2. The type of fibre produced depends on the method or reagent of precipitation.

3. The possible function of citrate in bone formation is discussed.

REFERENCES

Berendsen, H. J. C. (1962). *J. chem. Phys.* **36**, 3297.
Brown, P. C., Consden, R. & Glynn, L. E. (1958). *Ann. Rheum. Dis.* **17**, 196.
Courts, A. (1959). *Biochem. J.* **73**, 596.
Courts, A. (1961). *Biochem. J.* **81**, 356.
Courts, A. (1962). *Biochem. J.* **83**, 124.
Jackson, D. S. (1953). *Biochem. J.* **54**, 638.
Kelly, M., Little, K. & Courts, A. (1959). *Lancet*, ii, 1125.
Leach, A. A. (1958). *Biochem. J.* **69**, 429.
Little, K. (1959). *Brit. J. appl. Phys.* **10**, 225.
Little, K. (1961). *J. roy. micr. Soc.* **80**, 35.
Little, K., Kelly, M. & Courts, A. (1962). *J. Bone Jt Surg.* **44** B, 503.
Neuman, W. F. & Neuman, M. W. (1958). *The Chemical Dynamics of Bone Mineral*, p. 96. University of Chicago Press.
Partridge, S. M. (1948). *Biochem. J.* **43**, 387.
Reed, R. & Stainsby, G. (1961). *Bull. Leath. Res. Inst. Madras*, **8**, 90.

Biochem. J. (1963) **87**, 386

Microbial Growth on C_1 Compounds

5. ENZYME ACTIVITIES IN EXTRACTS OF *PSEUDOMONAS* AM1*

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Studies on the labelling of intermediates during the short-term incubation of methanol- and formate-grown *Pseudomonas* AM 1 with [^{14}C]methanol and [^{14}C]formate (Large, Peel & Quayle, 1961) showed that the first intermediate to acquire radioactivity from the respective radioactive growth substrates was serine. As a result of studies on the labelling patterns of radioactive compounds isolated from short-term incubations, a scheme was proposed (Large, Peel & Quayle, 1962*a*) for the formation of C_3 and C_4 compounds by a pathway involving (*a*) the hydroxymethylation of glycine to give serine, and (*b*) the carboxylation of a C_3 derivative derived from serine to give a C_4 dicarboxylic acid such as oxaloacetate or malate. Alternative hypothetical pathways were proposed by which the glycine required for this sequence of reactions could be formed. Later work (Large, Peel & Quayle, 1962*b*) showed that the mechanism by which C_4 units could be formed from C_3 units involved an irreversible carboxylation of phosphoenolpyruvate to oxaloacetate catalysed by a phosphoenolpyruvate carboxylase (EC 4.1.1.31). This paper reports an examination of extracts of *Pseudomonas* AM 1 to determine whether other enzymes implicated in the proposed pathway are present, and to compare their activities in the same organism grown on different substrates.

* Part 4: Large, Peel & Quayle (1962*b*).

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MATERIALS AND METHODS

Maintenance and growth of the organism. The maintenance and growth of cultures of *Pseudomonas* AM 1 on methanol were described by Peel & Quayle (1961). For formate-grown cells, 0·1 M-sodium formate was substituted for 0·5% (v/v) methanol as the carbon source.

Preparation of cell-free extracts. Methanol- and succinate-grown cells were disrupted by ultrasonic disintegration and formate-grown cells by crushing the frozen bacteria in a Hughes (1951) press; both procedures have been described by Large *et al.* (1961). The bacterial extracts were then centrifuged at 25 000*g* for 10 min. in the high-speed head of an International refrigerated centrifuge (model PR-2), and the supernatant was used for assay.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks. Sodium [^{14}C]formate was purified as described by Large *et al.* (1961). L-[^{14}C]Serine was used without further purification. 3-Phospho[^{14}C]glycerate was obtained by incubating extracts of formate-grown *Pseudomonas oxalaticus* with sodium hydrogen [^{14}C]carbonate and ribulose 1,5-diphosphate (Quayle & Keech, 1959*b*). D-[^{14}C]Glycerate was obtained from 3-phospho[^{14}C]glycerate by treatment with Polidase-S (Schwarz Laboratories Inc., New York, U.S.A.) according to the procedure of Benson, Bassham & Calvin (1951). The resulting [^{14}C]glycerate was purified by paper chromatography in butan-1-ol–propionic acid–water (47:22:31, by vol.) (Benson *et al.* 1950).

Chromatographic analysis and radioautography. The solvent systems of Kornberg (1958), phenol–90% (w/v) formic acid–water (500:13:167, w/v/v), and of Benson *et al.* (1950), butan-1-ol–propionic acid–water (47:22:31,

by vol.), were used either one-dimensionally or two-dimensionally as described by Large *et al.* (1961). The radioactive compounds were located by radioautography and the radioactivity was assayed directly with a mica end-window Geiger–Müller tube (General Electric Co.; type 2B2), and corrected for background activity. A total of 1000 counts was collected for each spot, giving a standard error of radio assay of $\pm 3\%$.

Identification of labelled compounds. The radioactive compounds on chromatograms were eluted from the paper with water and identified by co-chromatography with authentic compounds and the solvent systems described by Large *et al.* (1961).

Sprays. Carboxylic acids were located on chromatograms by spraying with ethanolic 0·2% (w/v) bromocresol green adjusted to pH 7 with aq. NH_3 solution, or by spraying with 1% (v/v) aniline and 1% (w/v) xylose in 95% (v/v) methanol followed by heating at 105° (Wood, 1958); amino acids were located by spraying with ethanolic 0·2% (w/v) ninhydrin containing 0·1% (v/v) of collidine and heating at 100°.

Protein determinations. Protein was estimated by the Folin–Ciocalteu method as described by Lowry, Rosebrough, Farr & Randall (1951).

Chemicals. DL-Tetrahydrofolic acid (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) (500 mg.) was diluted by the addition of 2 ml. of 0·01 M-mercaptoethanol in 50 mM-phosphate buffer, pH 7·5. The diluted solution was distributed among several Thunberg tubes, each of which was filled with O_2-free N_2, sealed and stored at −15°. These solutions were assayed by diluting 10 µl. to 60 ml. with 0·01 M-mercaptoethanol in 50 mM-phosphate buffer, pH 7·5. The spectrum of this diluted solution was read against 0·01 M-mercaptoethanol as blank on the Cary recording spectrophotometer. From the extinction at 297 mµ, the concentration of the original tetrahydrofolic acid solution was calculated from the molar extinction coefficient of tetrahydrofolate at this wavelength ($2{\cdot}2 \times 10^4$) (Hatefi, Talbert, Osborn & Huennekens, 1960).

Potassium *threo*-D$_S$-(+)-isocitrate was a gift from Dr H. B. Vickery, lithium hydroxypyruvate from Mr D. H. Williamson, tartronic semialdehyde from Dr A. M. Gotto and tetrahydropteroyl-γ-glutamyl-γ-glutamyl-γ-glutamate (tetrahydroteropterin) from Dr J. R. Guest.

Buffers. Phosphate buffers were prepared by mixing appropriate volumes of 1 M-KH_2PO_4 and 1 M-Na_2HPO_4 to the required pH and diluting to a final concentration of 0·5 M. Maleate buffer was prepared by dissolving sodium maleate in water and adjusting to pH 7·5 with HCl. Tris–HCl was prepared by dissolving the solid tris in water and adjusting to the required pH with conc. HCl.

