effects on the organism also include increased concentrations in blood, liver and other tissues, and involve quantities of vitamin C in excess of those lost by the adrenal. The increased concentration in the liver is probably associated with decreased catabolism of ascorbate: the results of Lahiri & Lloyd (1962) make it unlikely that loss of adrenal ascorbate involves oxidation (cf. Lloyd & Sinclair, 1953), and with the present results lend no support to the view that corticotrophin promotes the oxidation of ascorbate.

## SUMMARY

1. The effects of various procedures on the concentrations of ascorbate and of dehydroascorbate plus dioxogulonate in blood and various tissues of anaesthetized rats have been investigated.

2. Laparotomy, haemorrhage and administration of corticotrophin increase the concentrations in blood.

3. The rise can be seen when the adrenals are excluded from the circulation, the highest concentrations being in blood from the inferior vena cava above the diaphragm; the femoral arteriovenous difference becomes increasingly negative.

4. The concentrations of ascorbate in several tissues (liver, spleen and kidney) are significantly increased in corticotrophin-treated animals, and the accompanying fall in the liver content of dehydroascorbate plus dioxogulonate is consistent with diminished ascorbate catabolism.

5. There is no support for the view that corticotrophin promotes the oxidation of ascorbate.

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# Studies on the Biosynthesis of Porphyrin and Bacteriochlorophyll by *Rhodopseudomonas spheroides*

## 3. THE EFFECT OF THREONINE ON THE BIOSYNTHESIS OF HOMOSERINE AND METHIONINE*

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Threonine inhibits the biosynthesis of bacteriochlorophyll by illuminated suspensions of *Rhodopseudomonas spheroides* and greatly increases the excretion of coproporphyrin; ethionine is the only other compound tested that has a similar effect, and the biosynthesis of bacteriochlorophyll in the presence of threonine, or of ethionine, can be restored by the addition of homocysteine or methionine (Gibson, Neuberger & Tait, 1962*b*). Evidence was also presented that methionine donates its methyl group to form the methyl ester group of bacteriochlorophyll, and Tait & Gibson (1961) showed that chromatophore preparations catalyse the formation of magnesium protoporphyrin monomethyl ester from magnesium protoporphyrin and *S*-adenosylmethionine. The most likely explanation for the effect of ethionine was that its *S*-adenosyl derivative inhibits the

* Part 2: Gibson, Neuberger & Tait (1962*b*).

enzymic methylation, and this has now been demonstrated (K. D. Gibson, A. Neuberger & G. H. Tait, unpublished work).

Both threonine and methionine are synthesized from homoserine, and homoserine is formed from aspartic acid via aspartyl β-phosphate and aspartate β-semialdehyde (Black & Wright, 1955*a*, *b*, *c*). To explain the action of threonine in inhibiting the synthesis of bacteriochlorophyll it was proposed that this amino acid inhibits the action of, or represses the synthesis of, one or more of the enzymes responsible for the conversion of aspartic acid into homoserine, thus inhibiting the synthesis not only of threonine but also of methionine. Such a mechanism was indicated by the work of Doudney & Wagner (1952, 1953), who showed that the inhibition of growth in a mutant of *Neurospora crassa* caused by threonine could be reversed by methionine, homocysteine or homoserine. In *Escherichia coli* and in yeast Wormser & Pardee (1958) found that threonine synthesis is controlled by a feedback inhibition of homoserine kinase; this could not possibly control methionine synthesis also.

Stadtman, Cohen, Lebras & de Robichon-Szulmajster (1961) reported on the control of threonine synthesis in *E. coli* and *Saccharomyces cerevisiae*. In *E. coli*, which also synthesizes lysine from aspartate β-semialdehyde, they showed the existence of two distinct β-aspartokinases, one inhibited by threonine and the other by lysine; the presence of a third β-aspartokinase, inhibited by homoserine, was also postulated. In *S. cerevisiae*, where lysine is synthesized by another pathway not involving aspartic acid, there is only one β-aspartokinase which is both inhibited and repressed by threonine. However, in *Micrococcus glutamicus*, homoserine dehydrogenase, the enzyme catalysing the conversion of aspartate β-semialdehyde into homoserine, is strongly inhibited by threonine, and its synthesis is repressed by growth in the presence of methionine (Nara *et al.* 1961). In *Rps. spheroides* we have found that none of the enzymes involved in the conversion of aspartic acid into homoserine is repressed by addition of threonine to the growth medium. However, as with *M. glutamicus*, homoserine-dehydrogenase activity is strongly inhibited by threonine. For a preliminary communication see Gibson, Matthew, Neuberger & Tait (1961).

