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Fixation of Carbon Dioxide by Extracts of the Strict Autotroph *Thiobacillus denitrificans*

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Chemosynthetic bacteria have in common with photosynthetic organisms the ability to grow upon carbon dioxide as the sole source of carbon. In recent years much progress has been made towards the elucidation of the mechanism by which carbon dioxide is fixed during photosynthesis. Benson *et al.* (1950) and Fager, Rosenberg & Gaffron (1950) demonstrated that phosphoglyceric acid is the first stable fixation product; later, Calvin & Massini (1952) and Bassham *et al.* (1954) obtained evidence which suggested that ribulose 1:5-diphosphate is the carbon dioxide acceptor. Recently an enzyme has been found in extracts of *Chlorella* (Quayle, Fuller, Benson & Calvin, 1954) and spinach (Weissbach & Horecker, 1955) which carboxylates ribulose 1:5-diphosphate according to the equation:

The formation of ribulose 1:5-diphosphate from ribulose 5-phosphate and adenosine triphosphate (equation 2) has been shown by Weissbach, Smyrniotis & Horecker (1954a) and Hurwitz (1955):

Phosphoglycerate is reduced to triose phosphate, which is then converted into hexose diphosphate (HDP) by aldolase. Hexose monophosphate is formed by dephosphorylation of HDP.

The pathway by which pentose phosphate is regenerated from hexose monophosphate and triose phosphate without loss of fixed carbon dioxide

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involves the enzymes transaldolase and transketolase, which catalyse reactions (3)–(5) (Horecker & Smyrniotis, 1953, 1954, 1955; Horecker, Smyrniotis & Klenow, 1953; Horecker, Gibbs, Klenow & Smyrniotis, 1954; Racker, de la Haba & Leder, 1954; de la Haba, Leder & Racker, 1955).

Pentose phosphate isomerase catalyses the inter-conversion of ribulose 5-phosphate and ribose 5-phosphate (Horecker, Smyrniotis & Seegmiller, 1951; Axelrod & Jange, 1954).

In this paper the formation of phosphoglycerate from pentose phosphates and some aspects of the metabolism of sugar phosphates by extracts of a strict chemosynthetic autotroph are reported. The organism studied, *Thiobacillus denitrificans*, grows on a simple bicarbonate-mineral medium utilizing the energy obtained from the oxidation of thio-sulphate or sulphur, and can be cultivated on a

large scale with relative ease (Baalsrud & Baalsrud, 1952). A preliminary account of the carboxylation reaction has appeared elsewhere (Trudinger, 1955), and some experiments on the metabolism of phosphate esters have also been briefly reported (Trudinger, 1956).

Recently, Santer & Vishniac (1955) have also reported the synthesis of phosphoglycerate (PGA) from pentose phosphate by extracts of the chemo-autotroph, *Thiobacillus thioparus*.

MATERIALS AND METHODS

Organism and preparation of extracts

Isolation and culture of the organism. The organism was isolated from local garden soil by inoculation into the elective medium for *T. denitrificans* of Baalsrud & Baalsrud (1954), and incubation at 28°. After four subcultures into fresh medium, only one bacterial type, a small, motile, Gram-negative rod, could be detected microscopically. This was plated on to the same medium, solidified with 1.5% agar, and single colonies were transferred into liquid medium. The organism was subsequently cultured and maintained in the medium described by Baalsrud & Baalsrud (1952) but containing twice the amount of FeSO_4 recommended by these authors. Stock cultures were kept as stabs in 1.5% agar medium.

The organism grew aerobically in a bicarbonate-mineral medium containing thiosulphate, tetrathionate or sulphur, but developed anaerobically only after the addition of nitrate. Washed suspensions metabolized thiosulphate and sulphur (flowers of sulphur suspended in the reaction medium) with the consumption of O_2 and, anaerobically, liberated N_2 from nitrate in the presence of thiosulphate or sulphur. In bicarbonate medium the reduction of nitrate in the presence of thiosulphate was accompanied by an uptake of CO_2 which could be followed manometrically. The organism therefore had the typical properties of *T. denitrificans* (Baalsrud & Baalsrud, 1954).