Enzyme assays

All spectrophotometric assays were performed at 22±1°.

Formate–tetrahydrofolate ligase (ADP) (formyltetrahydrofolate synthetase, E.C. 6.3.4.3). This was assayed by incubating 65 µmoles of maleate buffer (pH 7·5), 50 µmoles of KF, 2·5 µmoles of ATP, 0·77 µmole of DL-tetrahydrofolate, 10 µmoles of mercaptoethanol, 40 µmoles of $MgCl_2$ and 10 µmoles (3 µc) of sodium [^{14}C]formate with varying amounts of extract in a total volume of 1·2 ml. for 20 min. at 30°. The reaction was stopped by the addition of 1 ml. of 3·5% (w/v) $HClO_4$. Samples (0·1 ml.) were applied to sand-blasted aluminium planchets (1 in. diameter) and, after drying, these samples (which were less than 0·2 mg./cm.^{2} in thickness) were assayed for radioactivity in a gas-flow counter (model D-47, Nuclear Instrument and Chemical Corp., Chicago, Ill., U.S.A.), which had an efficiency of 15% under the conditions used. Samples of the tracer solution were diluted 100-fold with 30 mM-NaOH; samples (0·1 ml.) were applied to planchets and assayed for radioactivity under the same conditions. Results were calculated as µm-moles of [^{14}C]formate incorporated in 20 min./mg. of protein. A linear relationship was found when these values were plotted against the amount of bacterial protein added, showing that the assay was performed under conditions of limiting enzyme concentration. From the slopes of these graphs, the specific activities of the enzymes were calculated in terms of µmoles of formate incorporated/hr./mg. of protein.

Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5). This was assayed by the procedure of Hatefi, Osborn, Kay & Huennekens (1957). DL-Serine (20 µmoles) or 5 µmoles of formaldehyde were incubated with 1 µmole of $NADP^+$, 0·8 µmole of DL-tetrahydrofolate, and 100 µmoles of maleate or phosphate buffer (pH 7·5) with extract in a total volume of 3 ml. in silica cuvettes (light-path 1 cm.). The increase in extinction at 340 mµ was followed on a Cary recording spectrophotometer against a blank containing all components except formaldehyde or serine.

Serine hydroxymethyltransferase (EC 2.1.2.1). This was assayed as described by Batt, Dickens & Williamson (1960). Extract, 30 µmoles of formaldehyde, 3 µmoles of tetrahydrofolate and 30 µmoles of glycine were incubated with 40 µmoles of potassium phosphate (pH 7·5) and 0·1 µmole of pyridoxal phosphate in a total volume of 1·5 ml. in Thunberg tubes under O_2-free N_2 for 30 min. After heating for 2 min. at 100° to stop the reaction, the serine formed was estimated by oxidative decarboxylation with periodate (Blakley, 1955) as follows. Samples were transferred to micro-manometer vessels (vol. about 5 ml.) containing 500 µmoles of potassium phosphate (pH 5·8) and in the side arm 150 µmoles of sodium metaperiodate. The gas phase was air. After equilibration at 30° the periodate was tipped in and evolution of CO_2 followed for 60 min.

Glycerate dehydrogenase (hydroxypyruvate reductase EC 1.1.1.29). This was assayed by the method of Stafford, Magaldi & Vennesland (1954). Silica cuvettes (3 ml.; light-path 1 cm.) contained 100 µmoles of phosphate buffer (pH 7·5 or 6·5), 0·4 µmole of NADH and extract in a total volume of 3 ml. Lithium hydroxypyruvate (2 µmoles) was added and the decrease in extinction at 340 mµ measured against a blank containing buffer and extract. The rate was corrected for the oxidation of NADH by extract in the absence of substrate.

Glyoxylate reductase (EC 1.1.1.26). This was assayed (Zelitch, 1955) as described above, save that 10 µmoles of sodium glyoxylate were substituted for hydroxypyruvate.

Glyoxylate carboligase. This was assayed according to the method of Kornberg & Gotto (1961), in which the anaerobic thiamine pyrophosphate-dependent evolution of CO_2 from glyoxylate is measured manometrically.

Citrate synthase (EC 4.1.3.7), *malate synthase* (EC 4.1.3.2) *and isocitrate lyase* (EC 4.1.3.1). These were assayed by the methods of Dixon & Kornberg (1959).

Isocitrate dehydrogenase (EC 1.1.1.42). This was assayed by measuring the reduction of $NADP^+$ by potassium *threo*-D_S-(+)-isocitrate spectrophotometrically at 340 mμ (Ochoa, 1948).

Malate dehydrogenase (EC 1.1.1.37). This was assayed by measuring the oxidation of NADH at pH 7 consequent on the addition of oxaloacetic acid (80% pure, C. F. Boehringer und Söhne, Mannheim, Germany) to a silica cuvette (3 ml.) containing phosphate buffer and extract (Ochoa, 1955).

Malate dehydrogenase (decarboxylating) (EC 1.1.1.40). This was assayed as described by Ochoa, Mehler & Kornberg (1948), the reduction of $NADP^+$ by malate being measured spectrophotometrically.

Preparation of acetyl-coenzyme A. Acetyl-coenzyme A was prepared from acetic anhydride and coenzyme A as described by Stadtman (1957).

Removal of folic acid cofactors. Extracts of formate-grown *Pseudomonas* AM 1 were applied to a small column (1 cm. × 3 cm.) of Dowex 1 (Cl^- form; ×8; 100–200 mesh) resin that had previously been washed with water to constant pH. The extract was allowed to run through at 4° until the column was drained (Kisliuk & Woods, 1960).

Boiled cell extracts. A heated extract of *Pseudomonas* AM 1 was prepared by heating 2 g. wet wt. of methanol-grown *Pseudomonas* AM 1 with 3 ml. of water at 95° for 3 min. followed by cooling and centrifuging. A similar heated extract of *Escherichia coli* PA 15, prepared as described by Guest, Helleiner, Cross & Woods (1960), was a gift from Mr F. G. Bull.

Formation of pyruvate from serine. Extracts of *Pseudomonas* AM 1 were incubated with 100 μmoles of tris–HCl (pH 7·5), 40 μmoles of DL-serine and, in addition, in some tubes pyridoxal phosphate (0·4 μmole), GSH (10 μmoles) and AMP (1 μmole) in a total volume of 1 ml. at 30° for 60 min. The reaction was stopped by adding 0·1 ml. of 6N-HCl. After centrifuging, the supernatant was treated with 0·2 ml. of saturated 2,4-dinitrophenylhydrazine in 2N-HCl; the resultant 2,4-dinitrophenylhydrazones were extracted into ethyl acetate and samples were chromatographed in butan-1-ol–ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (140:20:1:39, by vol.) (El Hawary & Thompson, 1953).

Quantitative estimation of the pyruvate formed was performed by stopping the incubation with 0·1 ml. of 50% (w/v) trichloroacetic acid. After centrifuging, 0·5 ml. of the supernatant was taken and to this were added 0·3 ml. of water and 0·33 ml. of saturated 2,4-dinitrophenylhydrazine in 2N-HCl. After 10 min. 1·67 ml. of 2·5N-NaOH was added and the mixture incubated for a further 9 min. The extinction values at 445 and 520 mμ for each tube were then read (Friedemann & Haugen, 1943).