## MATERIALS AND METHODS

*Chemicals.* $NAD^+$, NADH, $NADP^+$, NADPH and ATP were obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany; DL-homoserine and αε-diaminopimelic acid (60% *meso* and 40% L-isomer) from California Corp. for Biochemical Research, Los Angeles, U.S.A. and uniformly labelled L-aspartic acid (L-[U-$^{14}$C]aspartic acid) from The Radiochemical Centre, Amersham, Bucks. Aspartyl β-phosphate was prepared in solution as described by Black & Wright (1955*a*). The concentration was determined by reaction with neutral hydroxylamine solution and subsequent colorimetric estimation with ferric chloride reagent (Davie, Konigsberger & Lipmann, 1956) with succinohydroxamic acid as standard. Since the colour yield varied with the amount of free hydroxylamine present in the reaction mixture the results cannot be regarded as strictly quantitative, although they were satisfactory for comparative purposes. Aspartate β-semialdehyde was prepared as described by Neuberger & Tait (1962*a*). The material was only obtained in solution. The concentration of the aldehyde was estimated both by nitrogen determination and by enzymic reduction to homoserine in the presence of NADPH by using the change in extinction at 340 mμ. Both methods gave results in close agreement. Carbonate–bicarbonate buffers were made from $Na_2CO_3$ and $NaHCO_3$. The sources of other materials were as given by Gibson, Neuberger & Tait (1962*a*).

*Organisms.* The strain of *Rps. spheroides* was that used by Gibson *et al.* (1962*a*). The conditions of maintenance, growth, harvesting and determination of dry weight and bacteriochlorophyll content are also given in that paper. In this work all cultures were grown anaerobically in the light at 32–34° for 44 hr. in 'medium S' of Lascelles (1956).

*Preparation and assay of cell-free extracts.* Extracts of organisms were prepared as described by Gibson *et al.* (1962*a*) and used after centrifuging at 105 000*g* without purification for all enzyme assays.

The assays for the enzymes of homoserine biosynthesis are based on those described by Black & Wright (1955*a b*, *c*) and by Stadtman *et al.* (1961). Details are given in the Results section. In the spectrophotometric assays the extinction coefficient of NADH and NADPH was taken to be $6{\cdot}22 \times 10^6$ cm.$^2$/mole at 340 mμ (Horecker & Kornberg, 1948).

## RESULTS

### *Inhibition studies*

*β-Aspartokinase.* β-Aspartokinase activity was detected in cell-free extracts by measuring the amount of hydroxamate formed in the presence of aspartic acid, ATP and hydroxylamine (Table 1). The formation of hydroxamate depends on the presence of enzyme, aspartic acid and ATP, and is stimulated by the addition of $Mg^{2+}$ ions. The amount of hydroxamate formed is approximately proportional to the amount of enzyme added. Of a number of other amino acids tested in place of aspartic acid (L-glutamic acid, L-threonine, DL-homoserine, L-serine, L-methionine, L-lysine and diaminopimelic acid), only L-glutamic acid led to formation of hydroxamate, the amount being only a quarter of that formed by aspartic acid under comparable assay conditions. The results in Table 1 were obtained by following closely the conditions described by Black & Wright (1955*a*). In other experiments the assay method of Stadtman *et al.* (1961) was used. This method, which differs from the former one in the use of a much lower concentration of L-aspartic acid, was em-

ployed in an attempt to show an inhibition of enzyme activity by added amino acids, which might not have been observed with high concentrations of substrate. Table 2 shows that no significant inhibition was obtained with various amino acids, most of which are synthesized from aspartic acid via aspartyl β-phosphate.

*Aspartate β-semialdehyde dehydrogenase.* This activity was measured by following the oxidation of NADPH spectrophotometrically in the presence of aspartyl β-phosphate. The dehydrogenase in *Rps. spheroides* does not utilize NADH and is similar in this respect to that in baker's yeast (Black & Wright, 1955*b*). The rate of oxidation was linear for 5 min. under the conditions described in Table 3 and was proportional to the concentration of enzyme up to at least 0·5 mg. of protein/ml. The enzyme has a sharp optimum at pH 8·4 in 0·1 M-tris, and the activity is markedly reduced when tris is replaced by either phosphate or carbonate–bicarbonate buffer. From measurements of the rate of oxidation of NADPH in the presence of different concentrations of aspartyl β-phosphate an approximate $K_m$ for aspartyl β-phosphate of 67 μM at 37° has been calculated from the Lineweaver & Burk (1934) reciprocal plot. The reverse reaction was also demonstrated by incubating the enzyme with aspartate β-semialdehyde, inorganic phosphate and $NADP^+$; under these conditions NADPH was formed. $NADP^+$ was only slightly reduced in the absence of inorganic phosphate (Fig. 1).

