all sulphur-containing compounds: choline, betaine, formaldehyde, DL-serine and methanol, all compounds capable of yielding biologically active one-carbon fragments; DL-norleucine, which like ethionine is known to be a methionine antagonist; (Vaughan & Steinberg, 1959); acetic acid, acetaldehyde, ethanol, L-aspartic acid, L-asparagine, DL-tyrosine, L-glutamic acid, L-glutamine, DL-alanine, DL-tryptophan, DL-threonine, L-arginine, pyridoxine, pyridoxamine and β-hydroxybutyric acid and aminoacetone.

Some of these compounds slightly stimulated porphyrin production, some slightly depressed it, and others had no effect at all. The only one which had a marked effect was DL-threonine which stimulated porphyrin accumulation to almost the same extent as did ethionine and which also

stopped bacteriochlorophyll synthesis (Table 24).

TABLE 24

Comparison of the effects of DL-threonine and DL-ethionine in Mixture I

Anaerobically grown organisms illuminated in Mixture I under standard conditions for 22 hr. DL-Ethionine 10^{-3}M and DL-threonine 5×10^{-3}M where added.

Expt.	Additions	Dry weight of organisms (mg./ml.)	Porphyrin (µmoles/ml.)	Bacteriochlorophyll (µmoles/ml.)
I	None	2.16	18.0	36.2
I	DL-Ethionine	2.01	95.2	25.6
I	DL-Threonine	1.92	66.9	27.4
I	Non-illuminated control	1.10	0.0	27.4
II	None	2.30	16.2	32.1
II	DL-Ethionine	1.82	81.2	20.6
II	DL-Threonine	1.96	58.0	25.7
II	Non-illuminated control	1.06	0.0	23.3

Some of the compounds were also tested for an effect on porphyrin synthesis in Mixture II. Methionine, homocysteine, cysteine and glutathione stimulated porphyrin production as did ethionine. This shows that the ethionine effect on porphyrin synthesis from α-oxoglutarate and glycine (Mixture I) is different from that on porphyrin synthesis

from ALA (Mixture II).

The similarity of the ethionine and threonine effects is shown in Table 25 where it has been found that threonine stimulates porphyrin production in biotin deficient organisms in the absence of added biotin.

TABLE 25

Effect of replacing $(NH_4)_2HPO_4$ in Mixture I by amino acids on porphyrin synthesis by normal and biotin deficient organisms

Normal and biotin deficient (grown in 10^{-9} M biotin) organisms illuminated in Mixture I (no $(NH_4)_2PO_4$) under standard conditions for 20 hr. Nitrogen source as indicated (.006 M).

Nitrogen source	Porphyrin (μmoles/ml.)	
	Normal organisms	Biotin deficient
$(NH_4)_2HPO_4$	14.1	0.8
L-Asparagine	9.2	0.8
L-Glutamine	13.7	0.3
DL-Alanine	23.7	0.6
L-Arginine	9.8	0.4
DL-Methionine	3.5	4.0
DL-Ethionine	44.2	49.5
DL-Threonine	122.0	91.5

In another experiment using organisms which were more biotin deficient than those described above, although ethionine stimulated porphyrin synthesis in the absence of added biotin, threonine did not. Also

threonine did not give porphyrin production with nicotinic acid or thiamine deficient organisms whereas ethionine did as described on p. 82 .

Whereas ethionine stimulates porphyrin synthesis at a concentration of 10^{-4} M the lowest concentration of DL-threonine which is effective is 10^{-3} M. L- and DL-Threonine at 5×10^{-3} M give quantitatively the same porphyrin production.

Porphyrins formed on illumination of organisms in Mixture I in the presence and absence of ethionine and threonine were extracted and chromatographed in the lutidine-water system of Nicholas & Rimington (1949) No new porphyrins were found in the presence of ethionine and threonine, and none of the porphyrins normally present were absent. The major porphyrin under all conditions was coproporphyrin, confirming the results of Lascelles (1956). In addition the spectra of the porphyrin solutions obtained after illumination of organisms under the conditions described above were all identical over the range 355 - 610 mμ. These observations show that the estimation used for porphyrin (p. 18) is a valid measure of the quantitative differences in porphyrin excreted in the presence and absence of ethionine and threonine.

Conditions required for ethionine and threonine effects

The effect of omitting some of the components of Mixture I or of changing their concentrations has been tried to see what conditions are required for ethionine and threonine stimulation of porphyrin production.

I. Ammonium phosphate. The presence of $(NH_4)_2HPO_4$ was found by Lascelles (1956) to be essential for porphyrin production. As can be

TABLE 26

Requirement of metal ions in Mixture I for the ethionine effect

Organisms grown anaerobically in the light were illuminated in Mixture I (without Mg^{2+}, Ca^{2+} and Mn^{2+}) under standard conditions for 18 hr. The metals added back as stated; concentrations as in Mixture I; DL-ethionine 5×10^{-3}M where added.

Metals	DL-Ethionine	Porphyrin (μmoles/ml.)
None	-	0.6
Mg^{2+}, Ca^{2+}, Mn^{2+}	-	15.0
None	+	8.7
Mg^{2+}	+	88.5
Ca^{2+}	+	10.9
Mn^{2+}	+	5.3
Mg^{2+}, Ca^{2+}	+	79.0
Mg^{2+}, Mn^{2+}	+	97.0
Ca^{2+}, Mn^{2+}	+	6.7
Mg^{2+}, Ca^{2+}, Mn^{2+}	+	74.5

seen in Table 25 various amino acids can replace $(NH_4)_2HPO_4$ and the stimulation of porphyrin synthesis by ethionine and threonine does not require the presence of $(NH_4)_2HPO_4$.

2. Glycine. The presence of glycine in Mixture I is essential for porphyrin synthesis. Very little porphyrin is synthesised in its absence even when ethionine or threonine are added.

3. Metals. Lascelles (1956) in working out the composition of Mixture I found that Mg^{2+}, Ca^{2+} and Mn^{2+} are all required for porphyrin synthesis. In the presence of ethionine Mg^{2+} is the only one required for the stimulation of porphyrin production (Table 26).

Reversal of ethionine and threonine effects

Methionine and homocysteine have been found to inhibit the accumulation of free porphyrin in the presence of ethionine and threonine, and also to restore the bacteriochlorophyll synthesis (Table 27).

As shown in Table 27 methionine and homocysteine alone have little or no effect on porphyrin or bacteriochlorophyll production and homocysteine at 10^{-3} M does not completely stop porphyrin accumulation in the presence of ethionine. Homocysteine is ineffective at 10^{-4} M. Methionine at 10^{-4} M in some experiments completely reverses the ethionine effect and in others only partially reverses it.

Homoserine (10^{-3} M) has been found to reverse the threonine effect under conditions identical with those reported in Table 27. It inhibits porphyrin accumulation and restores bacteriochlorophyll synthesis. The combination of homoserine plus cysteine which might be expected to give

TABLE 27

Reversal of ethionine and threonine effects by methionine and homocysteine

Organisms grown anaerobically in the light were illuminated in Mixture I for 21 hr. under standard conditions. DL-Threonine (5×10^{-3}M), L-ethionine, L-methionine and DL-homocysteine (all at 10^{-3}M), added where stated.

Additions	Porphyrin (µmoles/ml.)	Bacteriochlorophyll (µmoles/ml.)
None	16.2	45.1
L-Methionine	12.8	53.8
DL-Homocysteine	15.8	49.8
L-Ethionine	77.0	26.4
L-Ethionine + L-Methionine	17.2	51.3
L-Ethionine + DL-Homocysteine	34.4	49.5
DL-Threonine	77.0	32.3
DL-Threonine + L-Methionine	19.0	53.0
DL-Threonine + DL-Homocysteine	18.1	55.0
Non-incubated control	0.0	26.5

homocysteine *in vivo* has a similar effect. Neither homoserine (10^{-3} M) nor homoserine (2×10^{-3} M) plus cysteine (10^{-3} M) reverse the ethionine effect, with ethionine at 10^{-3} M. Homoserine, and homoserine plus cysteine, have no effect on porphyrin and bacteriochlorophyll synthesis in Mixture I.

Various related compounds do not reverse the ethionine and threonine effects - cysteine, homocystine,, serine, pyruvate and norleucine. The following have only been tested against ethionine - choline, betaine, glutathione, D and L-penicillamine - they are all ineffective. Formaldehyde at 2.5×10^{-3} M stops porphyrin accumulation in the presence of ethionine and threonine, but it does not give an increase in bacteriochlorophyll; at 5×10^{-4} M it is ineffective.

Methionine in some experiments but not in others, has been shown to decrease porphyrin accumulation and increase bacteriochlorophyll synthesis when added to Mixture I.

Reversal of ethionine and threonine effects by iron

Lascelles (1956) showed that addition of Fe^{2+} to Mixture I decreased the amount of free porphyrin and increased the amount of bacteriochlorophyll formed. The increase of bacteriochlorophyll in the presence of iron was much less than the free porphyrin formed in its absence. The effect on porphyrin production of adding different amounts of Fe^{2+} to Mixture IS in the presence of different amounts of ethionine and threonine is shown in Table 28.

Organisms incubated aerobically in the dark in Mixture I do not

excrete porphyrins even on the addition of ethionine.

Ethionine has no effect in cell-free systems on ALA synthetase or ALA dehydrase, and ethionine and threonine, as shown on p. 60 , have no effect on L-alanine-ALA transaminase.

Ethionine has no effect on the accumulation of aminoacetone on illumination of organisms in Mixture I at low or high buffer concentrations, nor has increasing the buffer concentration, leading to aminoacetone accumulation any effect on bacteriochlorophyll synthesis in the absence of ethionine, or on porphyrin excretion in the presence of ethionine.