Transamination of serine. Extracts of formate-grown *Pseudomonas* AM 1 were incubated with 40 μmoles of sodium pyruvate or sodium α-oxoglutarate, 40 μmoles of DL-serine or glycine, 1 μmole of pyridoxal phosphate and 100 μmoles of phosphate, pH 7·5, in a total volume of 1 ml. for 60 min. at 30° under a layer of light petroleum to exclude air. The reaction was stopped by heating at 100° for 4 min. After removal of the denatured protein by centrifuging, samples were chromatographed on paper in the solvent systems (*a*), methanol–butanol-1-ol–water (2:2:1, by vol.) to separate serine and alanine, and (*b*), butan-1-ol–acetone–water–diethylamine (10:10:5:2, by vol.), to separate glycine, serine and glutamate (Hardy, Holland & Nayler, 1955). Standards containing exactly 1 μmole of the amino acid to be detected were also chromatographed on the same paper. After drying, the amino acids were located by ninhydrin spray and approximate quantitative assay was performed by elution of the spots in 70% (v/v) ethanol and colorimetric determination at 575 mμ, the chromatographed standards being used for comparison.

RESULTS

Formation of serine from glycine

The following reactions have been shown to operate (Rabinowitz, 1960) in the formation of serine from formate and glycine or formaldehyde and glycine.

(*a*) The tetrahydrofolate- and ATP-dependent activation of formate, catalysed by formate–tetrahydrofolate ligase (ADP) (tetrahydrofolate formylase; Whiteley, Osborn & Huennekens, 1959):

$$\text{Formate} + \text{ATP} + \text{tetrahydrofolate} \rightleftharpoons N^{10}\text{-formyltetrahydrofolate} + \text{ADP} + P_i \quad (1)$$

(*b*) The conversion of N^{10}-formyltetrahydrofolate into $N^{5,10}$-methenyltetrahydrofolate catalysed by methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9):

$$N^{10}\text{-Formyltetrahydrofolate} + H^+ \rightleftharpoons N^{5,10}\text{-methenyltetrahydrofolate} + H_2O \quad (2)$$

(*c*) The NADPH-dependent reduction of $N^{5,10}$-methenyltetrahydrofolate to $N^{5,10}$-methylenetetrahydrofolate, catalysed by methylenetetrahydrofolate dehydrogenase (Hatefi *et al.* 1957):

$$N^{5,10}\text{-Methenyltetrahydrofolate} + \text{NADPH} \rightleftharpoons N^{5,10}\text{-methylenetetrahydrofolate} + \text{NADP}^+ \quad (3)$$

(*d*) The hydroxymethylation of glycine by $N^{5,10}$-methylenetetrahydrofolate to give serine, catalysed by serine hydroxymethyltransferase (Blakley, 1955; Alexander & Greenberg, 1956; Huennekens, Hatefi & Kay, 1957):

$$N^{5,10}\text{-Methylenetetrahydrofolate} + \text{glycine} \rightleftharpoons \text{tetrahydrofolate} + \text{serine} \quad (4)$$

Cell-free extracts of *Pseudomonas* AM 1 have been examined for the presence of these enzymes.

Tetrahydrofolate-dependent fixation of formate by extracts. Specific activities of 0·65, 0·56 and 0·07 μmole of formate incorporated/hr./mg. of protein were obtained respectively for the tetrahydrofolate- and ATP-dependent fixation of [^{14}C]-formate by extracts of methanol-, formate- and succinate-grown cells.

When extracts of formate-grown *Pseudomonas* AM 1 were incubated with 9 μmoles of sodium formate, 5 μmoles of ATP, 0·5 μmole of DL-tetrahydrofolate and 100 μmoles of maleate buffer, pH 7·5, for 10 min., acidified with 0·05 ml. of 6N-

hydrochloric acid, and the spectrum of the test system was examined against a control containing all components except formate, an absorption maximum at 355 mμ was observed. Under these conditions this is characteristic of $N^{5,10}$-methenyltetrahydrofolate (Rabinowitz, 1960), which is formed under acid conditions from N^{10}-formyltetrahydrofolate according to reaction (2).

The nature of the products of the tetrahydrofolate-stimulated incorporation of [^{14}C]formate was also investigated by chromatography. Samples of the perchloric acid-treated reaction mixture (see the Materials and Methods section) were chromatographed one-dimensionally by the ascending technique in M-formic acid (Greenberg, Jaenicke & Silverman, 1955) and the radioactive spots were located by radioautography. The folic acid derivatives were located by their fluorescence in ultraviolet light. Three fluorescent products were obtained: α, R_F 0·39, bluish-white fluorescence, 50–75% of the incorporated radioactivity; β, R_F 0·6, very faint fluorescence, slightly radioactive; γ, R_F 0·77, bright-blue fluorescence, not radioactive. The R_F of α is very close to that quoted (0·37) for $N^{5,10}$-methenyltetrahydrofolate (Greenberg *et al.* 1955). When the spot was eluted with dilute acid, the spectrum of the resulting solution was similar to that of $N^{5,10}$-methenyltetrahydrofolate (Rabinowitz, 1960), having a peak between 320 and 360 mμ. The extinction of this peak agreed with that predicted for $N^{5,10}$-methenyltetrahydrofolate from the amount of [^{14}C]formate incorporated, on the basis of an extinction coefficient at 355 mμ of $2{\cdot}2 \times 10^4$ (Whiteley *et al.* 1959).

Methylenetetrahydrofolate dehydrogenase. Extracts of *Pseudomonas* AM1 were found to catalyse the tetrahydrofolate-dependent reduction of $NADP^+$ by serine or formaldehyde. This is consistent with the presence of the enzymes serine hydroxymethyltransferase and methylenetetrahydrofolate dehydrogenase catalysing the reversal of reactions (4) and (3) respectively. The overall reaction, followed spectrophotometrically as described in the Materials and Methods section, was dependent on extract, tetrahydrofolate and formaldehyde or serine. NAD^+ would not replace $NADP^+$, nor would tetrahydropteroyl triglutamate replace tetrahydrofolate. Formaldehyde reacts non-enzymically with tetrahydrofolate under these conditions to give $N^{5,10}$-methylenetetrahydrofolate (Kisliuk, 1957), so that in this case the methylenetetrahydrofolate-dehydrogenase activity only is measured, whereas with serine both this enzyme and serine hydroxymethyltransferase are involved. The specific activities obtained with methanol-, formate- and succinate-grown cells are given in Table 5. At the conclusion of the reaction, the system was acidified by the addition of 0·05 ml. of 6N-hydrochloric acid, to both test and blank cuvettes, and the effect on the test cuvette was noted by scanning the spectrum between 340 and 370 mμ. The acidification serves both to destroy the dihydronicotinamide nucleotide coenzyme (Hatefi *et al.* 1957) and also to push the equilibrium of reaction (2) to the right. Under these conditions, an absorption maximum at 355 mμ was observed. This corresponds to the presence of $N^{5,10}$-methenyltetrahydrofolate, which is the only tetrahydrofolate derivative to exhibit this particular absorption maximum.