Aspartate β-semialdehyde dehydrogenase was assayed in the presence of the same amino acids that were used with β-aspartokinase. None of these compounds led to the oxidation of NADPH when incubated with enzyme in the absence of aspartyl β-phosphate, and none of them strongly inhibited the activity of aspartate β-semialdehyde dehydrogenase (Table 3). When the amount of aspartyl β-phosphate in the assay system was decreased from 0·84 to 0·23 μmole exactly the same degree of inhibition was obtained.

*Homoserine dehydrogenase.* This activity was measured by following the reduction of $NADP^+$ in the presence of DL-homoserine. Like aspartate β-semialdehyde dehydrogenase the homoserine dehydrogenase of *Rps. spheroides* is specific for $NADP^+$ and does not utilize $NAD^+$; it is similar in this respect to the homoserine dehydrogenase in *M. glutamicus* (Nara *et al.* 1961), but different from the one in baker's yeast (Black & Wright, 1955*c*), which can use both nucleotides. The rate of reduction was linear for 5 min. under the conditions described in Fig. 2 and was proportional to the enzyme concentration up to at least 1·8 mg. of protein/ml. The optimum pH is 9·2 in 0·1 M-carbonate–bicarbonate buffer. Over the pH range

Table 1. *β-Aspartokinase activity in extracts*

For the assay, L-aspartic acid (100 μmoles), ATP (20 μmoles), hydroxylamine (200 μmoles), all neutralized to pH 8 with tris, $MgCl_2$ (20 μmoles) and enzyme extract as indicated, in a total volume of 1·0 ml., were incubated at 37° for 1 hr. The hydroxamate formed was estimated by adding 2·0 ml. of ferric chloride reagent (0·65 N-HCl; 0·37 M-$FeCl_3$; 0·31 N-trichloroacetic acid). After removal of protein by centrifuging, the extinction was measured at 540 mμ.

| Enzyme (mg. of protein) | Omissions | Hydroxamate (μmoles) |
|---|---|---|
| 0 | Enzyme | 0·00 |
| 0·50 | L-Aspartic acid | 0·26 |
| 0·50 | ATP | 0·18 |
| 0·75 | $MgCl_2$ | 0·84 |
| 0·50 | None | 1·42 |
| 0·75 | None | 1·86 |
| 1·00 | None | 2·44 |

Table 2. *Effect of amino acids on the activity of β-aspartokinase*

For the assay, L-aspartic acid (10 μmoles), ATP (10 μmoles), hydroxylamine (800 μmoles), all neutralized to pH 8 with tris, $MgCl_2$ (10 μmoles), enzyme (1·43 mg. of protein in Expt. I, 0·94 mg. of protein in Expt. II) and added amino acids, in a total volume of 1·0 ml., were incubated at 37° for 30 min. The hydroxamate formed was estimated by adding 2·0 ml. of ferric chloride reagent. After removal of protein by centrifuging, the extinction was measured at 540 mμ.

| Expt. | Additions | Amount (μmoles) | Hydroxamate (μmole) |
|---|---|---|---|
| I | None | — | 0·88 |
| | L-Threonine | 50 | 0·78 |
| | DL-Homoserine | 100 | 0·65 |
| | L-Serine | 50 | 0·90 |
| | L-Methionine | 50 | 0·80 |
| | L-Lysine | 50 | 0·91 |
| II | None | — | 0·72 |
| | Diaminopimelic acid | 60 | 0·73 |

Table 3. *Effect of amino acids on the activity of aspartate β-semialdehyde dehydrogenase*

For the assay, tris buffer, pH 8·1 (300 μmoles), aspartyl β-phosphate (0·84 μmole), enzyme (0·48 mg. of protein) and added amino acids, in a total volume of 3·0 ml., were incubated in cells with a 1 cm. light-path at 37° in a thermostatically controlled cell carriage. NADPH (0·33 μmole) was added and the extinction at 340 mμ was followed for 5 min.