The bacteria were grown on a large scale in 5 l. conical flasks filled to within 2 in. of the top with medium. A portion (100 ml.) of a 4-day culture was used as inoculum. Visible gas formation began after about 24 hr. and growth was complete in 4–5 days at 28°. The cells were harvested on the Sharples centrifuge and washed twice with 1% (w/v) KCl. Up to 1 g. dry wt. of cells was obtained from each flask.

Preparation and extraction of freeze-dried bacteria. A thick paste of bacteria in water was spread in a thin layer on the inner surface of a round-bottomed flask, frozen in an ethanol-solid CO_2 mixture and freeze-dried at approximately 0.5 mm. pressure. The dry powder was stored at 2° in *vacuo* over anhydrous CaCl_2 . For extraction approximately 1 g. of the freeze-dried powder was suspended in 10 ml. of 1% (w/v) KCl and left overnight at 2° under N_2 . The mixture was then centrifuged at 17 000 g for 30 min. at 0°. The supernatant so obtained was reddish brown and contained about 2 mg. of total N/ml. As far as the enzymes studied were concerned the dry powder remained stable for several months.

Preparation of extracts with the Hughes press. The bacteria were mixed with an equal volume of acid-washed powdered glass and crushed in the Hughes press (Hughes, 1951) which had been cooled to -60° with solid CO_2 . The mixture was

extracted with 4–5 vol. of 1% (w/v) KCl and centrifuged at 17 000 g for 30 min. at 0°.

Fractionation of CO_2 -fixing enzyme. All manipulations were carried out at 0°. Solid $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation was added to the crude extract from the Hughes press. After standing for 10 min. the precipitate was removed by centrifuging and discarded. Ammonium sulphate to 90% saturation was added to the supernatant, the precipitate collected by centrifuging at 17 000 g for 30 min., dissolved in a small volume of 1% (w/v) KCl and dialysed overnight against 1% (w/v) KCl with stirring. Next day the dialysed enzyme solution was centrifuged to remove a small amount of precipitated matter and fractionated further with $(\text{NH}_4)_2\text{SO}_4$. The fractions collected were usually those precipitating at 30–40, 40–50, 50–60 and 60–70% saturation of $(\text{NH}_4)_2\text{SO}_4$. These were dissolved in small volumes of 1% (w/v) KCl and dialysed overnight against the same solution.

Special chemicals

Glucose 6-phosphate (G 6-P). The crystalline barium salt, 98% pure by *Zwischenferment* assay, was obtained from the Sigma Chemical Co.

6-Phosphogluconate (6-PG). The barium salt was a gift from Dr M. G. Macfarlane. It gave a single phosphate spot on paper chromatograms.

Fructose 6-phosphate (F 6-P). This was prepared by hydrolysis of barium hexose diphosphate (HDP) with *n*-HBr (Neuberg, Lustig & Rothenberg, 1944) and isolated as the barium salt. The preparation contained about 5% each of HDP and inorganic phosphate.

Ribose 5-phosphate (R 5-P). This was prepared by acid hydrolysis of 5-adenylic acid and isolated as the barium salt (Khym & Cohn, 1953). On paper chromatograms the preparation showed one major phosphate spot and a trace of material corresponding to adenylic acid. Analysis showed no inorganic phosphate, 6.69% of bound phosphorus, 32.6% of ribose (orcinol test) and 1.3% of adenylic acid (260 m μ . absorption): theoretical for $\text{BaC}_5\text{H}_9\text{O}_8 \cdot 5\frac{1}{2}\text{H}_2\text{O}$, 6.62% of bound phosphate and 32% of ribose.

Hexose diphosphate. The barium salt, about 90% pure, was prepared from a yeast fermentation mixture by the method of Robison & Morgan (1930). The main impurity was inorganic phosphate.

Ribulose 1:5-diphosphate (RuDP). The barium salt, purity about 70%, was obtained from Dr J. Hurwitz.

3-Phosphoglycerate (3-PGA). The barium salt was prepared from a yeast-fermentation mixture according to Neuberg & Lustig (1942). The preparation gave a single phosphate spot on paper chromatograms and the amount of bound phosphate was over 98% of theoretical for 3-PGA.

2-Phosphoglycerate (2-PGA). The silver salt was donated by Dr W. Bartley.

Phosphoenolpyruvate (PEP). The silver-barium salt was a gift from Dr W. Bartley.

Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). These were prepared from pig liver and separated by gradient elution from Dowex 1 resin with sodium formate-formic acid mixtures (Horecker, private communication). Preparations containing 70–90% of either DPN or TPN were obtained free of TPN and DPN respectively. Another sample of TPN, 82% pure by *Zwischenferment* assay, was a Sigma Chemical Co. product. DPN was assayed with the ethanol-alcohol dehydrogenase system.

Adenosine triphosphate (ATP). Two samples were used: (a) barium ATP, purity 90%, prepared from rabbit muscle (LePage, 1949) and donated by the Biochemistry Department, University of Sheffield; and (b) dipotassium ATP from the Sigma Chemical Co.

Pyruvate 2:4-dinitrophenylhydrazone. This was prepared by the method of Goodwin & Williams (1952). The carbonate solution of the 2:4-dinitrophenylhydrazone was acidified with H_2SO_4 and extracted with ether. The residue, after evaporation of the ether, was used. The material gave two spots on paper chromatograms (cf. Isherwood & Cruickshank, 1954).

Ribulose. A solution of ribulose for use as a marker on paper chromatograms was made by hydrolysis of RuDP with phosphatase. Inorganic phosphate was removed by precipitation with barium and centrifuging. The resulting solution was treated with Dowex 50 (H form) to remove cations and freeze-dried. The residue was dissolved in a small volume of water.

Mannoheptulose. This was a gift from the Chemistry Department, University of Birmingham.

$\text{Na}_2^{14}\text{CO}_3$. Radioactive sodium carbonate, containing $130 \mu\text{C}/\text{mg.}$ of Na_2CO_3 , was obtained from the Radiochemical Centre, Amersham.

Preparation of sodium or potassium salts of phosphate esters. The barium salts were dissolved in a small volume of 0.1N-HCl and a slight excess of either Na_2SO_4 or K_2SO_4 was added. The precipitate was removed by centrifuging, washed with 0.1N-HCl, and the supernatant and washings were neutralized with either NaOH or KOH. Silver salts were suspended in a small volume of water and decomposed with H_2S . After centrifuging the supernatant was aerated for 30 min. and neutralized.

Zwischenferment. A preparation from baker's yeast (Oliver, 1955) was given by Mr I. T. Oliver. The preparation contained hexose monophosphate isomerase but had no action on either 6-PG or R 5-P.

Alcohol dehydrogenase. A preparation from baker's yeast (Racker, 1950) was given by Mr I. T. Oliver.

Acid phosphatase. This was prepared from potato-press juice by the method of Schramm & Flammersfeld (1947), except that the second fractionation with $(\text{NH}_4)_2\text{SO}_4$ was omitted. A water solution of the enzyme was freeze-dried and kept at 2° *in vacuo* over anhydrous CaCl_2 , under which condition it remained stable for at least 6 months. The enzyme was strongly inhibited by inorganic phosphate; for this reason, esters to be hydrolysed were generally first separated from inorganic phosphate by chromatography on paper. For hydrolysis, about 1 mg. of phosphatase was mixed with the ester in 0.01M acetate, pH 5.0, and incubated at 30° . Under these conditions 1–4 μmoles of 3-PGA, 6-PG, G 6-P, R 5-P or HDP were more than 90% hydrolysed in 60–90 min.; in general an incubation period of 5–7 hr. was used.

Analytical methods

Total nitrogen. This was determined by digestion of the sample according to Johnson (1941), followed by steam distillation of the ammonia formed into ethanolic boric acid (Conway & O'Malley, 1942) and titration with HCl.

Phosphate. Inorganic phosphate was measured by the method of Fiske & Subbarow (1925), and total phosphorus according to Allen (1940).

Triose phosphate. The inorganic phosphate liberated during incubation of the sample in N-NaOH for 20 min. at

room temperature was taken as a measure of triose phosphate (Lohmann & Meyerhof, 1934).

Phosphoenolpyruvate. This was measured by the liberation of inorganic phosphate in alkaline iodine solution (Lohmann & Meyerhof, 1934) and also by the keto acid produced on hydrolysis of the sample in N-HCl for 30 min. at 100° .

Total fructose. This was determined by reaction with resorcinol (Roe, 1934).

Total pentose. This was determined by reaction with orcinol (Meijbaum, 1939). The samples were heated in boiling water for 40 min. as recommended by Albaum & Umbreit (1947).