The much greater activity of formaldehyde over serine in this system is interesting. The contrary was observed in mammalian liver extracts by Hatefi *et al.* (1957). Probably it indicates that in the bacterial extracts reaction (4) is rate-limiting.

The methylenetetrahydrofolate dehydrogenase has been purified from methanol-grown cells some 17-fold, and its properties have been studied (P. J. Large, unpublished work).

Serine hydroxymethyltransferase. Extracts of *Pseudomonas* AM1 catalysed the formation of serine from glycine and formaldehyde. The amount of serine formed in 30 min., under the conditions described in the Materials and Methods section, showed a linear relationship with the amount of extract used. Serine formation was dependent on the presence of extract, formaldehyde, glycine and tetrahydrofolate (Table 1). Replacement of the

Table 1. *Formation of serine by extracts of* Pseudomonas AM1

Ultrasonic extracts of methanol-grown cells were incubated anaerobically with 30 μmoles of formaldehyde, 30 μmoles of glycine, 40 μmoles of potassium phosphate (pH 7·5), 0·1 μmole of pyridoxal phosphate, and reduced folic acid derivatives, as indicated, in a total volume of 1·5 ml. After incubation for 60 min. the reaction was stopped by heating and the serine formed estimated manometrically by periodate decarboxylation as described in the Materials and Methods section.

Expt. no.	System	Serine formed (μmoles/hr./mg. of protein; corrected for the boiled control)
1	Complete (3 μmoles of DL-tetrahydrofolate)	1·39
	No tetrahydrofolate	0·01
	Complete, but extract boiled	0
2	Complete (0·3 μmole of DL-tetrahydrofolate)	0·73
	Complete, but extract boiled	0
3	Complete (0·3 μmole of DL-tetrahydropteroyltriglutamate)	0·95
	No glycine	0
	No formaldehyde	0·016
	No tetrahydropteroyltriglutamate	0·031
	Complete, but extract boiled	0

latter by tetrahydropteroyltriglutamate did not significantly stimulate the rate. When the products of the reaction were chromatographed on paper in butanol–acetone–water–diethylamine, serine was found only in the complete system; the controls contained only glycine.

Comparative figures for the specific activity of serine hydroxymethyltransferase in cells grown on methanol, formate and succinate, under the assay conditions described in the Materials and Methods section, are given in Table 5.

Table 2. *Formation of serine from* [^{14}C]*formate by extracts of formate-grown* Pseudomonas *AM* 1

The complete system contained, in 1·7 ml.: 10 μmoles of glycine, 5 μmoles of ATP, 20 μmoles of $MgCl_2$, 2·5 μmoles of NADPH, 5 μmoles (10 μc) of sodium [^{14}C]formate, 50 μmoles of tris–HCl (pH 7·5), 0·1 μmole of pyridoxal phosphate, 0·2 ml. of an extract of heated cells of *Pseudomonas* AM 1 (see the Materials and Methods section) and extract of formate-grown *Pseudomonas* AM 1. Incubation was for 30 min. in air at 30°. The reaction was stopped by addition of 3 ml. of boiling ethanol; the resulting solutions were centrifuged and the supernatants concentrated at 60° under N_2 and chromatographed in butanol–propionic acid–water (see the Materials and Methods section). Serine and glyceric acid, the only radioactive products, were identified by co-chromatography.

Omission	Total products formed (counts/min./mg. of protein)	
	Serine	Glycerate
None	6450	440
ATP	1420	170
Boiled cell extract	6780	470
NADPH	150	0
Glycine	1040	240
None (NADPH replaced by NADH)	940	30
None (extract boiled)	0	0

Formation of serine from [^{14}C]*formate.* It has been shown that all the enzymes necessary for formation of serine from formate and glycine are present in extracts of *Pseudomonas* AM 1. When these substrates were allowed to interact in the presence of pyridoxal phosphate, ATP, NADPH and bacterial extract, serine synthesis took place (Table 2). The reaction was dependent on ATP, glycine and NADPH; the last cofactor could not be replaced by NADH. Addition of tetrahydrofolate or heated cell extract did not stimulate the reaction. In addition to serine, one other radioactive product, which contained less than 12 % of the incorporated radioactivity, was detected and was identified by co-chromatography as glyceric acid. The formation of this compound is discussed below.

The fact that neither tetrahydrofolate nor heated cell extract stimulated the reaction shows that extracts of *Pseudomonas* AM 1 must contain appreciable amounts of a natural folic acid cofactor. Treatment with Dowex 1 ion-exchange resin has been widely used (Rabinowitz & Pricer, 1956; Kisliuk & Woods, 1960) to remove folic acid cofactors from bacterial extracts. Accordingly, the effect of treating extracts of formate-grown *Pseudomonas* AM 1 with resin was examined.

Formation of [^{14}C]*serine by resin-treated extracts.* When Dowex 1-treated extract was incubated with [^{14}C]formate, there was a 94 % inhibition of the serine-forming activity of untreated extracts (Table 3). The ability of various reduced folic acid coenzymes to restore this activity was then investi-

Table 3. *Formation of serine from* [^{14}C]*formate by Dowex* 1*-treated extracts*

The complete system contained, in 2 ml.: 10 μmoles of glycine, 5 μmoles of ATP, 20 μmoles of $MgCl_2$, 2·5 μmoles of NADPH, 5 μmoles (10 μc) of sodium [^{14}C]formate, 50 μmoles of tris–HCl (pH 7·5), 0·1 μmole of pyridoxal phosphate, 10 μmoles of mercaptoethanol, Dowex 1-treated extract or untreated extract, with additions shown below. Incubation was for 30 min. at 30° in Thunberg tubes under N_2. The reaction was stopped with 3 ml. of boiling ethanol, the resulting solutions were centrifuged and the supernatants concentrated at 60° under N_2 and samples chromatographed in phenol–formic acid–water (see the Materials and Methods section). The only products detected were serine (identified by co-chromatography) and formylated folic derivatives.

Bacterial extract used	Additions	Products formed (counts/min./mg. of protein)	
		Serine	Folic derivatives
Untreated	None	4320	940
Dowex 1-treated	None	260	0
	Tetrahydrofolate (1·1 μmoles)	1070	18 250
	Tetrahydropteroyltriglutamate (0·3 μmole)	7830	13 420
	Tetrahydropteroyltriglutamate (0·1 μmole)	2280	3530
	Tetrahydropteroyltriglutamate (0·1 μmole) + tetrahydrofolate (0·5 μmole)	1330	8670
	Boiled cell extract of *Pseudomonas* AM 1 (0·2 ml.)	760	0
	Boiled cell extract of *Escherichia coli* PA 15 (0·2 ml.)	7370	0
Untreated, boiled	None	0	0

gated. It was found that tetrahydrofolate restored serine-forming activity very poorly: there was a considerable incorporation of label from [^{14}C]formate into formylated folic derivatives, but very little into serine. Tetrahydrofolate (0·55 mM) increased the activity of resin-treated extract four-fold, whereas 16-fold stimulation was required to bring the activity up to that of the untreated extract. In contrast, tetrahydropteroyltriglutamate was much more effective in promoting serine synthesis at the lower concentration of 0·15 mM. There was a 30-fold stimulation over the activity of the unsupplemented resin-treated extract. The effect of natural folic acid cofactors was also tested. A heated extract of *Pseudomonas* AM1 (prepared as described in the Materials and Methods section) was a poor source of cofactors, being less effective than tetrahydrofolate in restoring the endogenous amount of serine formation. A similarly prepared extract of *E. coli* PA15, which has been shown to be a good source of the natural folic acid cofactor for the biosynthesis of methionine methyl groups from serine (Guest *et al.* 1960), was almost as effective as 0·15 mM-tetrahydropteroyltriglutamate in reactivating the Dowex-treated extract, and in this case there was no accumulation of radioactivity in folic acid derivatives.