| Additions | Amount (μmoles) | $NADP^+$ formed (μmole/min.) |
|---|---|---|
| None | — | 0·0131 |
| L-Threonine | 50 | 0·0111 |
| L-Methionine | 25 | 0·0097 |
| L-Methionine | 50 | 0·0092 |
| L-Lysine | 33 | 0·0121 |
| L-Lysine | 66 | 0·0078 |
| DL-Homoserine | 100 | 0·0121 |
| Diaminopimelic acid | 33 | 0·0092 |
| Diaminopimelic acid | 84 | 0·0097 |

7·2–9·6 the nature of the buffer has no significant effect on the activity. The rates of the reaction in the presence of various concentrations of DL-homoserine are shown in Fig. 2. Assuming that the enzyme is specific for the L-isomers of the amino acids the $K_m$ value for L-homoserine has been calculated from these results to be 3·0 mM. From similar experiments the $K_m$ for $NADP^+$ was found to be 0·072 mM. The reverse reaction was also demonstrated by incubating the enzyme with L-aspartate β-semialdehyde and NADPH. NADH is not utilized in this reaction. The reaction rate does not vary greatly between pH 6 and 7 but the optimum in 0·1 M-phosphate lies between pH 6·4 and 6·7. The rate of oxidation of NADPH in the presence of different concentrations of substrate is shown in Fig. 3. From this, and three other similar experiments, a $K_m$ of 0·125 mM for L-aspartate β-semialdehyde was calculated.

The reaction in both directions is strongly inhibited by L-threonine. The rates were measured in the presence of various amounts of substrate and of L-threonine, and the results obtained were plotted in a number of ways (Dawes, 1956), one of which (Dixon, 1953) is shown in Figs. 4 and 5. In this way the inhibition by threonine was shown to be competitive against both homoserine and aspartate β-semialdehyde. With homoserine as substrate $K_i$ for L-threonine was found to be 0·42 mM, and, with L-aspartate β-semialdehyde as substrate, 0·15 mM.

L-Methionine, L-lysine and diaminopimelic acid do not inhibit either of the reactions catalysed by homoserine dehydrogenase. A small but significant inhibition of the reduction of aspartate β-semialdehyde, but not of the oxidation of homoserine, was observed with L-serine. All of these compounds were tested at concentrations between 50 and 100 μmoles/assay.

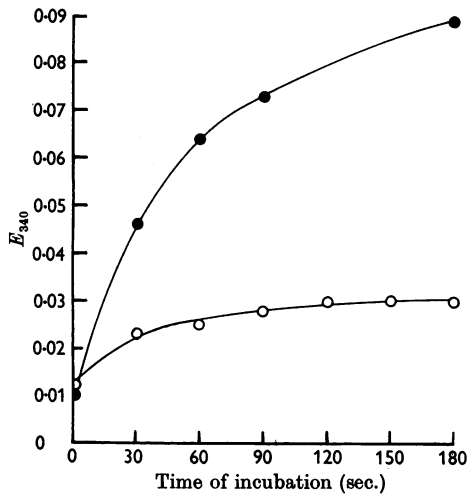


Fig. 1. Oxidation of L-aspartate β-semialdehyde. Tris buffer (300 μmoles), pH 9·0, $NADP^+$ (1 μmole) and L-aspartate β-semialdehyde (1·15 μmoles) in a total volume of 3·0 ml. were incubated at 37° in cells with a 1 cm. light-path in a thermostatically controlled cell carriage. Further additions: none, ○; $K_2HPO_4$ (100 μmoles), ●. Enzyme (0·43 mg. of protein) was added and the extinction at 340 mμ was followed.

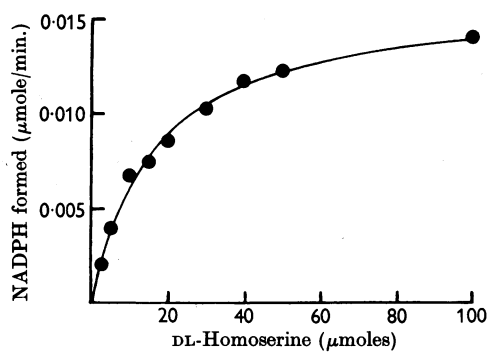


Fig. 2. Reaction rate of homoserine dehydrogenase and homoserine concentration. Carbonate–bicarbonate buffer (300 μmoles), pH 9·2, DL-homoserine (as stated), and $NADP^+$ (0·5 μmole) in a total volume of 3·0 ml. were incubated at 37° in cells with a 1 cm. light-path in a thermostatically controlled cell carriage. Enzyme (2·4 mg. of protein) was added and the extinction at 340 mμ was followed for 5 min.