Keto acids. The method of Goodwin & Williams (1952) was used.

Total carbohydrate. The anthrone method of Trevelyan & Harrison (1952) was used.

Spectrophotometry. Spectrophotometric measurements were made with the Unicam SP. 500 spectrophotometer.

Paper partition chromatography

Whatman no. 1 paper was used throughout, and development carried out at $20\text{--}25^\circ$. Unless otherwise stated descending development was used. The solvent ratios listed below are v/v unless otherwise stated.

Phosphate esters. Paper for the chromatography of phosphate esters was pretreated by washing consecutively with N-HCl (Mortimer, 1952), water and 0.2% ethylenediaminetetraacetate at pH 8.5 (Eggleson & Hems, 1952). After six further washings in water the papers were dried in air.

Initial separations were usually made with either diisopropyl ether–90% (w/v) formic acid (3:2) (Hanes & Isherwood, 1949) or ethyl acetate–acetic acid–water (3:3:1) (Mortimer, 1952). The first solvent was particularly useful in separating phosphate esters from inorganic phosphate. The following solvents were also used for identification of esters: methanol– NH_3 soln. (sp. gr. 0.88)–water (16:3:1) (Bandurski & Axelrod, 1951), *tert.*-pentanol–water–toluene–*p*-sulphonic acid (60 ml.:30 ml.:2 g., upper phase) (Hanes & Isherwood, 1949), isobutyric acid–N- NH_3 soln. (5:3) containing 0.001M ethylenediaminetetraacetic acid (Krebs & Hems, 1953) and 80% aqueous ethanol containing acetate, pH 3.5 (Cohen & Scott, 1950). 2- and 3-PGA were separated by the technique of Cowgill (1955) except that, instead of washing the papers with molybdate, 0.5% sodium molybdate was added to the solvent system [ethanol–water–90% (w/v) formic acid (70:29:1)].

Areas containing phosphate were revealed by spraying the papers with a mixture of perchloric acid, ammonium molybdate and HCl (Hanes & Isherwood, 1949), followed by heating at 100° for 3 min. and exposure to H_2S .

Reducing carbohydrates. These were separated with ethyl acetate–pyridine–water (2:1:2, upper phase) (Jermyn & Isherwood, 1949). Reducing areas were located by spraying the papers with AgNO_3 in acetone, exposing to NH_3 fumes and heating for 10 min. at 80° (Dedonder, 1952). Fructose was detected as a red spot by spraying with resorcinol in ethanol acidified with HCl (Forsyth, 1948). The differential spray of Klevstrand & Nordal (1950), consisting of orcinol and trichloroacetic acid (TCA) in *n*-butanol, was also used. With this spray heptulose appears as a blue spot and fructose as a yellow spot (Benvenue & Williams, 1951). Ribulose gives a brown colour (Weissbach *et al.* 1954a).

Organic acids. These were separated with either pentan-1-ol-5M aqueous formic acid (1:1, upper phase) (Buch, Montgomery & Porter, 1952) or *n*-butanol-propionic acid-water, prepared according to Benson *et al.* (1950). Acid areas were located by spraying with 0.04% (w/v) bromophenol blue in ethanol (Buch *et al.* 1952).

2:4-Dinitrophenylhydrazones. The solvent systems *n*-butanol saturated with water and *n*-butanol-ethanol-water (50:10:40, upper phase) (Cavallini, Frontali & Toschi, 1949) were used. In general sufficient material was applied to the paper to be visible without further treatment. Faint spots were intensified by spraying the paper with 10% (w/v) aqueous NaOH (Rice, Keller & Kirchner, 1951).

Radioactivity. Radioactive areas on paper chromatograms were detected by one of two methods. (a) The papers were exposed to Ilford, Industrial G, X-ray film for 3-4 weeks. The film was then developed in Kodak D. 19b X-ray film developer and fixed in Kodak F. 54A fixing solution. The limit of detection by this method was about 100 counts/min. of $^{14}\text{C}/\text{cm}^2$ applied to the paper. (b) With one-way chromatograms, radioactive areas were also detected by cutting the paper into 0.5 cm. $\times$ 2.0 cm. strips and determining the relative radioactivity in each strip with the Geiger-Müller counter.

Since the paper exhibited a variable self-absorption the quantitative determination of ^{14}C on chromatograms was made by eluting the radioactive area with water, plating and counting as described in the next section.