TABLE 28

Effect of Fe^{2+} on porphyrin accumulation in Mixture I in the presence and absence of ethionine and threonine

Organisms grown anaerobically in the light were illuminated under standard conditions in Mixture IS for 20 hr. Additions as stated.

Additions	Porphyrin (μmoles/ml.)			
		Fe^{2+}		
	-	10^{-6} M	10^{-5} M	10^{-4} M
-	11.6	4.4	1.7	0.9
DL-Ethionine (2.5×10^{-5} M)	32.2	18.7	7.9	4.2
DL-Ethionine (10^{-3} M)	51.2	37.5	20.7	17.6
DL-Ethionine (5×10^{-3} M)	44.6	47.5	48.9	48.9
DL-Threonine (5×10^{-3} M)	50.0	-	24.5	-

THE ORIGIN OF THE METHYL ESTER GROUP OF BACTERIOCHLOROPHYLL

The chlorophyll and bacteriochlorophyll molecules have a methyl ester group on the propionic acid side chain of ring C (Fig. 2), that is on the side chain which forms the isocyclic ring in the complete molecule. Very little is known about the biogenesis of methyl ester groups in nature. The enzymic formation of methoxy groups and of N-methyl groups have been shown, in every case where they have been studied enzymically, to involve S-adenosylmethionine as the methyl donor.

The origin of the methyl group in chlorophyll has been studied previously by Green, Altman, Richmond & Salomon (1957) who showed that chlorophyll isolated from cultures of Chlorella vulgaris grown in the presence of ^{14}C-formate was labelled in the methyl ester group, but not in the rest of the molecule.

The results reported in the last section with ethionine and threonine suggest that these compounds are blocking the synthesis of bacteriochlorophyll by interfering with some metabolic reaction of methionine. It was thought to be important to obtain some results on the role played by methionine in bacteriochlorophyll synthesis. In the experiments to be reported the carotenoids of Rps. spheroides have also been investigated. The two major carotenoids, the yellow and red carotenoids, each contain one methoxy group per molecule (Stanier, 1959) (Fig. 2), which by analogy with other methoxy groups is probably donated by S-adenosylmethionine.

Experimental. Anaerobically grown Rps. spheroides were illuminated at 2 mg. dry wt./ml. in Mixture I for 18 hr. at 34°. Under these conditions the dry weight of the organisms doubles, as does the amount of bacteriochlorophyll and carotenoids (see Fig. 21). The incubation mixture, total volume 100 ml. also contained 10^{-4} M L[methyl-C^{14}]methionine (0.2 μc/mole). A control was set up having an identical composition but being only 50 ml. in volume. This control was kept at 0° instead of being illuminated.

At the end of the illumination portions of the experimental and control were removed for estimation of bacteriochlorophyll and carotenoid by the method of Cohen-Bazire et al. (1957). The rest was harvested, washed with water and made into a thick suspension which was crushed in the Hughes Press.

Extraction and purification of the pigments. This was done by a modification of the method of Komen (1956). The pigments were extracted from the broken organisms with acetone. To the acetone extract was added 5 ml. of light petroleum (b.p. 60 - 80°). The acetone was removed by passing the solution through a large volume of water; the light petroleum solution, containing all the pigments, was dried over $MgSO_4$, filtered and taken to dryness under reduced pressure. The residue was dissolved in the minimum of light petroleum containing a trace of carbon tetrachloride to facilitate the solution of the bacteriochlorophyll.

Sucrose (commercial sugar) was powdered in a mortar and sieved (40 mesh). A thick sludge was made in light petroleum and a column

6 x 0.5 in. prepared and allowed to settle. The pigment solution was applied to the column. Elution with light petroleum brought off the yellow, then the red carotenoid. These were collected together. The bacteriochlorophyll which remained as a blue band at the top of the column during elution with light petroleum, was removed by adding acetone to the light petroleum (light petroleum: acetone, 9:1 v/v).

This bacteriochlorophyll preparation is pure as judged by the spectrum between 400 mμ and 830 mμ being identical with that given by Koman (1956).

Degradation of purified bacteriochlorophyll and isolation of fragments. The method used follows closely that given by Fischer & Stern (1940) who showed that alkaline hydrolysis degrades bacteriochlorophyll to bacteriochlorin e_6, phytol, methanol and Mg^{2+}.

Purified bacteriochlorophyll, 1.78 mg., was dissolved in 1.5 ml. of 24% methanolic KOH, and the solution refluxed under nitrogen for 3 hr. The methanol was distilled off, collected in a receiver cooled in dry ice, and kept at -20° until required. This distillate contains the methanol derived from the methyl ester group of bacteriochlorophyll. The residue was redissolved in methanol, and ether and water added. The ether phase was removed and the aqueous phase re-extracted with ether. The combined ether extracts were dried down to give phytol. The aqueous phase was acidified to pH 1.5 with HCl and extracted with ether to remove all the green pigment. The ether extracts, which contain the bacteriochlorin, were dried down. On paper chromatography in 2:6 lutidine-water (5:3.5 v/v) (Falk, Dresel, Benson

TABLE 29

Quantities of pigments synthesised during illumination with ^{14}C-methionine

Component	Experimental (µg/100 ml.)	Non-illuminated control (µg/50 ml.)	Amount synthesised during illumination (µg.)
Before purification			
Bacteriochlorophyll	9400	2175	5050
Carotenoids	1970	497	570
After purification			
Bacteriochlorophyll	3560	320	
Carotenoids	951	154	

& Knight, 1956), more than one porphyrin spot was obtained. This probably means that some of the bacteriochlorin has been further degraded. The complete fraction was used for estimation of radioactivity.

Determination of radioactivity

Solid counting. All samples were plated out as solutions on 2 sq. cm. planchettes. The solutions were dried, leaving a uniform layer of solid. The counting was done at infinite thinness, unless otherwise stated, in a Geiger end-window counter. Standards and background counts were done in all experiments. The standard gave approximately the same count in all experiments. A linear increase in counts with increasing amounts of bacteriochlorophyll was obtained up to 300 µg/2 sq. cm. confirming that the counts have actually been done at infinite thinness.

Liquid scintillation counting. Solutions made to 0.5 ml. were mixed with 5 ml. of dioxane-methanol-ethylene glycol-naphthalene (100:10:2:6 by vol.) containing 0.3% 2:5-diphenyloxazole and 0.02% 1:4 bis-2(5-phenyloxazolyl)benzene (Bray, 1960). The radioactivity was measured in the Panax liquid scintillation counter (discriminator 12 volts, E.H.T. 1480 volts). The standard gave approximately the same value in all experiments.

Results. The synthesis of pigments on illumination and the recovery on purification is described in Table 29.

The purified pigments were counted at infinite thinness (Table 30).

From these results the specific radioactivity of the pigments synthesised during the course of the experiment has been calculated.

TABLE 30

Radioactivity of purified pigments

Solid counting at infinite thinness

Sample	µg.	Counts/300 sec. (corrected for background)
Bacteriochlorophyll	89	817
	178	1508
Bacteriochlorophyll (control)	320	16
Carotenoid	95	2397
	190	4420
Carotenoid (control)	154	286

assuming that there is no metabolic turnover of pigment during the illumination. Methionine (0.2 µc/µmole) has also been counted at infinite thinness under identical conditions.

Bacteriochlorophyll	3022 counts/µmole/min.
Carotenoid	5586 counts/µmole/min.
Methionine	28970 counts/µmole/min.

The bacteriochlorophyll was degraded and the fractions counted in the scintillation counter (Table 31). The specific radioactivity of the methyl ester group can be obtained since a given volume of the methanol distillate will contain the methanol derived from a known weight of bacteriochlorophyll.

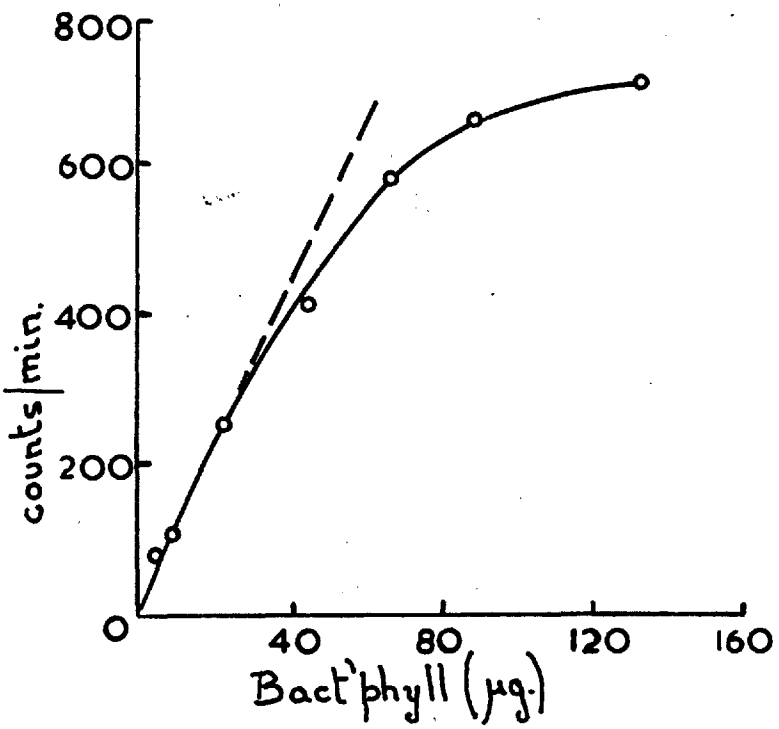


Fig. 22 Measurement of radioactivity of bacteriochlorophyll by scintillation counting

TABLE 31

Liquid scintillation counting of fragments of bacteriochlorophyll

Sample	Bacteriochlorophyll from which the fragment was derived (µg.)	Radioactivity (corrected for background) counts/2 min.
Bacteriochlorophyll	178	1461
Bacteriochlorophyll	356	1611
Bacteriochlorin from	1780	21
Phytol from	1780	248
Methanol from	237	5357
Methanol from	474	9848

As can be seen the pigment solutions quench the scintillation. The bacteriochlorophyll has therefore been counted at low concentrations (Fig. 22). The curve is linear at low concentrations and this has been used to calculate a specific radioactivity for bacteriochlorophyll. These data give a direct comparison between the radioactivity of the bacteriochlorophyll and the fragments derived from it.