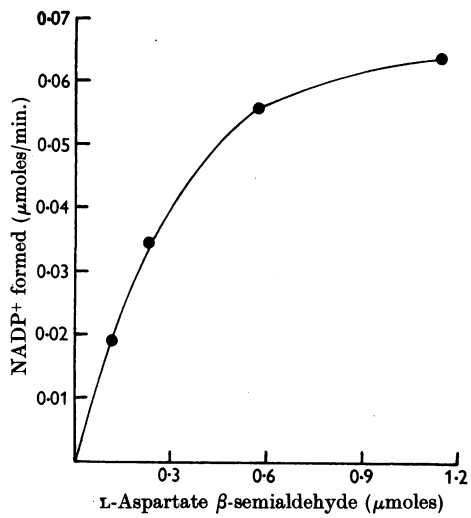


Fig. 3. Reaction rate of homoserine dehydrogenase and L-aspartate β-semialdehyde concentration. Phosphate buffer (300 μmoles), pH 6·7, L-aspartate β-semialdehyde (as stated) and NADPH (0·66 μmole) in a total volume of 3·0 ml. were incubated at 37° in cells with a 1 cm. light-path in a thermostatically controlled cell carriage. Enzyme (0·54 mg. of protein) was added and the extinction at 340 mμ was followed for 2 min.

*Repression studies*

To determine whether the synthesis of any of the enzymes described above is repressed when organisms are grown in the presence of any of the amino acids synthesized from aspartate β-semialdehyde, experiments were carried out in which the organisms were grown in 'medium S' of Lascelles (1956) to which the amino acids had been added. In previous work (Gibson *et al.* 1962*b*) the effects of threonine were investigated in a medium in which only slight growth occurs. In the experiments described here the organisms were grown in 'medium S' from a small inoculum to the end of the log phase. Threonine exerts the same effects on growth and bacteriochlorophyll synthesis under these conditions as it does under those employed previously. Thus at a concentration of 0·5 mM the effects are marginal; at 3 mM both growth and the concentration of bacteriochlorophyll are reduced by 50%, and at 10 mM the reductions are still greater. Addition of methionine (1 mM) or homoserine (5 mM) overcomes these effects. L-Lysine and L-methionine at concentrations up to 5 mM have no effect on growth, whereas diaminopimelic acid increases the rate of growth appreciably. The concentration of bacteriochlorophyll in the organisms is not affected by any of the last three amino acids.

In these experiments the activities of the three enzymes which convert aspartic acid into homoserine were measured (Table 4). The results show clearly that none of the four amino acids has more than a slight effect on the activities of the three enzymes. Similarly there is little or no effect on the activity of the L-threonine dehydrogenase, whose properties are described more fully by Neuberger & Tait (1962*b*).

It was reported by Gibson *et al.* (1962*b*) that the addition of threonine or ethionine to organisms illuminated in a medium containing α-oxoglutarate and glycine ['mixture I' of Lascelles (1956)] inhibits the synthesis of bacteriochlorophyll and stimulates the excretion of porphyrin. In an experiment performed exactly as described by Gibson *et al.* (1962*b*), in which both threonine and ethionine showed the effects mentioned above, lysine and diaminopimelic acid had no effect on the synthesis of porphyrin or bacteriochlorophyll.

*Effect of threonine on the conversion of aspartic acid into methionine in whole organisms*

The results described above and by Gibson *et al.* (1962*b*) suggest that threonine inhibits bacteriochlorophyll synthesis by interfering with the synthesis of methionine. If this is so it should be possible to demonstrate a decrease in the incorporation of radioactivity from [$^{14}$C]aspartic acid into free methionine in organisms illuminated in the presence of threonine.

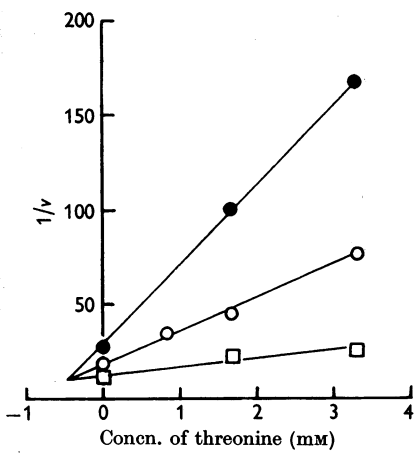


Fig. 4. Inhibition by L-threonine of the oxidation of homoserine by homoserine dehydrogenase. DL-Homoserine (●, 1·66 mM; ○, 5·0 mM; □, 16·6 mM), L-threonine (as indicated), tris buffer (300 μmoles), pH 8·4, and $NADP^+$ (0·5 μmole) in a total volume of 3·0 ml. were incubated at 37° in cells with a 1 cm. light-path in a thermostatically controlled cell carriage. Enzyme (3·25 mg. of protein) was added and the extinction at 340 mμ was followed. The initial reaction velocity, *v*, is expressed as the increase in extinction at 340 mμ in 5 min.