It is thus clear that tetrahydrofolate is much less effective as a cofactor for serine formation from glycine and formate than the untreated extract, tetrahydropteroyltriglutamate or other natural cofactors such as those contained in boiled extracts of *E. coli* PA15. Tetrahydrofolate inhibited the cofactor activity of tetrahydropteroyltriglutamate (Table 3). Tetrahydrofolate (0·25 mM) produced 42% inhibition of the serine-forming activity of 0·05 M-tetrahydropteroyltriglutamate. This may be compared with the observation (Kisliuk & Woods, 1960; Jones, Guest & Woods, 1961) that tetrahydrofolate is a competitive inhibitor of the formation of the methionine methyl group when tetrahydropteroyltriglutamate or a boiled cell extract of *E. coli* PA15 is the source of the cofactors.

Conversion of serine into C_3 *and* C_4 *compounds*

Formation of pyruvate from serine. When extracts of *Pseudomonas* AM1 were incubated with DL-serine, the 2,4-dinitrophenylhydrazone of pyruvic acid could be isolated from the reaction mixture (see the Materials and Methods section). However, quantitative estimations of the rate of conversion of serine into pyruvate by crude extracts showed that the conversion was very slow (57 μm-moles of pyruvate formed/hr./mg. of protein). The pH optimum for pyruvate formation was 8·0.

Transamination of serine. Extracts of *Pseudomonas* AM1 catalysed a transamination between serine and α-oxoglutarate or pyruvate. Under the conditions used (see the Materials and Methods section) approximately 1·03 μmoles of glutamate were formed/hr./mg. of protein from α-oxoglutarate and 1·30 μmoles of alanine/hr./mg. of protein from pyruvate, in the presence of serine. This indicates that extracts can bring about the reaction:

$$HO\cdot CH_2\cdot CH(NH_2)\cdot CO_2H + R\cdot CO\cdot CO_2H \rightleftharpoons HO\cdot CH_2\cdot CO\cdot CO_2H + R\cdot CH(NH_2)\cdot CO_2H \quad (5)$$

where R = $HO_2C\cdot CH_2\cdot CH_2$ or CH_3.

The extracts could catalyse a transamination between glycine and α-oxoglutarate, giving glutamate and presumably glyoxylate. The rate of formation of glutamate was found to be 1·37 μmoles/hr./mg. of protein.

Enzymic reduction of hydroxypyruvate and glyoxylate. Hydroxypyruvate reductase (glycerate dehydrogenase) (Stafford *et al.* 1954) and glyoxylate reductase (Zelitch, 1955) were tested for spectrophotometrically as described in the Materials and Methods section. Extracts of *Pesudomonas* AM1 were found to catalyse the reduction of both hydroxypyruvate and glyoxylate by NADH:

$$HO\cdot CH_2\cdot CO\cdot CO_2H + NADH + H^+ \rightleftharpoons HO\cdot CH_2\cdot CH(OH)\cdot CO_2H + NAD^+ \quad (6)$$

$$HCO\cdot CO_2H + NADH + H^+ \rightleftharpoons HO\cdot CH_2\cdot CO_2H + NAD^+ \quad (7)$$

Glycerate was characterized as the product of hydroxypyruvate reduction by cochromatography with authentic [^{14}C]glycerate.

The specific activity with respect to these substrates in extracts of formate-grown cells was 41·6 μmoles of NADH oxidized/hr./mg. of protein for hydroxypyruvate and 4·6 μmoles/hr./mg. of protein for glyoxylate, both at pH 7·5. The values for methanol- and succinate-grown cells are given in Table 5.

To see whether both hydroxypyruvate and glyoxylate reduction is carried out by the same enzyme, as appears probable in plant tissue (Stafford *et al.* 1954; Zelitch, 1955; Holzer & Holldorf, 1957), an extract of methanol-grown cells was fractionated by precipitation with ammonium sulphate in an attempt to separate the two activities. It was found that both activities were present in the protein fraction precipitated between 60 and 80% saturation of ammonium sulphate. The purification was the same for each (2·1-fold) and the ratio, specific activity with hydroxypyruvate/specific activity with glyoxylate, was the same, namely 4·3, both for the crude and the ammonium sulphate fraction. These data indicate that in *Pseudomonas* AM1 extracts the two activities are due to the same enzyme. Since the enzyme shows much higher activity with hydroxypyruvate than with glyoxylate it is probable that the former substrate is the natural one.

The pH optimum in crude extract for the NADH-linked reduction of hydroxypyruvate (6) was found to be 4·5 (Fig. 1). A low pH optimum is to be expected in view of the proton requirement for the reaction.

The specificity of the reaction for substrate and nicotinamide nucleotide coenzymes was also examined. NADPH was much less effective than NADH, the specific activity at pH 6·5 for the reduction of hydroxypyruvate being respectively 63·9 μmoles/hr./mg. of protein for NADH and 24 μmoles/hr./mg. of protein with NADPH. With glyoxylate, the figures were 3·45 μmoles/hr./mg. of protein with NADH, and with NADPH there was no detectable activity. Extracts of *Pseudomonas* AM 1 were found to reduce tartronic semialdehyde, but much less readily than hydroxypyruvate (Fig. 2): the tartronic semialdehyde-reductase activity was 4·6 % of the hydroxypyruvate-reductase activity with NADH, at equimolar substrate concentration. This may be contrasted with tartronic semialdehyde reductase from *Pseudomonas ovalis* Chester, which reduces both tartronic semialdehyde and hydroxypyruvate, but the former much faster than the latter (Gotto & Kornberg, 1961).

Conversion of L-*serine into glycerate.* The ability of extracts of formate-grown *Pseudomonas* AM 1 to catalyse the formation of glycerate from L-serine has been demonstrated (Table 4). The amount of [^{14}C]glycerate formed was increased 13-fold in the presence of sodium pyruvate and NADH. This is consistent with the glycerate having been formed by coupling of reactions (5) and (6). These results explain the appearance of [^{14}C]glycerate together with [^{14}C]serine in the experiments on the incorporation of [^{14}C]formate into serine (Table 2).