1 µmole bacteriochlorophyll	9800	counts/min.
Methanol from 1 µmole bacteriochlorophyll	9920	" "
Phytol from 1 µmole bacteriochlorophyll	64	" "
Bacteriochlorin from 1 µmole bacteriochlorophyll	6	" "

The bacteriochlorin value is probably low due to quenching. Solid counting of the total bacteriochlorin gave 12 counts/min. while solid

counting of the total phytol gave 8 counts/min.

These results prove conclusively that under the experimental conditions used the methyl group of methionine is incorporated specifically into the methyl ester group of bacteriochlorophyll, but not into the rest of the molecule.

A comparison of the specific radioactivities of the added methionine and of the pigments formed is given in Table 32. The values for specific radioactivities of the pigments have been calculated assuming no turnover during illumination.

TABLE 32

Specific radioactivities of methionine added, and of the pigments formed in its presence

Experiment I is the one described in detail above. Experiment II was done under identical conditions using 10^{-4} M methionine (0.2 μc/μmole) and 5×10^{-4} M methionine (0.04 μc/μmole). All radioactivities determined at infinite thinness.

Expt.	Conc. of methionine	Specific radioactivities counts/μmole/min.		
		Methionine	Bacteriochlorophyll	Carotenoids
I	10^{-4} M	28970	3022	5586
II	10^{-4} M	28970	3600	-
II	5×10^{-4} M	5800	1883	

Further experiments have been done in conjunction with Dr. K.D. Gibson to see if the enzyme step in methylation can be directly

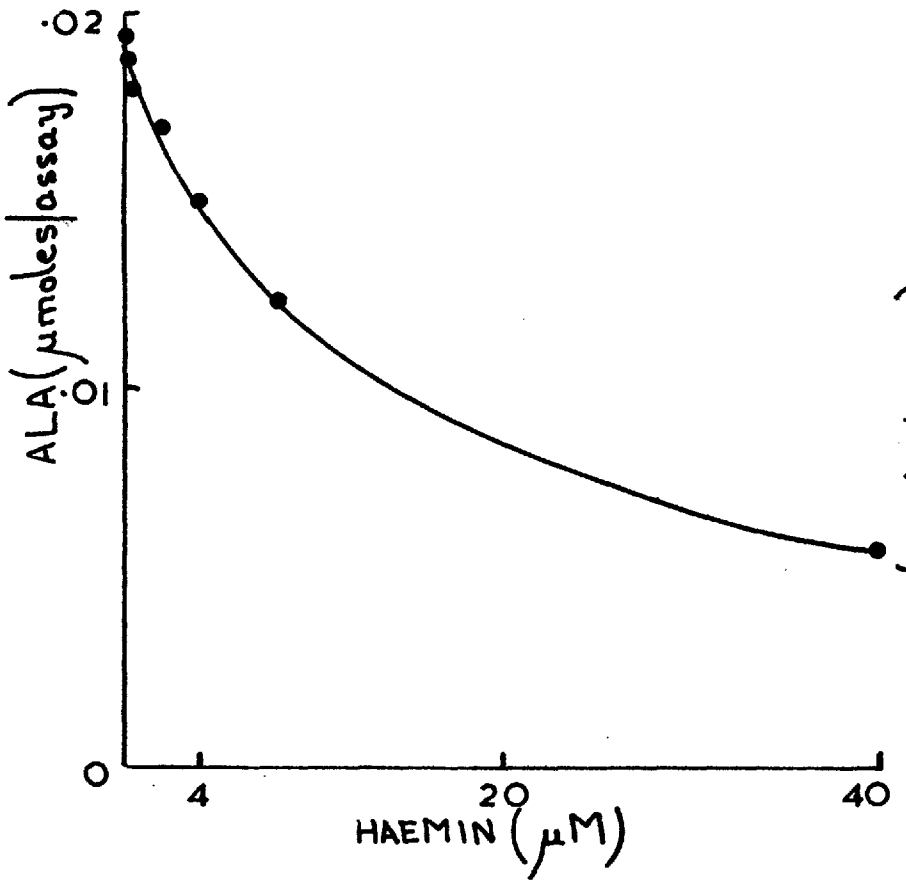


Fig. 23 Effect of haemin on ALA synthetase activity

Assay exactly as described on p. 19.
Crude enzyme extract (= 0.15 mg. protein).
Haemin as stated.

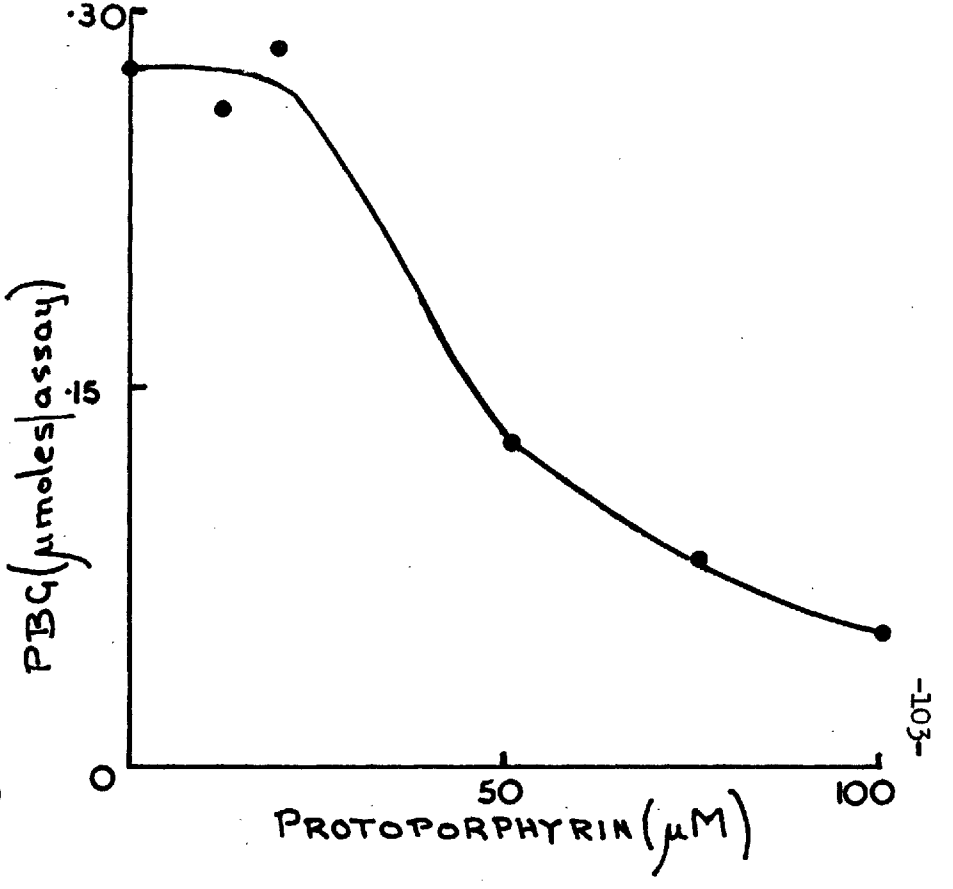


Fig. 24 Effect of protoporphyrin on ALA dehydrase activity

Assay exactly as described on p. 20.
Crude enzyme extract (= 3.0 mg. protein). Protoporphyrin as stated.

demonstrated.

Whole organisms were illuminated with ALA or protoporphyrin or haemin or Mg protoporphyrin in the presence of ^{14}C-methionine. Broken cell homogenates were also incubated with the substrates stated above in the presence of radioactive methionine or radioactive S-adenosyl-methionine. The porphyrins formed were isolated but no radioactive porphyrin was found. These experiments are being continued.

EFFECT OF PORPHYRINS OF ALA SYNTHETASE AND ALA DEHYDRASE ACTIVITIES IN ENZYME EXTRACTS

Having made protoporphyrin, Mg protoporphyrin and haemin it was thought of interest to test them for an effect on ALA synthetase and ALA dehydrase in cell-free extracts since Lascelles (1960a) showed that haemin, when added to growing organisms, represses the synthesis of ALA synthetase and ALA dehydrase.

Haemin was found to inhibit ALA synthetase activity (Fig. 23). Protoporphyrin, protoporphyrin dimethyl ester, Mg protoporphyrin and Mg protoporphyrin dimethyl ester at a concentration of 50 μM were inactive.

Protoporphyrin inhibited ALA dehydrase (Fig. 24). Haemin and Mg protoporphyrin at a concentration of 50 μM were inactive.

DISCUSSION

DISCUSSION

Aminoacetone. The first reports of the possible role of aminoacetone in metabolism came from Shemin (1955) and Nemeth, Russell & Shemin (1957). On administration to rats of aminoacetone labelled with ^{14}C in the carbon atom bearing the amino group, they found that label appeared in respiratory CO_2, urinary formate and in the ureido groups of uric acid. This was exactly analagous to the findings with ALA on the basis of which the succinate-glycine cycle was postulated (Shemin & Russell, 1953). Mauzerall & Granick (1956) then detected a small amount of an aminoacetone-like aminoketone in human urine. In studying ALA synthetase, Gibson et al. (1958) and Kikuchi et al. (1959) showed that the enzyme was not specific for succinyl-CoA, but would condense glycine with other acyl-CoA compounds, at a lower rate than with succinyl-CoA, to form aminoketones.