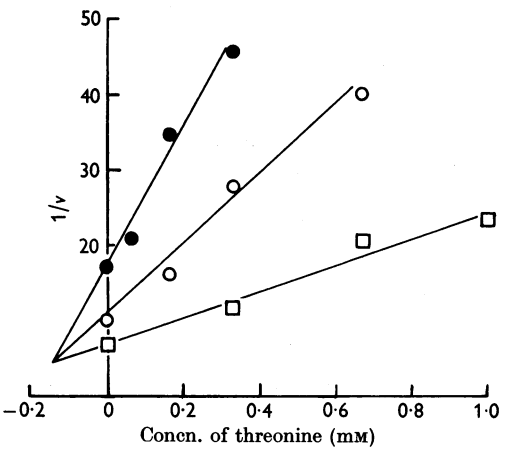


Fig. 5. Inhibition by L-threonine of the reduction of aspartate β-semialdehyde by homoserine dehydrogenase. L-Aspartate β-semialdehyde (●, 38 μM; ○, 76 μM; □, 192 μM), L-threonine (as indicated), phosphate buffer (300 μmoles), pH 6·7, and NADPH (0·33 μmole) in a total volume of 3·0 ml. were incubated at 37° in cells with a 1 cm. light-path in a thermostatically controlled cell carriage. Enzyme (0·36 mg. of protein) was added and the extinction at 340 mμ was followed. The initial reaction velocity, *v*, is expressed as the decrease in extinction at 340 mμ in 2 min.

Organisms were illuminated at 2 mg. dry wt./ml. in 'mixture I' of Lascelles (1956) for 20 hr. at 32–34°. The incubation mixtures (total volume 80 ml.) also contained 0·5 mM-L-[U-$^{14}$C]aspartic acid (0·04 $\mu$C/$\mu$mole), and various amounts of L-threonine. After illumination the organisms were harvested by centrifuging. The organisms were washed with 0·05M-phosphate buffer, pH 7·4, and suspended in about 3 ml. of water, and in some experiments a known amount of L-methionine was added. The organisms were then disrupted in the Hughes (1951) press. The resulting suspension was diluted to 10 ml. with water, and trichloroacetic acid was added to give a final concentration of 5% (w/v). The mixture was centrifuged and the precipitate was re-extracted with 10 ml. of 5% (w/v) trichloroacetic acid. The combined supernatants were extracted twice with 10 ml. of ether to remove the trichloroacetic acid and evaporated to dryness under reduced pressure. The amino acids were extracted from the solid residue with 10 ml. of acetone containing 5% of 6N-HCl (Smith, 1958). This solution was evaporated to dryness and the residue dissolved in a small volume of water. This amino acid solution was applied to Whatman no. 1 or 3 MM paper and 30% (v/v) $H_2O_2$ was added in an amount sufficient to wet the spot. Two-dimensional chromatography was performed with butan-1-ol–acetic acid–water (12:3:5, by vol.) as the first solvent and phenol–water (4:1, w/v) as the second solvent. The amino acids were detected by dipping the papers in 0·05% (w/v) ninhydrin solution in acetone. The spots corresponding to methionine sulphoxide and methionine sulphone were cut out and the amino acids eluted. The eluates were combined, evaporated to dryness, and the residue was dissolved in a small volume of water. Portions were estimated for amino acids by the quantitative ninhydrin method (Spies, 1957) and other portions were counted at infinite thinness in a Nuclear–Chicago gas-flow counter with a Micromil end-window.