Conversion of [^{14}C]*glycerate into phosphoglycerate.* Extracts of *Pseudomonas* AM 1 were shown to catalyse the phosphorylation of glycerate to 3-phosphoglycerate:

$$\text{Glycerate} + \text{ATP} \rightarrow \text{3-phosphoglycerate} + \text{ADP} \quad (8)$$

by incubation with ATP and [^{14}C]glycerate. The complete reaction system contained, in 1 ml.: 50 μmoles of tris hydrochloride (pH 7·5), 4 μmoles of magnesium chloride, 5 μmoles of GSH, 10 μmoles of potassium fluoride, 5 μmoles of ATP, sodium D-[^{14}C]glycerate (1000 counts/min.) and extract (containing 4 mg. of bacterial protein). Incubation was

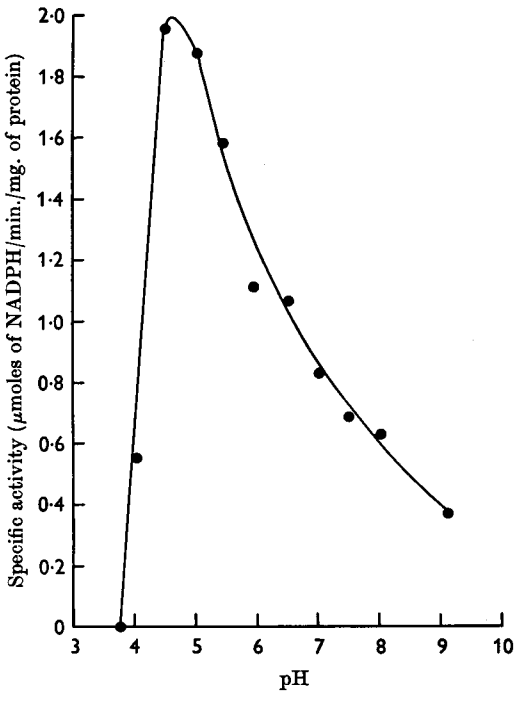


Fig. 1. pH optimum for the reduction of hydroxypyruvate by extracts of methanol-grown *Pseudomonas* AM 1. Silica cuvettes (light-path, 1 cm.) contained: 100 μmoles of buffer, 0·4 μmole of NADH (in test cuvette only) and extract, in a total volume of 3 ml. Rate of decrease in extinction at 340 mμ on addition of 2 μmoles of lithium hydroxypyruvate to the test cuvette was followed on the Cary recording spectrophotometer. Sodium acetate–acetic acid buffers were used below pH 5·5, sodium phosphate–potassium phosphate buffers above pH 5·5.

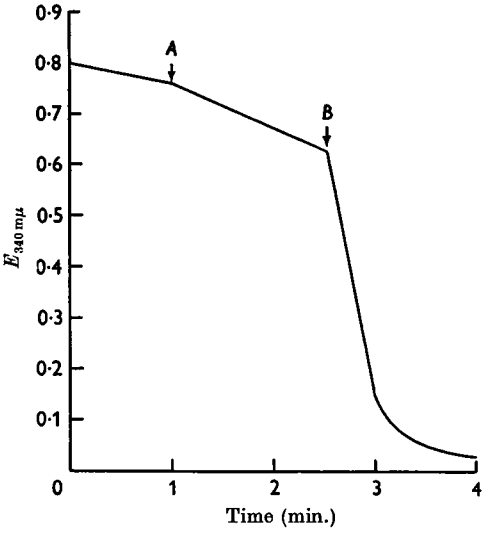


Fig. 2. Substrate specificity of hydroxypyruvate reduction by crude extracts. Silica cuvettes (light-path, 1 cm.) contained 100 μmoles of phosphate buffer (pH 6·5), 0·2 μmole of NADH (in test cuvette only) and extract, in a total volume of 1 ml. At *A*, 2 μmoles of tartronic semialdehyde were added to the test cuvette; at *B*, 2 μmoles of lithium hydroxypyruvate. Change of extinction at 340 mμ was followed in a Cary recording spectrophotometer.

Table 4. *Conversion of* [^{14}C]*serine into glycerate by extracts of formate-grown* Pseudomonas *AM* 1

The system contained, in 1·3 ml.: extract of formate-grown *Pseudomonas* AM1 (containing 1·2 mg. of protein), 60 μmoles of phosphate buffer (pH 7·5), 0·1 μmole of pyridoxal phosphate and 0·34 μmole (1 μc) of L-[^{14}C]serine with the additions shown below. Incubation was for 30 min. at 30° under N_2, and the reaction stopped by addition of 3 ml. of boiling 95% (v/v) ethanol. The deproteinized solutions were concentrated at 60° and samples chromatographed two-dimensionally (see the Materials and Methods section). Products were identified by co-chromatography.

Tube no.	Addition	Radioactive compounds (10^{-3} counts/min./mg. of protein)	
		Serine	Glycerate
1	None	53·9	0·23
2	Sodium pyruvate (4 μmoles)	40·4	0·55
3	Sodium pyruvate (4 μmoles) + NADH (3 μmoles)	40·16	7·02
4	None; boiled extract	50·9	0

Table 5. *Enzymic activities of extracts of methanol-, formate- and succinate-grown* Pseudomonas *AM* 1

Assay methods are reported in the Materials and Methods section.

Enzyme	Growth substrate: Specific activity (μmoles of product formed/hr./mg. of protein)			Ratio of specific activities	
	Methanol	Formate	Succinate	Methanol-grown / Succinate-grown	Formate-grown / Succinate-grown
Tetrahydrofolate formylase	0·65	0·56	0·07	9·3	8·0
Methylenetetrahydrofolate dehydrogenase (formaldehyde as substrate)	24·2	22·0	13·1	1·85	1·68
Methylenetetrahydrofolate dehydrogenase (serine as substrate)	0·38	0·54	0·28	1·36	1·93
Serine hydroxymethyltransferase	2·56	1·78	0·82	3·12	2·17
Glycerate dehydrogenase	94·2	41·6	21·0	4·5	1·98
Glyoxylate reductase	4·36	4·6	1·97	2·22	2·33
Glyoxylate carboligase	0	0	0	—	—
Malate synthase	1·17	1·74	2·5	0·46	0·69
Isocitrate lyase	0	0	0	—	—
Citrate synthase	4·05	2·25	7·01	0·58	0·32
Isocitrate dehydrogenase	—	10·4	20·0	—	0·52
Malate dehydrogenase	54·8	44·2	75·1	0·73	0·59
Malate dehydrogenase (decarboxylating)	0	0	0	—	—

for 60 min. at 30° and was terminated by adding 2 ml. of boiling ethanol. The deproteinized solutions were chromatographed two-dimensionally and the products located by radioautography. The identity of 3-phosphoglycerate was established by co-chromatography with an authentic sample in methanol–90% (w/v) formic acid–water (16:3:1, by vol.) (Bandurski & Axelrod, 1951). In the presence of ATP, 540 counts/min. of 3-phospho-[^{14}C]glycerate were formed and 15 counts/min. of [^{14}C]glycerate remained unchanged. When ATP was left out of the incubation mixture, no radioactive 3-phospho[^{14}C]glycerate could be detected and 935 counts/min. of unchanged [^{14}C]glycerate were recovered.

Enzymes of the tricarboxylic acid cycle

The presence of malate dehydrogenase, isocitrate dehydrogenase and citrate synthase was demonstrated in extracts of *Pseudomonas* AM1. The specific activities of these enzymes in extracts of cells grown on methanol, formate and succinate are given in Table 5. An enzyme catalysing the carboxylation of phosphoenolpyruvate to oxaloacetate has been found in methanol-grown *Pseudomonas* AM1 (Large *et al.* 1962*b*). No malate dehydrogenase (decarboxylating) activity could be found in cell-free extracts.