The interest in aminoacetone was increased when Elliott (1958, 1959, 1960a) identified it as a product of the aerobic incubation of Staphylococcus aureus in a medium containing threonine or in one containing glycine and glucose. Elliott (1959) postulated that aminoacetone could be metabolised by a cyclic pathway similar to the succinate-glycine cycle for ALA, and identified the first step of this cycle (Elliott, 1960b) by finding an enzyme in ox-plasma which oxidatively deaminated aminoacetone to methylglyoxal. More recently, Urata & Granick (1961) described the formation of aminoacetone from pyruvate and glycine, and from threonine in liver mitochondria, and also detected the

breakdown of aminoacetone in liver.

Mijovic & Walker (1960) identified aminoacetone as a constituent of the antibiotic micrococcin P.

These reports indicate that aminoacetone may be an important natural metabolite, and when a similar aminoketone was detected as a product of Rps. spheroides' metabolism it was of interest of identify and study it. Another reason for such a study is that being an analogue of ALA it was thought that the aminoketone might play some role in the control of porphyrin and bacteriochlorophyll synthesis.

The amount of aminoketone accumulating in the medium when Rps. spheroides is incubated aerobically in the dark or anaerobically in the light in a medium containing α-oxoglutarate, fumarate and glycine is increased greatly by raising the buffer concentration to 0.2 M and keeping the pH below 7. Aerobically a further increase in aminoketone is obtained by raising the Mn^{2+} concentration. The aminoketones formed aerobically and anaerobically were purified and identified as aminoacetone. The identification was achieved by showing on gas chromatography that the acid formed on periodate oxidation of the aminoketones was acetic acid.

It is not known why the modified incubation conditions are effective in giving an accumulation of aminoacetone. They may be blocking further metabolism of aminoacetone, or giving an increased rate of synthesis.

The mechanism of aminoacetone synthesis was thought to be from acetyl-CoA and glycine, since these organisms contain an active ALA

synthetase. But if acetate replaces α-oxoglutarate and fumarate for aerobic incubation no aminoacetone accumulates. This implies either that aminoacetone is not synthesised from acetyl CoA and glycine or that in the presence of acetate it is metabolised as fast as it is formed. Another puzzling feature is that aerobically in the dark no ALA or porphyrin is formed from α-oxoglutarate, fumarate and glycine. It would be imagined that if ALA synthetase is forming aminoacetone under these conditions, ALA ought to be produced also. It may be that ALA is metabolised rapidly via the succinate-glycine cycle or that under these conditions insufficient succinyl-CoA is available; or aminoacetone may be synthesised by some mechanism not involving ALA synthetase.

These findings are analagous to those of Urata & Granick (1961) who showed that aminoacetone, but no ALA, was formed when glycine plus any one of a number of the intermediates of the tricarboxylic acid cycle was added to liver homogenates.

Aminoacetone accumulates on aerobic incubation of organisms in a medium containing threonine. The pathway of formation is clear under these conditions since a threonine dehydrogenase has been found in cell-free extracts. Such an enzyme was postulated by Elliott (1960a) to account for his results with Staph. aureus, but this is the first time its presence has been demonstrated. The enzyme has been partially purified and some of its properties studied. The enzyme requires DPN^+. TPN^+ is inactive and no aminoacetone is formed in the absence of DPN^+. For every mole of DPN^+ reduced to DPNH approximately one mole of aminoacetone is formed. Desamino-DPN^+ is inactive as a cofactor, but

3-acetylpyridine-DPN$^+$ has 40% the activity of DPN$^+$.

These two analogues of DPN$^+$ have been tested on a number of enzymes (Pullman, Colowick & Kaplan, 1952; Kaplan, Ciotti & Stolzenbach, 1956). With some of the enzymes they are as effective as DPN$^+$, with some they are less effective, and with some they are completely inactive.

The enzyme is active with L-threonine and D-allothreonine, the two isomers of α-amino-β-hydroxybutyric acid which have the D configuration on the hydroxyl-bearing carbon atom. The other two isomers, D-threonine and L-allothreonine are inactive as substrates and do not inhibit the reaction with L-threonine and D-allothreonine. D-Allothreonine is acted on at about 40% the rate of L-threonine.

Elliott (1960a) suggested that the reaction occurred in one of two ways (p. 35), either the dehydrogenation or the decarboxylation taking place first. 1-Aminopropan-2-ol, the expected intermediate of threonine decarboxylation, is not converted to aminoacetone by this enzyme, and does not inhibit the conversion of threonine to aminoacetone. This indicates that threonine is first dehydrogenated to α-amino-β-oxobutyric acid, which is then decarboxylated to aminoacetone. No enzyme need be postulated for the decarboxylation since Laver *et al.* (1959) have found that α-amino-β-oxobutyric acid is very unstable under physiological conditions. The possibility remains that the decarboxylation occurs simultaneously with the dehydrogenation on the enzyme surface by a concerted reaction similar to that proposed by Ingraham (1959).

Serine and Y-amino-β-hydroxybutyric acid are inactive as substrates

or inhibitors, but bicarbonate inhibits the reaction markedly. From the data available it would appear that the groups of threonine which are essential for action of the enzyme are the methyl group, the carboxyl group and the correct configuration on the hydroxyl-bearing carbon atom. Having the amino group in a cisoid-configuration with the hydroxyl group, as in D-allothreonine, reduces the rate of the reaction as compared with L-threonine where the amino group and the hydroxyl group are in a transoid-configuration to one another.

The threonine dehydrogenase is strongly inhibited by Ag^{2+} and p-chloromercuribenzoate and partially inhibited by iodoacetamide which suggests that a thiol group in the enzyme is essential for activity. Hg^{2+} and Pb^{2+} react with aminoacetone non-enzymically at pH 8.8, as does Mn^{2+}, so that it is difficult to tell whether they also inhibit the enzyme. Inhibition by EDTA, 50% inhibition at 5.6×10^{-5} M, and by 8-hydroxyquinoline suggest a metal requirement. In support of this Mg^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+} stimulate the activity slightly. Addition of pyridoxal phosphate does not increase the activity and L-penicillamine, which has been shown by Du Vigneaud, Kuchinskas & Horvath (1957) and others to inhibit pyridoxal phosphate enzymes, has no effect. Cyanide inhibits the enzyme; since the enzyme does not appear to contain pyridoxal phosphate the inhibitory action of cyanide may be related to the metal requirement. Complete elucidation of the requirements will have to await further purification of the enzyme.

At 37° K_M for L-threonine is 7.4×10^{-2} M and for DPN^+ it is 1.7×10^{-4} M showing that the enzyme has a low affinity for L-threonine.

Some of these findings have already been published (Neuberger & Tait, 1960).

Aminoacetone, although an analogue of ALA, does not appear to have any effect on the synthesis or metabolism of ALA. It does not inhibit ALA synthetase, and it is not an inhibitor or a substrate of ALA dehydrase or L-alanine-ALA transaminase. Aminoacetone, added to organisms illuminated in Mixture I, has no marked effect on porphyrin or bacteriochlorophyll synthesis, and increasing the buffer concentration in Mixture I, which leads to accumulation of aminoacetone has no effect on porphyrin or bacteriochlorophyll synthesis. Threonine, which inhibits bacteriochlorophyll synthesis and stimulates accumulation of porphyrins, was thought to be acting through being converted to aminoacetone, but in view of the results reported above this does not appear to be so. Another explanation for the effect of threonine will be discussed later.

The metabolism of aminoacetone has not been investigated, but Rps. spheroides may be a suitable organism in which to study it. Since it has the ability to synthesise relatively large amounts of aminoacetone it presumably also has the ability to metabolise large amounts.

β-Hydroxybutyric acid dehydrogenase. An active DPN^+ requiring β-hydroxybutyric acid dehydrogenase has been found in cell-free extracts. This enzyme reduces DPN^+ about 120 times faster than the threonine dehydrogenase in crude enzyme extracts, and it was thought that the threonine dehydrogenase activity might be due to this enzyme since threonine is an α-amino substituted β-hydroxybutyric acid. The

difference in rates of reaction could be explained by steric hindrance due to the amino group of threonine. For both activities to be due to the same enzyme it would have to be a D-β-hydroxybutyric acid dehydrogenase. This was at first assumed to be so since under suitable conditions Rps. spheroides accumulate large amounts of poly-D-β-hydroxybutyric acid (Doudoroff & Stanier, 1959), and has been confirmed by the recent report of Doudoroff, Merrick & Contopoulou (1961) who have described a D-β-hydroxybutyric acid dehydrogenase, with very similar properties to the one reported here, in the related microorganism Rhodospirillum rubrum.

Some of the early results did suggest that both activities were due to one enzyme. No separation of activities was obtained on partial purification (p. 51), both reactions were inhibited by bicarbonate (p. 51), the pH optima were close, and the specific activities were increased by approximately the same amount in biotin deficient organisms (Table 16). Like the activity towards threonine that with β-hydroxybutyric acid was inhibited by Hg^{2+} and p-chloromercuribenzoate, indicating an essential thiol group, and by EDTA, suggesting a metal requirement. Further evidence indicates that two different enzymes are involved. Deamino-DPN^+ is inactive with both substrates, but 3-acetylpyridine-DPN^+ has 40% of the activity of DPN^+ with threonine, and only 8% of the activity of DPN^+ with β-hydroxybutyric acid. The Michaelis constant for DPN^+ is also different. For threonine dehydrogenase activity it is 1.7×10^{-4}M and for β-hydroxybutyric acid dehydrogenase it is 4.0×10^{-5}M. Further work is required before it

can be stated definitely that two different enzymes are involved.

The properties found for the β-hydroxybutyric acid dehydrogenase are very similar to those reported by Doudoroff et al. (1961). The D-β-hydroxybutyric acid dehydrogenase which they found, and purified about 50-fold is specific for DPN^+, is protected by Mg^{2+} and DPN^+ from inactivation and is inhibited by EDTA. At 30° they give a K_M for D-β-hydroxybutyric acid of 8.4×10^{-4} M and for DPN^+ 7×10^{-5} M, which is very close to the results reported here of 6.25×10^{-4} M and 4.0×10^{-5} M respectively at 37°.