The results giving the values for the radioactivity in non-protein methionine after illumination are shown in Table 5. In the presence of threonine there is a large decrease in the incorporation of

Table 4. *Effect of growth in the presence of amino acids on the activities of enzymes*

Organisms were grown in 'medium S' of Lascelles (1956), with the additions stated, as described in the Materials and Methods section. The preparation of the enzyme extract is also described in the Materials and Methods section. $\beta$-Aspartokinase was assayed as described in Table 1 for the experiments with threonine, and as described in Table 2 for the other experiments. Aspartate $\beta$-semialdehyde dehydrogenase was assayed as described in Table 3 and homoserine dehydrogenase as described in Fig. 2 with a concentration of DL-homoserine of 50 $\mu$moles/assay. All activities are expressed as $\mu$moles/mg. of protein/hr.

| Expt. | Additions to growth medium | Concn. (mM) | $\beta$-Aspartokinase | Aspartate $\beta$-semialdehyde dehydrogenase | Homoserine dehydrogenase |
|---|---|---|---|---|---|
| I | None | — | 1·56 | 1·77 | 0·33 |
| | DL-Threonine | 0·5 | 1·45 | 1·77 | 0·32 |
| | DL-Threonine | 3 | 1·32 | 1·74 | 0·29 |
| | DL-Threonine | 10 | 1·47 | 1·50 | 0·30 |
| II | None | — | 1·68 | 1·41 | 0·49 |
| | L-Methionine | 0·5 | 1·34 | 1·74 | 0·41 |
| | L-Methionine | 2 | 1·38 | 1·68 | 0·40 |
| | L-Methionine | 5 | 1·26 | 1·38 | 0·41 |
| III | None | — | 1·19 | 1·59 | 0·51 |
| | L-Lysine | 0·5 | 1·34 | 1·71 | 0·43 |
| | L-Lysine | 3 | 1·24 | 1·32 | 0·43 |
| | L-Lysine | 10 | 1·28 | 1·56 | 0·43 |
| IV | None | — | 1·46 | 0·84 | 0·40 |
| | Diaminopimelic acid | 0·5 | 1·25 | 0·84 | 0·37 |
| | Diaminopimelic acid | 2 | 1·21 | 0·84 | 0·37 |
| | Diaminopimelic acid | 5 | 1·12 | 0·72 | 0·36 |

Table 5. *Conversion of* [$^{14}$C]*aspartic acid into methionine in the presence and absence of threonine*

The conditions are described in the text.

| Expt. | Concn. of threonine present in the medium (mM) | Methionine added after illumination ($\mu$moles) | Methionine recovered | |
|---|---|---|---|---|
| | | | $\mu$moles | Counts/min./ $\mu$mole |
| I | 0 | 1·0 | 0·16 | 1650 |
| | 5 | 1·0 | 0·26 | 980 |
| II | 0 | 5·0 | 2·0 | 130 |
| | 5 | 5·0 | 2·4 | 15 |
| | 10 | 5·0 | 2·3 | 10 |
| III | 0 | 0 | 0·23 | 1900 |
| | 5 | 0 | 0·29 | 500 |

radioactivity into this amino acid. Although other explanations are possible the findings are completely compatible with the hypothesis put forward above.

## DISCUSSION

The work reported here shows that the biosynthesis of homoserine in *Rps. spheroides* follows the pathway previously demonstrated in yeast by Black & Wright (1955*a*, *b*, *c*). The enzymes which convert aspartic acid into homoserine have remarkably similar properties in both organisms, except that in *Rps. spheroides* the homoserine dehydrogenase can utilize only NADP, whereas the corresponding enzyme from yeast utilizes both NADP and NAD and in fact has a greater affinity for the latter. Nara *et al.* (1961) found that the homoserine dehydrogenase from *M. glutamicus* also was specific for NADP. The work reported here is consistent with the postulate that threonine inhibits the production of bacteriochlorophyll in *Rps. spheroides* by interfering with the synthesis of methionine. Indeed it shows that this action is due at least in part to the competitive inhibition of homoserine dehydrogenase by threonine and is not mediated by repression of any of the enzymes involved in the conversion of aspartic acid into homoserine. The inhibition of growth by threonine can also be explained in this way, since growth, like bacteriochlorophyll synthesis (Gibson *et al.* 1962*b*), can be restored by the addition of homoserine or methionine.