Measurement of radioactivity

In samples containing much solid material, such as incubation mixtures, organic material was converted into CO_2 by wet combustion (Van Slyke & Folch, 1940). The CO_2 liberated was trapped in 3N-NaOH and converted into BaCO_3 by mixing the alkaline solution with excess of 5% (w/v) BaCl_2 . The BaCO_3 was collected on Whatman no. 40 filter paper disks, washed with water and acetone and dried under an infrared lamp. The ^{14}C content of the BaCO_3 was found with an end-window Geiger-Müller counter. Corrections for self absorption were made from a standard curve. This technique has been described in more detail by Ormerod (1956).

The ^{14}C in samples containing less than 0.1 mg. of solid matter (e.g. eluates from paper chromatograms) was generally determined by plating the sample directly on to copper disks, drying and counting (Ormerod, 1956). Self absorption was negligible and good agreement was found between values obtained by this method and those by wet combustion.

The ^{14}C in the carboxyl group of glyceric acid was determined by oxidation of the acid with sodium metaperiodate at pH 5.8. Under these conditions glyceric acid is converted into formaldehyde, formic acid and CO_2 , the CO_2 arising from the carboxyl group (Sprinson & Chargaff, 1946; Sakami, 1950). Carrier glyceric acid was mixed with the radioactive sample, NaIO_4 added and the mixture aerated with CO_2 -free air for 2 hr. at room temperature. Carbon dioxide was trapped in NaOH, converted into BaCO_3 and counted as described above.

Activities were corrected for background and are expressed as counts/min. at infinite thinness. Unless otherwise stated, the standard counting error was less than $\pm 3\%$.

General experimental procedures

$^{14}\text{CO}_2$ fixation experiments. These were carried out in Warburg manometers equipped with double side-bulb cups. To prevent losses of $^{14}\text{CO}_2$ during equilibration and gassing, the $\text{Na}_2^{14}\text{CO}_3$ was placed in one side bulb and other substrates in the second side bulb. The buffer systems used were such that the change in pH on addition of $\text{Na}_2^{14}\text{CO}_3$ was less than 0.1 unit. At the end of the reaction the cups were removed from the manometers and 5% (w/v) of TCA was added. The cup contents were then aerated for 30 min. to remove residual CO_2 . Control experiments showed that the retention of radioactive CO_2 was negligible after this treatment.

Isolation of 'phosphate esters'. Reaction mixtures were deproteinized with 5% (w/v) of TCA and centrifuged, and the supernatants brought to pH 8.2 with NaOH. Barium acetate was then added to give a concentration of about 0.2M and the mixture poured into 4 vol. of absolute ethanol. The precipitate was removed by centrifuging, washed with absolute ethanol and dried *in vacuo*. The precipitate was redissolved in a small volume of 0.1N-HCl and passed through a column of Dowex 50 (H form) to remove barium. (Generally a small portion of the barium precipitate failed to redissolve in HCl. This was removed by centrifuging before treatment with Dowex.) The resulting solutions were then freeze-dried and stored at -20° until required. The dried material is referred to as the 'phosphate ester fraction', but also contained organic material other than phosphate esters.

RESULTS

Fixation of $^{14}\text{CO}_2$ by extracts of *Thiobacillus denitrificans*

Fixation by crude extracts. Crude dialysed extracts of *T. denitrificans* prepared in the Hughes press or by extraction of freeze-dried bacteria, were found to fix $^{14}\text{CO}_2$ in the presence of Mg^{2+} and ATP.

Table 1. Fixation of $^{14}\text{CO}_2$ by extracts of *Thiobacillus denitrificans*

Expt. 1. The reaction mixture (4 ml.) contained: 0.1M aminotrihydroxymethylmethane (tris, pH 7.1), 0.5% of KCl, 6 μmoles of ATP, 40 μmoles of MgCl_2 , 2.5 μmoles of NaHCO_3 , 1.6 μmoles of $\text{Na}_2^{14}\text{CO}_3$ (500 000 counts/min.) and 7.3 mg. of enzyme N (Hughes press extract, dialysed for 20 hr. against 1% (w/v) KCl at 2°). Incubation for 3 hr. under O_2 -free N_2 at 30° . Expt. 2. As Expt. 1 except that mixture contained 1.25 μmoles of NaHCO_3 and 4.95 mg. of enzyme N.