This enzyme is probably of great importance in the metabolism of Rps. spheroides and related species in producing DPNH and acetoacetate from D-β-hydroxybutyric acid formed from breakdown of the storage polymer. This polymer accumulates on growth of the organism in acetate, butyrate and β-hydroxybutyrate and can account for 20% or more of the dry weight. In organisms grown in malate or succinate this polymer does not accumulate, a polyglucose compound being formed instead (Stanier, Doudoroff, Kunisawa & Contopoulou, 1959). However, the specific activity of β-hydroxybutyric acid dehydrogenase in organisms grown in acetate is the same as in organisms grown in malate (Table 19).

The increase in the specific activities of threonine dehydrogenase and β-hydroxybutyric acid dehydrogenase in biotin deficiency is very marked (Table 16), and the activities increase as the biotin deficiency increases. The effect is specific for biotin deficiency, no changes in these specific activities occur in nicotinic acid or thiamine deficiency (Table 18). The reason for this is not known. It may be

due to non-synthesis of repressors of these enzymes in biotin deficiency, or be an attempt on the part of the organisms to adapt to biotin deficiency.

L-Alanine-ALA transaminase. ALA transaminases have been reported by Kowalski et al. (1957) and Kowalski et al. (1959) in mammalian tissues and by Bagdasarian (1959) in C. diphtheriae. These studies were done from ALA and an α-ketoacid, α-oxoglutarate and pyruvate, and the amino acid formed was detected by paper chromatography. This is a relatively tedious method. In the present studies the reaction has been carried out in the reverse direction and the ALA formed estimated directly by the specific and sensitive colour reaction of Mauzerall & Granick (1956).

Of the amino acids tested only L-, D- and β-alanine have an appreciable activity in this system, and only L-glutamic acid inhibits L-alanine-ALA transaminase. Partial purification has not separated the activities towards L-alanine and β-alanine, although this does not imply that only one enzyme is involved. The activity towards D-alanine in crude enzyme extracts is due to an alanine racemase, and there does not appear to be a D-alanine-ALA transaminase.

The enzyme does not form aminoacetone from methylglyoxal when incubated with alanine, glutamic acid or aspartic acid, and aminoacetone does not inhibit ALA transamination. Pyruvate, which is a product of the transamination between γδ -dioxovaleric acid and alanine, inhibits ALA production, as does excess γδ -dioxovaleric acid.

Methylglyoxal, although not a substrate, also inhibits ALA production. Whether this inhibition is of physiological importance is not known, but it is possible that methylglyoxal formed from aminoacetone accumulates in the organisms under certain conditions.

Shemin (1955) suggested that the succinate-glycine cycle controls the amount of ALA available for porphyrin synthesis, but there are no quantitative data to support this. Pyruvate added to a crude enzyme system forming ALA lowered the amount of ALA produced (Fig. 19). This was thought to be due to removal of ALA by transamination. Oxaloacetate did not have this effect, but α-oxoglutarate did, and there is only a weak glutamic acid-ALA transaminase activity in extracts. The pyruvate inhibition may not be due to transamination since alanine does not reverse the inhibition. α-Oxoglutarate inhibition of ALA synthesis has been noted by Laver et al. (1958), Kikuchi et al. (1958) and Granick (1958), who also noted pyruvate inhibition. These effects may be due to removal of ALA by transamination, or more probably to competitive inhibition of ALA synthetase.

The importance of the L-alanine-ALA transaminase and of the succinate-glycine cycle as a whole in controlling porphyrin synthesis will depend on the rate of these reactions relative to the rate of ALA synthesis and its metabolism to porphyrin, and on the importance of the products of the succinate-glycine cycle for other metabolic pathways. No direct estimates have been made, but Kowalski et al. (1959) have shown that the ALA transaminase is more active in tissues with a high nucleic acid turnover than in those with a low nucleic acid turnover. This is

possibly related to the requirement of one-carbon fragments for nucleic acid synthesis. Rps. spheroides in common with other microorganisms probably contains large amounts of L- and D-alanine in its cell-walls (Work, 1957) and one of the pathways for synthesis of alanine in these organisms may be the ALA transaminase.

Under conditions where the specific activity of ALA synthetase is greatly reduced, in biotin deficiency and growth in acetate, the ALA transaminase activity is not affected. This may be one of the reasons for the reduced formation of porphyrin pigments under these conditions.

Further studies on the role of L-alanine-ALA transaminase and of the succinate-glycine cycle in controlling porphyrin biosynthesis are indicated. Rps. spheroides may be a suitable organism for such studies since porphyrin and bacteriochlorophyll synthesis can be altered under different growth conditions. By detecting the incorporation of label from ALA into various cell components under different conditions, it may be possible to make an estimate of the quantitative importance of the succinate-glycine cycle in metabolism and in controlling porphyrin and bacteriochlorophyll synthesis.

Role of biotin in porphyrin and bacteriochlorophyll synthesis. Lascelles (1956) reported that when organisms, after growth aerobically in the dark in malate (Medium S) were illuminated in a medium containing α-oxoglutarate and glycine (Mixture I) they excreted porphyrins into the medium, the major porphyrin being coproporphyrin III. Organisms grown in a medium deficient in biotin, nicotinic acid or thiamine did not

excrete porphyrins when illuminated in Mixture I unless the missing vitamin was added. Normal and deficient organisms formed porphyrin at equal rates when incubated in a medium containing ALA, which argued that the vitamins were required for ALA synthesis, not for its conversion to porphyrins. Lascelles (1956) thought that nicotinic acid and thiamine were required for the conversion of α-oxoglutarate to succinyl-CoA by α-oxoglutarate dehydrogenase, but she had no explanation for the role of biotin.

The ALA synthetase reaction is thought to involve a decarboxylation, and since most of the biotin enzymes so far described catalyse carboxylations, it was thought that biotin might be involved in the condensation of succinyl-CoA and glycine to form ALA.

Lascelles' (1956) results were confirmed and in addition it was found that biotin was required before biotin deficient organisms formed porphyrin on illumination in a medium containing succinate and glycine. Since succinyl-CoA is formed from succinate and α-oxoglutarate by two different enzymes, neither of which involve biotin, so far as is known, this was taken to mean that biotin was involved in the ALA synthetase reaction.

It was noted that normal organisms illuminated in the α-oxoglutarate and glycine medium or in the succinate and glycine medium grew and formed bacteriochlorophyll as well as excreting porphyrin, and that biotin deficient organisms required the addition of biotin to do this. Organisms illuminated in the ALA medium did not grow or form bacteriochlorophyll. Lascelles (1959) reported that adaptation involving

protein synthesis was required for formation of bacteriochlorophyll when aerobic dark-grown organisms were transferred to the light, and it was thought that lack of biotin might be stopping this. Normal and biotin deficient organisms grown anaerobically in the light were therefore used for the illuminations and the results obtained were the same as those with organisms grown aerobically in the dark.

The next step was to compare ALA synthetase activities in extracts from normal and biotin deficient organisms. ALA synthetase activity is reduced in biotin deficiency; the greater the deficiency, the lower the enzyme activity (Table 16). ALA dehydrase is not reduced except in extreme deficiency. L-Alanine-ALA transaminase is also not reduced, and threonine and β-hydroxybutyric acid dehydrogenases are increased. Recently Lascelles (1960b) has confirmed that ALA synthetase activity is reduced in biotin deficiency and has found that ALA dehydrase and ribulose diphosphate carboxylase are not affected. This is not a long list of enzymes but it does show that the reduction in ALA synthetase activity appears to be specific, and was originally taken as evidence that ALA synthetase was a biotin enzyme.

Attempts to reactivate the ALA synthetase in extracts of biotin deficient organisms by addition of biotin, biotin plus ATP, or boiled extracts of normal organisms failed. This was not surprising since no other biotin enzymes have been reactivated under conditions where protein synthesis is absent.

Attempts were made to reactivate the ALA synthetase <u>in vivo</u> under conditions which were known to restart porphyrin and bacteriochlorophyll

synthesis, that is on illumination of biotin deficient organisms in Mixture I plus biotin, but these also failed. Although it indicated that ALA synthetase was not a biotin enzyme, this negative result is important since it shows that the level of ALA synthetase activity is not the primary factor determining whether or not porphyrins are formed under these conditions.

A crude preparation of avidin inhibited the ALA synthetase. The effect appeared to be specific. Other egg-white proteins were inactive; avidin did not inhibit the threonine dehydrogenase and did not interfere with the ALA colour reaction. The inhibition by avidin could not be reversed by excess succinyl-CoA or pyridoxal phosphate. In other studies it was found that although avidin inhibits biotin enzymes, avidin pre-treated with biotin does not inhibit (Halenz & Lane, 1960). With ALA synthetase, on the other hand, avidin pre-incubated with a large excess of biotin still inhibited the activity. This contradiction was resolved when it was found that purer samples of avidin did not inhibit the ALA synthetase. This showed that it was some other egg-white protein in the impure avidin preparation which was inhibiting the enzyme, and that ALA synthetase did not contain biotin. The nature of the inhibition by impure avidin has not been investigated.

In many of the experiments reported in a review on biotin by Lichstein (1951) and in a number of studies since then a role for biotin in an enzyme reaction has been proposed on the basis of its specific activity being reduced in biotin deficiency and on the basis of inhibition by crude avidin preparations. The results reported here show

that many of these early findings must be accepted with caution. Recently, with improved techniques and purification of enzymes, biotin has been identified as a constituent of a number of enzymes, and its role in enzyme reactions more firmly established (Lynen et al., 1959).