Feed-back controls exerted on the enzymes of the biosynthetic pathways for homoserine, threonine and methionine have now been reported in a number of organisms and there are some notable differences. In yeast and *E. coli* the activities of β-aspartokinase (Stadtman *et al.* 1961) and homoserine kinase (Wormser & Pardee, 1958) are decreased by threonine. In *E. coli* the conversion of homoserine into methionine is decreased by methionine (Rowbury & Woods, 1961). In *M. glutamicus* homoserine dehydrogenase is inhibited by threonine and repressed by methionine, as shown by Nara *et al.* (1961), who also produced indirect evidence for an inhibition of aspartate β-semialdehyde dehydrogenase by threonine. In *Rps. spheroides* only homoserine dehydrogenase is inhibited by threonine and none of the enzymes involved in the synthesis of homoserine is repressed by threonine, or inhibited or repressed by methionine. It appears that in most bacteria, but not in yeasts, lysine is synthesized from aspartic acid via aspartate β-semialdehyde and diaminopimelic acid (Gilvarg, 1960, 1962; Yugari & Gilvarg, 1961). It is probable that diaminopimelic acid and lysine are synthesized by *Rps. spheroides* in this way since at least one of the enzymes has been detected in extracts (Gilvarg, 1960). However, with *Rps. spheroides* lysine and diaminopimelic acid have at most a slight inhibitory effect on growth, on production of bacteriochlorophyll or on the synthesis or activity of any of the enzymes which convert aspartic acid into homoserine. This implies that feed-back control, if it exists, of the synthesis of diaminopimelic acid and lysine in these organisms is exerted on the conversion of aspartate β-semialdehyde into lysine rather than on the formation of aspartate β-semialdehyde.

It is impossible to assess completely the physiological significance of the inhibitory effect of threonine reported here. Homoserine dehydrogenase has almost the same affinity for threonine as for its substrate aspartate β-semialdehyde, and this suggests that the intracellular concentration of threonine could influence considerably the synthesis of methionine. On the other hand a relatively large amount of threonine must be added in whole-cell experiments to produce inhibition of growth and of bacteriochlorophyll production, and it is unlikely that such high concentrations of threonine are present inside the cell during growth in media not containing threonine. The physiological importance of this mechanism must therefore remain in doubt.

## SUMMARY

1. The presence of β-aspartokinase, aspartate β-semialdehyde dehydrogenase and homoserine dehydrogenase has been demonstrated in extracts of *Rhodopseudomonas spheroides*. The properties of these enzymes are very similar to those found in yeast by Black & Wright (1955*a*, *b*, *c*), except that homoserine dehydrogenase in *Rps. spheroides* is specific for NADP.

2. Threonine has no significant inhibitory effects on β-aspartokinase or aspartate β-semialdehyde dehydrogenase. However, it competitively inhibits both the forward and reverse reactions catalysed by homoserine dehydrogenase. At 37° and at pH 6·7 the $K_m$ for L-aspartate β-semialdehyde is 0·125 mM and the $K_i$ for L-threonine is 0·15 mM.

3. Threonine does not repress the synthesis of any of the three enzymes which convert aspartic acid into homoserine. Methionine, lysine and diaminopimelic acid neither inhibit nor repress any of these enzymes.

4. When organisms were illuminated in the presence of [$^{14}$C]aspartic acid there was considerable incorporation of radioactivity into the intracellular unbound methionine. This incorporation was markedly decreased when threonine was added to the illumination medium.

5. It is concluded that threonine inhibits the synthesis of methionine in *Rps. spheroides* by

competitively inhibiting homoserine dehydrogenase. This is the most probable explanation for the previous observation that threonine inhibits the biosynthesis of bacteriochlorophyll and leads to an accumulation of porphyrins (Gibson *et al.* 1962*b*).

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# The Formation of Cyclic Acetals during the Acid Hydrolysis of Lysoplasmalogens

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It has been recognized for some years that the fraction of tissue phospholipids stable to mild alkaline and acid treatment contained other phospholipids besides sphingomyelin (Brante, 1949; Dawson, 1954). In brain the amount of this non-sphingomyelin lipid was much reduced if the tissue lipids were initially precipitated with trichloroacetic acid, and the choline:phosphorus ratio of the fraction then approached unity (Dawson, 1954).

In a method developed by Dawson (1960) for the determination of individual phospholipids by successive chemical hydrolyses, the amount of non-sphingomyelin phospholipid in the fraction stable to mild hydrolysis by both alkali and acid was roughly proportional to the amount of plasmalogen present in the original phospholipid mixture. This suggested that such unknown phospholipid material might be formed from the plasmalogens during either the mild alkaline or the acid hydrolyses. The present paper describes the chemical characterization of one of the unknown phospholipids as a cyclic acetal of glycerylphosphorylethanolamine and presents evidence showing that it, together with free glycerylphosphorylethanolamine, is formed from ethanolamine lysoplasmalogen (alkylvinyl ether of glycerylphosphorylethanolamine) during mild acid hydrolysis. It also shows that the formation of the cyclic acetal can be considerably reduced by the addition of $Hg^{2+}$ ions to the hydrolysing reagent. These results have been briefly reported by Davenport & Dawson (1961). Similar conclusions have also been reached by Pietruszko & Gray (1962).