Expt.	Additions (μmoles)	^{14}C fixed (counts/min.)	Change
1	None	46 800	—
	R 5-P	93 700	46 900
	6-PG	53 800	7 000
	G 6-P	48 100	1 300
	HDP	75 000	28 200
	5-Adenylic acid	37 200	-9 600
2	DL-Malate (12 μmoles)	42 000	-4 800
	None	9 100	—
	R 5-P	42 000	22 900
	3-PGA	8 700	-400
	Pyruvate	11 100	2 000

Fixation of $^{14}\text{CO}_2$ was increased from two to four times by the addition of R 5-P (Table 1). Fixation was also increased by HDP and to a smaller extent by 6-PG. The addition of G 6-P, 3-PGA and pyruvate had little or no effect, and a slight inhibition was observed on the addition of either 5-adenylic acid or malate. In each case all the ^{14}C fixed was in the supernatant after precipitation of protein with TCA and centrifuging.

The fixation in the presence of R 5-P was much reduced in the absence of either ATP or Mg^{2+} (Table 2). Magnesium could be replaced by 10 mM- Mn^{2+} but Co^{2+} had little effect at this concentration and 0.1 mM- Fe^{2+} inhibited fixation by about 50%. Weissbach, Smyrniotis & Horecker (1954b) have shown that fixation of $^{14}\text{CO}_2$ by crude extracts of spinach in the presence of R 5-P was increased by the addition of TPN. With crude extracts of *T. denitrificans* addition of TPN doubled the 'blank' fixation but had no effect on the increase in $^{14}\text{CO}_2$ fixed due to R 5-P. Cysteine, glutathione, cocarboxylase, and DPN were also without effect.

The phosphate ester fraction was isolated from the protein-free supernatant of a fixation experiment and was found to contain about 92% of the total ^{14}C fixed (Table 3). Barium was removed from the precipitate by treatment with Dowex 50 and the resulting solution freeze-dried without neutralization. A considerable amount of radioactivity was lost at this stage. If the Dowex-treated material

was neutralized before freeze-drying this loss was prevented, which suggested that the loss was due to evaporation of volatile acids. However, since the main interest at this stage was in non-volatile phosphate esters and since large amounts of salt affected subsequent chromatography, neutralization was omitted. The freeze-dried material was chromatographed on paper with ethyl acetate-acetic acid-water, and radioactive areas were located by exposing the dry chromatograms to X-ray film. The position of each area relative to 3-PGA was noted, and the areas were eluted and their ^{14}C content was determined. The results are shown in Fig. 1. Recoveries of fixed ^{14}C during the fractionation are shown in Table 3.

Fig. 1. Products of CO_2 fixation. The freeze-dried material from the experiment described in Table 3 was chromatographed on paper (see text). The open columns show the counts/min. in the radioactive fractions from the reaction mixture without R 5-P, and the solid columns show the values obtained in the presence of R 5-P.

Table 2. Cofactors for $^{14}\text{CO}_2$ fixation in the presence of ribose 5-phosphate

The reaction mixture contained 6 μmoles of R 5-P; other conditions were as for Table 1. Enzyme N: Expt. 1, 3.34 mg.; Expt. 2, 6.5 mg.

Expt.		^{14}C fixed (counts/min.)
1	Complete system	25 000
	ATP omitted	3 450
	MgCl_2 omitted	870
	R 5-P omitted	7 000
2	Complete system	67 500
	MgCl_2 omitted	2 900
	MnSO_4 added in place of MgCl_2	79 000
	CoSO_4 added in place of MgCl_2	9 750

Table 3. Fractionation of products of $^{14}\text{CO}_2$ fixation

The reaction mixture (5 ml.) contained: 0.5% of KCl, 0.1M tris (pH 7.1), 10 μmoles of R 5-P, 4.8 μmoles of $\text{Na}_2^{14}\text{CO}_3$ (1 500 000 counts/min.), 30 μmoles of MgCl_2 , 10 μmoles of ATP and 8.6 mg. of enzyme N (Hughes press extract dialysed for 20 hr. against 1% (w/v) KCl at 2°). This was incubated at 30° under O_2 -free N_2 and the supernatant then fractionated as described in the text.