Organisms grown under conditions of biotin deficiency contain lower concentrations of bacteriochlorophyll and carotenoid than normal organisms, as do nicotinic acid and thiamine deficient organisms and those grown with acetate instead of malate. Organisms grown anaerobically in the light in acetate are very pale pink, while those grown in malate are deep red. In extracts made from these organisms ALA synthetase activity was reduced as compared with non-deficient organisms grown in malate (Tables 18, 19). In acetate grown organisms the ALA dehydrase activity was also greatly reduced. The other enzymes tested were not reduced. Lascelles (1959) reported that organisms grown aerobically in the dark in malate contained less pigment and less ALA synthetase and ALA dehydrase than organisms grown anaerobically in the light in the same medium.

Thus, under a variety of conditions which are unfavourable for pigment synthesis, ALA synthetase and sometimes ALA dehydrase activities are reduced. It is not known whether reduction in the enzyme levels stops bacteriochlorophyll synthesis or whether inhibition of bacteriochlorophyll synthesis leads to accumulation of an inhibitor or a repressor of the enzymes. Some inhibition may operate since the response to biotin in Mixture I to form porphyrin and bacteriochlorophyll does not involve an increase in ALA synthetase activity, as assayed in cell-free systems.

Alternatively both the low pigment levels and the low enzyme levels under these unfavourable conditions may be due to the inability of the organisms to form succinyl-CoA, glycine or pyridoxal phosphate. The low enzyme activities could be explained if these enzymes are unstable in the absence of their substrates or cofactors. This instability has been shown for a number of other enzymes (Pardee, 1959). ALA synthetase in cell-free extracts of Rps. spheroides is very unstable, and the same may be true in vivo.

Further attempts to explain the action of biotin have been unsuccessful. Chromatophores from biotin deficient organisms were compared with those from normal organisms, and on a protein basis the only components present in reduced amounts were bacteriochlorophyll and carotenoid. Mevalonic acid which is probably the precursor of phytol and carotenoids and whose synthesis may be blocked in biotin deficiency could not replace biotin. This is possibly due to the inability of mevalonic acid to penetrate into the organisms. An inhibition of mevalonic acid synthesis could explain the reduction in both bacteriochlorophyll and carotenoid levels in biotin deficiency.

Effects of haemin and protoporphyrin. Lascelles (1960a) showed that addition of haemin, but not of protoporphyrin or Mg protoporphyrin to growing organisms repressed the synthesis of ALA synthetase and ALA dehydrase. ALA did this also, probably due to its conversion to haemin. This is an example of negative feedback repression which has already

been established for a number of other biosynthetic pathways. In this study haemin, but not protoporphyrin or Mg protoporphyrin, inhibits ALA synthetase activity in cell-free extracts at a relatively low concentration, and protoporphyrin, but not haemin or Mg protoporphyrin inhibits ALA dehydrase (p. 104). This is an example of negative feedback inhibition, which has also been demonstrated for a number of other biosynthetic pathways. The important point is that both inhibition and repression operate. It is not known whether the repression is mediated by an initial inhibition of the action of these enzymes, whether the two effects are unrelated, or whether the differences between haemin and protoporphyrin action in whole cells and in cell-free extracts are significant.

These effects are important in photosynthetic bacteria, since they can also stop, or at least limit, synthesis of bacteriochlorophyll which quantitatively as well as biologically is the most important porphyrin pigments in these organisms.

The role of haemin and protoporphyrin in controlling pigment synthesis aerobically in the dark, and in the various growth conditions described above is not known, but accumulation of haemin and protoporphyrin may be one of the factors responsible for the reduced levels of ALA synthetase, ALA dehydrase, and pigments. In particular, under aerobic growth conditions in the dark or in the light, the high oxygen tension may favour oxidation of the porphyrinogens to porphyrins. If the porphyrinogens, not the porphyrins, are intermediates in cytochrome and bacteriochlorophyll synthesis oxidation to porphyrins will lead to

their accumulation, to inhibition of cytochrome and bacteriochlorophyll synthesis, and to repression and inhibition of ALA synthetase and ALA dehydrase. Unfortunately the oxidation levels at which the tetrapyrroles are converted to cytochromes and bacteriochlorophyll has not yet been established.

Lascelles (1956, 1959) reported that addition of iron to organisms illuminated in Mixture I stopped coproporphyrin excretion and increased bacteriochlorophyll synthesis. The coproporphyrin formed in the absence of iron was much greater than the extra bacteriochlorophyll and haemin formed in its presence. The reduction in coproporphyrin excretion can be explained by the conversion of iron to haemin, which inhibits ALA and PBG synthesis. The requirement of iron for bacteriochlorophyll synthesis may be due to its requirement for the conversion of coproporphyrinogen to protoporphyrinogen.

Origin of the methyl ester group in bacteriochlorophyll. The inhibition of bacteriochlorophyll synthesis by ethionine at concentrations where it did not appreciably affect growth indicated that it was inhibiting some essential reaction involving methionine. A likely site was thought to be the formation of the methyl ester group of bacteriochlorophyll. This methyl group is probably added to protoporphyrin or Mg protoporphyrin (Fig. 1). The only work on the origin of the methyl ester group of chlorophyll is that of Green et al. (1957) who found label in the methyl ester group, but not in the rest of the molecule when Chlorella were grown in the presence of ^{14}C-formate.

Methoxy groups and N-methyl groups, in all cases where they have been studied are donated by S-adenosylmethionine (Axelrod & Tomchick, 1958; Axelrod, 1961) but the enzymic formation of methyl ester groups does not appear to have been studied. In fact, methyl ester groups are comparatively rare in nature, the only other compounds found which contain them are a few of the alkaloids (Robinson, 1955) and pectin (Pigman & Goepp, 1948).

In this study using [methyl-^{14}C]methionine the radioactivity of the carotenoids has also been measured. The two major carotenoids of Rps. spheroides (Fig. 2) contain one methoxy group per molecule, which, by analogy with other methoxy groups, is probably donated by S-adenosylmethionine.

The label from [methyl-^{14}C]methionine was found solely in the methyl ester group, but not in the rest of the bacteriochlorophyll molecule.

That this methyl group is being donated directly by methionine, and not after methionine has lost its methyl group to the pool of one-carbon fragments is strongly indicated by the small dilution in radioactivity and by the fact that the specific radioactivity of the carotenoids is only slightly greater than that of the bacteriochlorophyll (Table 32). It must be pointed out that the specific radioactivities of the pigments have been calculated assuming that no turnover takes place during the illumination and this may not be justified.

Attempts in conjunction with Dr. Gibson to investigate the methylation enzymically have failed so far, but further work is being

done.

Effect of ethionine and threonine on porphyrin and bacteriochlorophyll synthesis. In experiments to investigate whether protein synthesis was involved in the response of biotin deficient aerobically grown organisms to biotin, p-fluorophenylalanine and ethionine were added and the organisms illuminated in Mixture I. p-Fluorophenylalanine inhibited the response to biotin, that is no growth or porphyrin and bacteriochlorophyll synthesis occurred. Ethionine, while it inhibited growth and bacteriochlorophyll synthesis, stimulated an excretion of porphyrin into the medium. This effect was noted with ethionine in the absence of added biotin and with non-deficient organisms. In the absence of ethionine, adaptation involving synthesis of ALA synthetase and ALA dehydrase is required before aerobic dark grown organisms form pigment on incubation anaerobically in the light in Mixture I (Lascelles, 1959).

Using light grown organisms this effect was also noted. In particular Fig. 21 shows that addition of 5×10^{-3} M ethionine to normal organisms completely stops bacteriochlorophyll and carotenoid synthesis, but only partially stops growth. The amount of porphyrin excreted in the presence of ethionine is greater by a factor of two than the sum of the porphyrin and bacteriochlorophyll formed in its absence. This is a reproducible finding, in other experiments the difference is sometimes three or fourfold. Ethionine also stimulates porphyrin excretion by nicotinic acid and thiamine deficient organisms in the absence of added vitamin.

All these results suggest that ethionine is not simply stopping bacteriochlorophyll synthesis, but is actually stimulating porphyrin synthesis possibly by relieving some inhibition which is normally present. The more biotin deficient the organisms, the less porphyrin is formed in response to ethionine and this may be related to the ALA synthetase content of the organisms.

A large number of compounds were tested, but the only one which had an activity similar to ethionine was threonine. Like ethionine, it inhibits bacteriochlorophyll synthesis and stimulates porphyrin excretion when added to organisms illuminated in Mixture I, and it also stimulates porphyrin excretion by biotin deficient organisms in the absence of added biotin.

Ethionine stimulates porphyrin excretion when organisms are illuminated in ALA. This is not a specific effect, since methionine, cysteine, glutathione and homocysteine do this also. Since illumination in ALA is different from illumination in Mixture I in that no growth or bacteriochlorophyll synthesis occurs, the effect of ethionine in the two media is probably different.

The porphyrin solutions formed on illumination of organisms in Mixture I in the presence and absence of ethionine and threonine all have the same spectra between 355 and 610 mμ. Extraction and chromatography of the porphyrins showed that not only is coproporphyrin the major component, but all the minor components are the same, under all conditions. Therefore the estimation of porphyrin based on coproporphyrin is a valid measure of the quantities of porphyrin excreted in the

presence and absence of ethionine and threonine.

On illumination of organisms in Mixture I all the components are essential for porphyrin and bacteriochlorophyll synthesis (Lascelles, 1956, 1959). In the presence of ethionine or threonine no ammonia is required, and with ethionine Mg^{2+} is the only metal required for maximum porphyrin excretion. This is a further indication that ethionine and threonine are "uncoupling" porphyrin synthesis.

No effect of ethionine or threonine could be demonstrated in cell-free systems. Ethionine did not affect ALA synthetase or ALA dehydrase and ethionine and threonine did not transaminate with γδ-dioxovaleric acid or inhibit the L-alanine-ALA transaminase reaction.