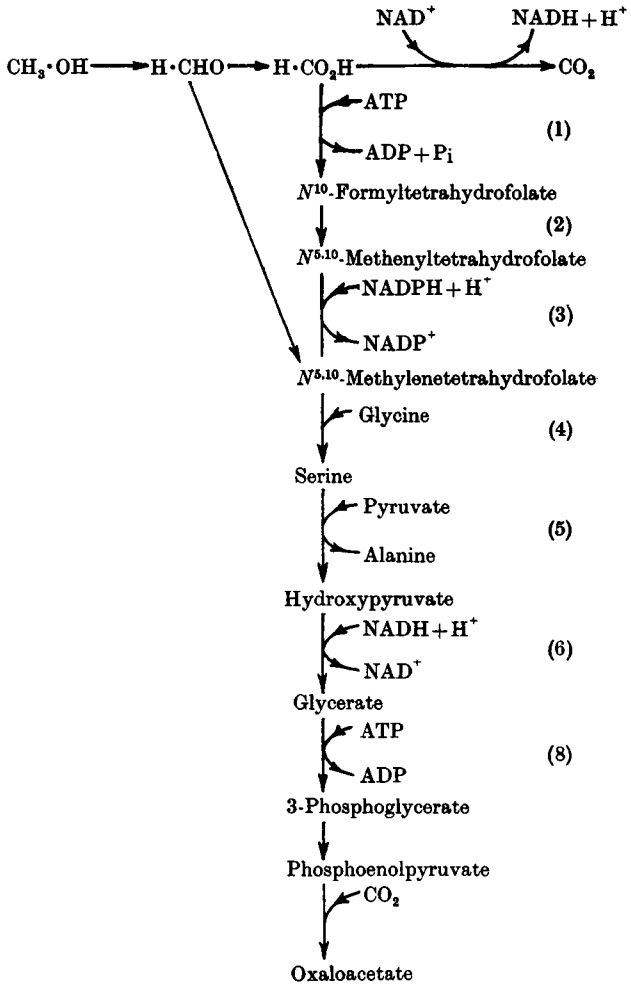
Enzymes of the glyoxylate cycle

Isocitrate lyase and malate synthase were assayed by the procedure of Dixon & Kornberg (1959). Isocitrate lyase was not found in extracts of cells grown on any substrate. Malate synthase was present in all extracts (Table 5).

DISCUSSION

The presence of enzymes that, acting in concert, can convert glycine and formate or formaldehyde into 3-phosphoglycerate has been demonstrated in

extracts of *Pseudomonas* AM1. The reaction sequence is set out in Scheme 1. In this sequence, hydroxypyruvate is synthesized from serine by transamination with pyruvate. An alternative reaction in which serine is deaminated by serine dehydratase (Yanofsky & Reissig, 1953; Benziman, Sagers & Gunsalus, 1960) proceeded only slowly in extracts; rates of transamination with α-oxoglutarate or pyruvate were respectively increased 18-fold and 23-fold. Hence transamination with pyruvate is considered to be the most likely reaction, although the role of the alternative reactions *in vivo* may well be a significant one also. Transamination of serine with pyruvate has been demonstrated previously in dog liver (Sallach, 1956) and human liver (Hedrick & Sallach, 1960). The possibility that conversion of serine into phosphoglycerate might occur via a reversal of the scheme found in *Salmonella typhimurium* LT-2 (Umbarger & Umbarger, 1962), in which phosphoglycerate is converted into serine via phosphohydroxypyruvate and phosphoserine, has not yet been examined.



Scheme 1. Reactions leading to the formation of 3-phosphoglycerate from C_1 compounds and glycine. Numerals in parentheses refer to equations in the text.

The sequence of reactions demonstrated in extracts of *Pseudomonas* AM1 represents a scheme for carbohydrate synthesis from glycine and C_1 units. Isotope experiments with whole cells support the suggestion that such a scheme plays a major role in the microbial synthesis of cell constituents from C_1 growth substrates (Large *et al.* 1961, 1962*a*). If this is indeed the case, it may be predicted that when the organism is grown on a substrate such as succinate, the metabolism of which is not suspected to involve any reactions other than those of the tricarboxylic acid cycle and its ancillary processes, the specific activity of the enzymes involved in the scheme should be lower than when the organism is grown on C_1 compounds. In growth on succinate the enzymes in question would be involved only in the synthesis of particular cell constituents, e.g. serine and glycine, whereas in growth on C_1 compounds it is proposed that the bulk of the cell carbon passes through the sequence of reactions. That this prediction is borne out may be seen in Table 5, where enzyme activities in methanol- and formate-grown cells are compared with those in succinate-grown cells. Of the enzymes tested, only the first five had specific activities greater in formate- and methanol- than in succinate-grown cells. These five enzymes are precisely the ones suggested in Scheme 1 as playing a key role in the formation of C_3 compounds from methanol or formate. Their increased activity in cells grown on C_1 compounds is thus evidence in favour of their special importance in this type of metabolism.

The increased activity of tricarboxylic acid-cycle enzymes in the succinate-grown organism is consistent with this cycle furnishing both energy and carbon skeletons for cellular constituents during growth on this substrate. In contrast, growth on methanol or formate, where these substrates are probably oxidized directly by specific oxidative enzymes, results in a lower activity of the tricarboxylic acid-cycle enzymes as the cycle here serves only as a source of cellular constituents (Krebs, Gurin & Eggleston, 1952).

The suggestion (Large *et al.* 1961, 1962*a*) that serine hydroxymethyltransferase plays a key role in the bacterial formation of C_3 compounds is not new. Kaneda & Roxburgh (1959) suggested that the enzyme is the principal mechanism for the assimilation of carbon by *Pseudomonas* PRL-W4 grown on methanol and the same enzyme has been shown to play a key role both in the formation of C_3 compounds and in energy-generation in the anaerobe

Diplococcus glycinophilus grown on glycine (Sagers & Gunsalus, 1961; Klein & Sagers, 1962). The absence of the enzyme glyoxylate carboligase (Table 5) indicates that other mechanisms for the conversion of C_2 into C_3 units observed in organisms grown on the C_2 substrates, glycollate, glycine and oxalate (Kornberg & Elsden, 1961), and on allantoin (Valentine, Bojanowski, Gaudy & Wolfe, 1962), are not operative in this organism.

The suggested key role of tetrahydrofolate formylase in the assimilation of carbon by formate-grown *Pseudomonas* AM1 may be contrasted with the other known case where it plays a central function in the economy of a cell, namely providing the sole source of ATP by acting in reverse during purine fermentation by *Clostridium cylindrosporum* (Rabinowitz & Pricer, 1956). It may be noted that in growth on formate by *P. oxalaticus*, which involves a chemoautotrophic metabolism, the enzyme plays no central role (Quayle & Keech, 1959*a*, *b*).

The mechanism by which methanol enters the proposed Scheme 1 is not known. It seems likely that it is first oxidized to formaldehyde, which could then combine either enzymically or non-enzymically with a tetrahydrofolate coenzyme giving an active C_1 unit at the right oxidation state for serine synthesis.

This work has not elucidated the mechanism of the synthesis of glycine, or its precursors, from C_1 compounds. The absence of isocitrate lyase is evidence that formation of glyoxylate by cleavage of isocitrate is not involved.

SUMMARY

1. The following enzymes have been found in extracts of methanol-, formate- and succinate-grown *Pseudomonas* AM1 and their activities measured: tetrahydrofolate formylase, methylene-tetrahydrofolate dehydrogenase, serine hydroxymethyltransferase, glycerate dehydrogenase, glyoxylate reductase, malate synthase, citrate synthase, isocitrate dehydrogenase and malate dehydrogenase. No glyoxylate carboligase, isocitrate lyase or malate dehydrogenase (decarboxylating) could be detected in the organism grown on any of the substrates.

2. Transamination of serine with α-oxoglutarate and pyruvate has been observed in extracts of *Pseudomonas* AM1.

3. Cell-free extracts of *Pseudomonas* AM1, fortified with suitable cofactors, catalyse the conversion of formate and glycine to give serine and glycerate as major products. In the presence of adenosine triphosphate the extracts catalyse the phosphorylation of glycerate to phosphoglycerate.

4. It is proposed that assimilation of carbon by *Pseudomonas* AM1 growing on C_1 growth substrates proceeds via the reactions shown in Scheme 1. This contention is supported by the finding that the activities of those enzymes directly implicated in the scheme are higher in methanol- or formate-grown cells than in the succinate-grown cells. The mechanism of synthesis of glycine from C_1 compounds, necessary for operation of this Scheme of reactions, is not known.

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REFERENCES

Alexander, N. & Greenberg, D. M. (1956). *J. biol. Chem.* **220**, 775.
Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.
Batt, R. D., Dickens, F. & Williamson, D. H. (1960). *Biochim. biophys. Acta*, **45**, 571.
Benson, A. A., Bassham, J. A. & Calvin, M. (1951). *J. Amer. chem. Soc.* **73**, 2970.
Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W. (1950). *J. Amer. chem. Soc.* **72**, 1710.
Benziman, M., Sagers, R. D. & Gunsalus, I. C. (1960). *J. Bact.* **79**, 474.
Blakley, R. L. (1955). *Biochem. J.* **61**, 315.
Dixon, G. H. & Kornberg, H. L. (1959). *Biochem. J.* **72**, 3P.
El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
Friedemann, F. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Gotto, A. M. & Kornberg, H. L. (1961). *Biochem. J.* **81**, 273.
Greenberg, G. R., Jaenicke, L. & Silverman, M. (1955). *Biochim. biophys. Acta*, **17**, 589.
Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). *Biochem. J.* **76**, 396.
Hardy, T. L., Holland, D. O. & Nayler, J. H. C. (1955). *Analyt. Chem.* **27**, 971.
Hatefi, Y., Osborn, M. J., Kay, L. D. & Huennekens, F. M. (1957). *J. biol. Chem.* **227**, 637.
Hatefi, Y., Talbert, P. T., Osborn, M. J. & Huennekens, F. M. (1960). *Biochem. Prep.* **7**, 89.
Hedrick, J. L. & Sallach, H. J. (1960). *Biochim. biophys. Acta*, **41**, 531.
Holzer, H. & Holldorf, A. (1957). *Biochem. Z.* **329**, 292.
Huennekens, F. M., Hatefi, Y. & Kay, L. D. (1957). *J. biol. Chem.* **224**, 435.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.

Jones, K. M., Guest, J. R. & Woods, D. D. (1961). *Biochem. J.* **79**, 566.
Kaneda, T. & Roxburgh, J. M. (1959). *Biochim. biophys. Acta*, **33**, 106.
Kisliuk, R. L. (1957). *J. biol. Chem.* **227**, 805.
Kisliuk, R. L. & Woods, D. D. (1960). *Biochem. J.* **75**, 467.
Klein, S. M. & Sagers, R. D. (1962). *J. Bact.* **83**, 121.
Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
Kornberg, H. L. & Elsden, S. R. (1961). *Advanc. Enzymol.* **23**, 401.
Kornberg, H. L. & Gotto, A. M. (1961). *Biochem. J.* **78**, 69.
Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.
Large, P. J., Peel, D. & Quayle, J. R. (1961). *Biochem. J.* **81**, 470.
Large, P. J., Peel, D. & Quayle, J. R. (1962*a*). *Biochem. J.* **82**, 483.
Large, P. J., Peel, D. & Quayle, J. R. (1962*b*). *Biochem. J.* **85**, 243.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Ochoa, S. (1948). *J. biol. Chem.* **174**, 133.
Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 735. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Ochoa, S., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.
Peel, D. & Quayle, J. R. (1961). *Biochem. J.* **81**, 465.
Quayle, J. R. & Keech, D. B. (1959*a*). *Biochem. J.* **72**, 623.
Quayle, J. R. & Keech, D. B. (1959*b*). *Biochem. J.* **72**, 631.
Rabinowitz, J. C. (1960). In *The Enzymes*, vol. 2, p. 185. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Rabinowitz, J. C. & Pricer, W. E. (1956). *J. Amer. chem. Soc.* **78**, 1513.
Sagers, R. D. & Gunsalus, I. C. (1961). *J. Bact.* **81**, 541.
Sallach, H. J. (1956). *J. biol. Chem.* **223**, 1101.
Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. 3, p. 931. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Stafford, H. A., Magaldi, A. & Vennesland, B. (1954). *J. biol. Chem.* **207**, 621.
Umbarger, H. E. & Umbarger, M. A. (1962). *Biochim. biophys. Acta*, **62**, 193.
Valentine, R. C., Bojanowski, R., Gaudy, E. & Wolfe, R. S. (1962). *J. biol. Chem.* **237**, 2271.
Whiteley, H. R., Osborn, M. J. & Huennekens, F. M. (1959). *J. biol. Chem.* **234**, 1538.
Wood, T. (1958). In *Chromatographic Techniques*, p. 207. Ed. by Smith, I. London: William Heinemann Ltd.
Yanofsky, C. & Reissig, J. L. (1953). *J. biol. Chem.* **202**, 567.
Zelitch, I. (1955). *J. biol. Chem.* **216**, 553.

Biochem. J. (1963) **87**, 396

Changes in the Mucopolysaccharide Composition of Bovine Heart Valves with Age

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The morphology of human heart valves changes throughout the average lifetime. Some of the changes, recognizable in all four types of heart valve in the absence of disease, are associated with the aging process (Lev & McMillan, 1961). Preparatory to studies on human valves, we have investigated some of the age-dependent biochemical properties of bovine heart valves. The biosynthetic activity of bovine heart valves declines continuously with increasing age of the animal (Boström, Moretti & Whitehouse, 1963). This also happens in another connective tissue, cartilage, the mucopolysaccharide composition of which changes with age (Whitehouse & Boström, 1961, 1962). We have therefore examined the composition of each of the heart valves from a large number of new-born calves and old cattle and found distinct differences in their mucopolysaccharide content.

Three acidic mucopolysaccharides have been identified as constituents of pooled bovine and porcine heart valves (Deiss & Leon, 1955; Meyer, Davidson, Linker & Hoffman, 1956), namely hyaluronate, chondroitin sulphate C and derman sulphate (also known as chondroitin sulphate B or 'β-heparin'). Some new fractionation procedures involving the use of Rivanol (6,9-diamino-2-ethoxyacridine lactate) were developed for the quantitative isolation of small amounts of these polysaccharides from heart valves.

A brief report of this investigation has been published (Whitehouse & Moretti, 1963).

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