Organisms incubated aerobically in the dark in Mixture I do not form porphyrins or bacteriochlorophyll and ethionine has no effect under these conditions.

The ethionine and threonine effects are reversed by methionine and homocysteine and the threonine effect by homoserine. The ethionine effect is not reversed by homoserine or by homoserine plus cysteine, perhaps because too low concentrations were used. This reversal means that the porphyrin excretion is reduced to the level excreted in the absence of ethionine, and bacteriochlorophyll and carotenoids are synthesised. Methionine, alone in some experiments, stimulated bacteriochlorophyll synthesis and reduced porphyrin excretion, but homocysteine and homoserine had no effect in the absence of ethionine or threonine. Serine, glutathione, cysteine and norleucine did not reverse the ethionine or threonine effects.

Methionine and homocysteine as well as restarting bacteriochlorophyll synthesis in the presence of ethionine and threonine appear to be reintroducing the inhibition which ethionine and threonine abolish since the sum of the porphyrin and the bacteriochlorophyll formed in their presence is less than the porphyrin excreted with ethionine or threonine alone.

These results indicate that both ethionine and threonine are blocking some reaction of methionine. Since norleucine is inactive it is probable that the reaction involves transfer of the methyl group. It has been found that the methyl ester group of bacteriochlorophyll is most probably donated directly by methionine, and it is very likely that this is the reaction which ethionine and threonine inhibit.

The mode of action of ethionine is clear since in a number of studies it has been shown to compete with methionine for ATP and to form S-adenosylethionine, which donates its ethyl group to form an ethylated instead of a methylated compound (Parks, 1958). Alternatively the compound may not accept the ethyl group.

The mode of action of threonine is not so obvious. The best explanation appears to be that threonine is blocking the de novo synthesis of methionine. Both compounds are synthesised from aspartic acid via homoserine (Meister, 1957) and addition of threonine may exert a feedback control on the early steps of the common biosynthetic pathway which will inhibit de novo synthesis of both compounds. There is no sound experimental evidence for this except a report by Doudney & Wagner (1952, 1953) who showed that growth inhibition of a mutant of

Neurospora crassa by threonine could be reversed by methionine, homocysteine or homoserine. On the other hand the control of methionine synthesis in E. coli involves only the part of the pathway from homoserine to methionine, both cystathionase and homocysteine methylase being repressed by addition of methionine to the growth medium (Rowbury & Woods, 1961a and b). It would be of interest to investigate the nature of the threonine effect further to see whether it actually involves repression or inhibition of the early enzymes in the pathway of homoserine biosynthesis.

The probable role of iron in inhibiting porphyrin excretion in Mixture I has been discussed above. The effect of iron is exactly opposite to the effects of ethionine and threonine. Iron and ethionine antagonise one another (Table 28). At low concentrations of ethionine, iron decreases porphyrin excretion as it does in the absence of ethionine, but at high concentrations of ethionine iron has no effect.

If the effect of ethionine in stimulating porphyrin excretion is being mediated solely by its ability to inhibit formation of the methyl ester group in bacteriochlorophyll, it ought to be inactive in Chlorobium whose chlorophyll does not contain a methyl ester group (Holt & Hughes, 1961; Holt & Morley, 1960). Similarly ethionine ought to have no effect on porphyrin synthesis in organisms which do not form chlorophyll. This is probably true since a large number of papers have appeared recently on the effects of ethionine in animals, and in none of these is any mention made of a derangement in porphyrin synthesis.

Control mechanisms for pigment synthesis. Before discussing hypotheses to account for the results obtained it must be noted that all the experiments with ethionine and threonine have been done under one set of conditions, namely illumination in Mixture I. This medium was devised by Lascelles (1956) specifically to give porphyrin excretion, and this excretion may mean that under these conditions the organisms have already lost some of their control over porphyrin synthesis even without the addition of ethionine or threonine. Iron can control porphyrin excretion in Mixture I (Lascelles, 1956), but the results with ethionine and threonine indicate that another control mechanism is operating under these conditions.

To account for the stimulation of porphyrin excretion by ethionine and threonine it can be postulated that not only do they inhibit bacteriochlorophyll and carotenoid synthesis by interfering with methylation, but they also inhibit the formation of one of the intermediates in bacteriochlorophyll synthesis which can act as a feedback inhibitor. The point of action of the inhibitor may be either one of the early steps in porphyrin synthesis, ALA synthetase or ALA dehydrase, or the first step leading to bacteriochlorophyll. This, according to Granick (1961) is the addition of Mg^{2+} or the methyl ester group to protoporphyrin (Fig. 1). For the purposes of this hypothesis it must be assumed that the Mg^{2+} is incorporated first and that the inhibitor is Mg protoporphyrin monomethyl ester or one of the later intermediates, most of which have not been identified. Alternatively, the inhibitor

may be some other methylated compound, but on the basis of feedback control, elucidated for a number of pathways, this is unlikely.

The presence of an inhibitor is also indicated by the finding that the response to biotin in Mixture I, giving growth and porphyrin and bacteriochlorophyll synthesis, does not involve any increase in ALA synthetase activity, as assayed in cell-free extracts.

To account for the effect of ethionine under conditions where pigment synthesis does not normally occur on illumination, that is in vitamin deficient organisms and in those previously grown aerobically in the dark, it must be assumed that the block in bacteriochlorophyll synthesis is after the synthesis of the inhibitor which therefore accumulates. It must also be assumed that the inhibitor is being destroyed and resynthesised on illumination in Mixture I. Ethionine and threonine then inhibit its resynthesis. The reason why ethionine does not act aerobically in the dark may be that other inhibitors are present under these conditions, or that the postulated inhibitor is stable, or that porphyrin and bacteriochlorophyll synthesis is absent, not due to the presence of inhibitors but to the absence of substrates. At low oxygen tension in the dark bacteriochlorophyll is synthesised (Lascelles, 1959) and it would be of interest to see whether ethionine can stimulate porphyrin excretion under these conditions.

Methionine and homocysteine reverse the ethionine and threonine effect probably by restoring synthesis of the inhibitor.

The point of action of the inhibitor cannot be decided on the evidence available. If it acts on the synthesis or metabolism of ALA

its removal will give increased porphyrin synthesis. In the absence of added iron most of the porphyrin will be excreted as coproporphyrin and any protoporphyrin formed will be converted to Mg protoporphyrin, which unlike haemin and protoporphyrin does not inhibit or repress ALA synthetase and ALA dehydrase, and coproporphyrin excretion will continue. This explains the requirement for Mg^{2+}. The inhibition of the ethionine effect by Fe^{2+} is probably due either to its requirement for coproporphyrinogen metabolism, or to its forming haemin from protoporphyrin. The reason why Fe^{2+} does not inhibit porphyrin excretion at high ethionine concentrations cannot be explained satisfactorily. It may be that ethionine also inhibits formation of protoporphyrin and haemin, but there is no evidence for this.

If the inhibitor blocks the formation of Mg protoporphyrin, its removal will allow this step to proceed. In the presence of Mg^{2+}, Mg protoporphyrin will be formed, less protoporphyrin and haemin will accumulate, and the inhibition on ALA synthetase and ALA dehydrase will be lifted. The inhibition by Fe^{2+} is probably due to its competing with Mg^{2+} for available protoporphyrin, although this is not a completely satisfying explanation.

Alternatively the postulated inhibitor may act at both sites. That it is an inhibitor rather than a repressor is shown by the fact that ethionine can stimulate porphyrin excretion in the absence of protein synthesis.

Haemin is already known to act as a repressor of ALA synthetase and ALA dehydrase, and haemin and protoporphyrin as inhibitors of the

activities of these enzymes, so it may be more likely for the postulated inhibitor to act at some other step. However, Kikuchi et al. (1958) reported a heat-labile inhibitor of ALA synthetase in extracts of aerobic dark grown Rps. spheroides but not in extracts of organisms grown anaerobically in the light. This could not be confirmed by Lascelles (1959). The heat-lability argues against its being haemin. Thus it may well be that there are a number of inhibitors or repressors acting on the early steps of porphyrin synthesis, their relative importance depending on the growth conditions.

This explanation of the reason for the results with ethionine and threonine may not be correct, and further work will have to be done to test it. The stimulation of porphyrin excretion may not be a direct consequence of the inhibition of bacteriochlorophyll synthesis and it may not even be a direct consequence of inhibition of methylation. A number of reports have appeared which indicate that some of the biological effects of ethionine are not directly related to its competition with methionine (Levy, Montanez & Dunn, 1955; Stekol, Mody, Bedrak, Keller & Perry, 1959).

The above hypothesis may explain some other results on pigment synthesis. Lascelles (1959) noted that addition of protein synthesis inhibitors, other than ethionine, to Rps. spheroides not only inhibited growth, but also inhibited porphyrin excretion and bacteriochlorophyll synthesis. This can be explained by accumulation of haemin, in the absence of protein to form cytochromes, and accumulation of bacteriochlorophyll intermediates in the absence of protein to synthesise

chromatophores. The reason for reduced enzyme levels in vitamin deficiency may also be due to accumulation of inhibitors and repressors. The response to the vitamin, giving growth, removes the inhibitors and repressors and allows porphyrin and bacteriochlorophyll synthesis.

In other growth conditions which are unfavourable for pigment synthesis, high oxygen tension in the dark or in the light, and high light intensity, the combined accumulation of haemin and the postulated inhibitor may account for the reduced levels of ALA synthetase and ALA dehydrase.

It is not known what is the primary cause of inhibition of pigment synthesis under any of these conditions and it is probable that the accumulation of the inhibitors and repressors so far identified, and the reduction in enzyme levels are secondary features, being the consequence rather than the cause of the inhibition of pigment synthesis. It is also probable that pigment synthesis is inhibited in different ways under different conditions.

The availability of succinyl-CoA under some conditions may be the primary reason for failure of porphyrin pigment synthesis, but there is no direct evidence for this. The results of Elsden & Ormerod (1956) with R. rubrum, and there is no reason to suppose that Rps. spheroides is different, show that aerobically in the dark the metabolism of all substrates is by the tricarboxylic acid cycle-acetate, butyrate, pyruvate, propionate, malate, fumarate and succinate, but that anaerobically in the light only acetate, butyrate and pyruvate are metabolised by the tricarboxylic acid cycle. The anaerobic metabolism

of propionate, malate and succinate is not fully known, but it may be by glycolysis or the hexosemonophosphate shunt. Stanier et al. (1959) find that the anaerobic light metabolism of acetate and butyrate leads to accumulation of poly-β-hydroxybutyric acid, but that anaerobic light metabolism of propionate, succinate and malate lead to accumulation of a glucose polymer. It is also known that very little pigment is formed at high oxygen tensions in the dark, irrespective of the substrate, and that anaerobically in the light, while growth on acetate gives organisms with a low pigment content, growth in malate gives highly pigmented organisms. From these results it would appear that, when the major pathway of metabolism is the tricarboxylic acid cycle pigment synthesis is not favoured, but that pigment synthesis is favoured by the other pathway. That is high oxygen tension in the light or in the dark favours the tricarboxylic acid cycle metabolism of malate and succinate, while low oxygen tension in the light or in the dark favours an alternative metabolism for these compounds which makes succinyl-CoA available for porphyrin synthesis.

The effect of increasing the light intensity, which stops pigment synthesis (Cohen-Bazire et al., 1957) may act in the same way by leading to oxidation of the electron-transport carriers and so favouring the tricarboxylic acid cycle.

Additional proof for this argument is that aerobically in the dark Rps. spheroides form aminoacetone but no ALA from α-oxoglutarate, fumarate and glycine. The aminoacetone is probably formed from acetyl-CoA and glycine which indicates that the ALA synthetase is active under

these conditions, and that absence of ALA is due to absence of succinyl-CoA. The absence of available succinyl-CoA may also explain the results of Urata & Granick (1961) who found that aerobic incubation of glycine plus any one of a number of the tricarboxylic acid cycle intermediates with liver homogenates gave accumulation of aminoacetone but not of ALA.

The absence of succinyl-CoA under aerobic growth conditions and in growth in acetate may be the reason for the reduced levels of ALA synthetase and ALA dehydrase since these enzymes may be unstable in vivo or be synthesised at a reduced rate in the absence of their substrates, as has been shown for a number of other enzymes (Pardee, 1959). This may also be the reason for inhibition of pigment synthesis and reduced enzyme levels in vitamin deficiency.

Inhibition of carotenoid synthesis must also be accounted for if this hypothesis is correct, but this will have to await further experiments.

It may be that the stimulation of porphyrin excretion by ethionine and threonine can be explained by their making more succinyl CoA available for porphyrin synthesis.

ALA synthetase is a pyridoxal phosphate requiring enzyme and Gibson (1958) showed that pyridoxal phosphate had to be added to crude undialysed extracts of Rps. spheroides before any ALA was synthesised. The level of pyridoxal phosphate may control not only the activity of this enzyme, but also its absolute amount, if the enzyme is unstable in the absence of its cofactor.

of its cofactor.

The importance of alternative pathways of metabolism for ALA have not been fully explored, but they may play a role in the control of porphyrin synthesis.

Sano & Granick (1961) proposed that porphyrin synthesis in liver can be controlled by the distribution of the enzymes of porphyrin synthesis between the mitochondria and the cytoplasm. This probably does not apply in Rps. spheroides, since all the enzymes up to that forming coproporphyrinogen at least, are most probably in the cytoplasm.

Cohen-Bazire et al. (1957) consider that the inhibition of pigment synthesis in anaerobically growing organisms by addition of oxygen or by increasing the light intensity is caused by the oxidation of a carrier of the electron transport chain. At first sight this is rather a vague hypothesis. However, there are a number of oxidative and reductive steps in both bacteriochlorophyll and carotenoid synthesis - oxidation is required for the conversion of coproporphyrinogen to protoporphyrin, for formation of the isocyclic ring, and for the conversion of the C_{40} terpene intermediate to the more highly conjugated carotenoids; reduction is required in rings B and D of the porphyrin molecule to form bacteriochlorophyll, and for conversion of the unsaturated C_{20} terpene to phytol. A delicate balance is probably required in the oxidation-reduction state of the electron carriers for all these steps to occur.

Organisms grown aerobically in the dark do not contain chromatophores (Schachman, Pardee & Stanier, 1952) and this may be the reason for absence of pigment under these conditions. Recently Cohen-Bazire &

Kunisawa (1960) have suggested that partially formed chromatophores may be present aerobically in the dark. There may be some regulatory mechanism for chromatophore synthesis and under some conditions their absence may be the primary reason for failure of pigment synthesis.

At low oxygen tension in the dark pigments are synthesised (Lascelles, 1959). This is an important observation since it shows that pigment synthesis, or chromatophore synthesis, is not dependent on light, but is more closely linked to the oxidation state of the organisms.

It is not known which steps in bacteriochlorophyll and carotenoid synthesis occur outside the chromatophores, but it is possible that the last few steps occur in these particles. In the absence of chromatophores the accumulation of intermediates may account for the reduced enzyme levels.

Non-synthesis of the chromatophores cannot account for the inhibition of pigment synthesis by increasing the light intensity under anaerobic conditions since Cohen-Bazire & Kunisawa (1960) have shown that organisms grown at different light intensities contain approximately equal amounts of chromatophores, the only difference being in their bacteriochlorophyll and carotenoid content, which is lower at high light intensities.

It must be noted that changes in the levels of carotenoids under different growth conditions parallel changes in bacteriochlorophyll. The explanation for this is not known because very little is known about the details of carotenoid biosynthesis. These pigments are linked chemically since the phytol group and the carotenoids are both probably

synthesised from mevalonic acid. Thus a primary inhibition of carotenoid synthesis by high oxygen tension, high light intensity or vitamin deficiency could also inhibit phytol and bacteriochlorophyll synthesis and thus cause the observed changes in ALA synthetase and ALA dehydrase activities by accumulation of intermediates.

The carotenoid molecule (Fig. 2) is probably highly susceptible to oxidation, or to reduction, or to photochemical changes. As shown by Van Niel (1947) oxygen irreversibly converts carotenoid Y to carotenoid R in Rps. spheroides. Oxygen or high light intensity may lead to the accumulation of some carotenoid which inhibits de novo carotenoid and phytol synthesis.

The above discussion shows that there are a number of possible mechanisms for the control of pigment synthesis, and that their importance may depend on the growth conditions. The main obstacle to their elucidation is the incomplete knowledge of the metabolic pathways in Rps. spheroides, and of the intermediates in bacteriochlorophyll and carotenoid synthesis.

SUMMARY

SUMMARY

1. Rhodopseudomonas spheroides has been found to produce relatively large amounts of an aminoketone under suitable incubation conditions. This aminoketone has been purified and identified as aminoacetone. Although it is an analogue of δ-aminolaevulic acid, aminoacetone has no effect on any of ALA's enzyme reactions. Aminoacetone has no significant effect on porphyrin or bacteriochlorophyll biosynthesis in whole organisms.

2. A DPN^+ requiring enzyme has been found in extracts which converts threonine to aminoacetone. This enzyme has been partially purified and its specificity and properties studied. A β-hydroxybutyric acid dehydrogenase has also been discovered, and its properties studied. These two enzymic activities, although they are very similar, are probably due to two different enzymes.

3. An L-alanine-δ-aminolaevulic acid transaminase has been found in extracts. Its properties have been studied, and its possible role in the control of porphyrin and bacteriochlorophyll biosynthesis is discussed.

4. The effect of biotin deficiency on porphyrin and bacteriochlorophyll biosynthesis has been studied in whole cells, and the results of Lascelles (1956) have been confirmed and extended. Of various enzymes tested the only one whose specific activity is reduced in biotin deficiency is ALA synthetase. Although impure avidin inhibits ALA synthetase activity, purified avidin has no effect. This suggests that ALA synthetase is not

a biotin containing enzyme. This conclusion is strengthened by the finding that, under other growth conditions which are unfavourable for pigment synthesis, the activities of ALA synthetase and ALA dehydrase are reduced, while the activities of other enzymes tested are unaffected. These findings do, however, suggest that the organisms have some mechanism for controlling the activities of these enzymes.

5. Ethionine and threonine, when added to organisms illuminated in Mixture I, stop bacteriochlorophyll biosynthesis and stimulate the production of porphyrins. The amount of porphyrin produced in the presence of ethionine or threonine is greater than the sum of the porphyrin and bacteriochlorophyll formed in their absence by a factor of three or four. Ethionine and threonine also give porphyrin production with biotin deficient organisms and ethionine with nicotinic acid and thiamine deficient organisms, in the absence of added vitamins. A large number of related and unrelated compounds have been tested, but none of them shows these effects. The ethionine and threonine effects are specifically reversed by methionine and homocysteine, which strongly suggests that some reaction of methionine is being blocked. The threonine effect, but not the ethionine effect, is also reversed by homoserine.

6. The origin of the methyl ester group of bacteriochlorophyll has been investigated. The results show that it is most probably formed directly from the methyl group of methionine. This is probably the reaction with which ethionine and threonine interfere.

7. In cell-free extracts haemin inhibits ALA synthetase activity: protoporphyrin and Mg protoporphyrin have no effect. Protoporphyrin inhibits ALA dehydrase: haemin and Mg protoporphyrin have no effect.

8. These results, together with the results of other workers, have been used to discuss possible ways in which Rps. spheroides controls its porphyrin and bacteriochlorophyll biosynthesis.

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