	^{14}C fixed/ml. (counts/min.)		
	No R 5-P (a)	With R 5-P (b)	(b) - (a)
Total	20 400	46 900	26 500
Phosphate esters before freeze-drying	19 200	42 900	23 700
Phosphate esters after freeze-drying	9 500	24 700	15 200
Sum of counts/min. in eluates from chromatogram (see Fig. 1)	9 000	23 700	—

Eight radioactive areas were found, one of which (VI) was subsequently separated into two compounds by chromatography with *tert*-pentanol-water-toluene-*p*-sulphonic acid (see below). Three compounds (V, VI B and VII) were of immediate interest since the amount of ^{14}C in these was increased five- to seven-fold in the presence of R 5-P. The radioactivity in the other compounds was increased to a lesser extent by R 5-P.

Identification of compound (V). After correcting for the blank fixation about 50% of the radioactivity in the freeze-dried material was present in compound (V). Compound (V) had the same R_f as phosphoglycerate in ethyl acetate-acetic acid-water, and its identity was further confirmed by two-dimensional chromatography with carrier 3-PGA, and with *isopropyl* ether-formic acid and methanol- NH_3 soln.-water as solvents. The main radioactive area was coincident with PGA (Fig. 2a). A small radioactive area running slightly ahead of PGA and equal to about 1% of the total counts applied to the paper, was also present. This did not reproduce in the photograph but its position is shown by the solid line. Its position was that given by PEP and it may have been due to contamination by compound (VI). Compound (V) also ran as PGA in the solvents ethanol-acetate pH 3.5, *isobutyric* acid- NH_3 soln. and *tert*-pentanol-water-toluene-*p*-sulphonic acid. A sample of compound (V) was mixed with authentic 3-PGA and hydrolysed with phosphatase. The bulk of the inorganic phosphate was removed by the addition of a small amount of barium acetate, and centrifuging, and the supernatant treated with Dowex 50 (H form) to remove cations. There remained 95% of the ^{14}C in the supernatant. The latter was concentrated to a small volume by distillation from the frozen state and run on a two-dimensional chromatogram with pentan-1-ol-formic acid-water and *n*-butanol-propionic acid-water. Most of the radioactivity corresponded to glyceric acid, located by spraying with bromophenol blue (Fig. 2b). The two smaller radioactive areas appeared to be due to a breakdown of glyceric acid either during development in the first solvent or during the intermediate drying, since when the glyceric acid was eluted and rechromatographed under the same conditions the two contaminating spots reappeared.

Fig. 2. For legend see bottom of next column.

Fig. 2. Chromatography and radioautography of compound V and its dephosphorylation product. (a) Photographic reproduction of a radioautograph of a two-dimensional chromatogram of compound (V) (9000 counts/min.) with carrier 3-PGA developed 1, with *isopropyl* ether-formic acid (ascending); 2, with methanol- NH_3 soln.-water (descending). After the radioautograph was made (exposure for 3 weeks) the chromatogram was sprayed with acid-molybdate reagent. The 3-PGA area (dotted line) has been superimposed on the radioautograph. The solid line outlines a faint radioactive area which did not appear on reproduction. (b) Photographic reproduction of a radioautograph of a two-dimensional chromatogram of compound (V) (15000 counts/min.), and carrier 3-PGA chromatographed after treatment with phosphatase (see text). Development: 1, pentan-1-ol-5M formic acid (ascending); 2, butanol-propionic acid-water (descending). After radioautography (exposure for 3 weeks) acid areas (dotted line) were located by spraying with bromophenol blue.

The solvent systems described above did not distinguish between 2- and 3-PGA. Fig. 3 shows the result of a chromatogram of compound (V) developed with ethanol-formic acid containing sodium molybdate. Both 2- and 3-PGA were found in a ratio approximating to that reported for the phosphoglyceromutase equilibrium (Meyerhof & Kiessling, 1935).

Identification of compound (VIB). The position of compound (VI) on the chromatogram developed with ethyl acetate-acetic acid-water corresponded to that given by PEP. The radioactive material also moved as PEP with the solvents ethanol-acetate pH 3.5 and methanol-NH₃ soln.-water. However, on chromatograms developed with *tert.*-pentanol-water-toluene-*p*-sulphonic acid, compound (VI) separated into two parts, the faster-moving component, compound (VIB), corresponding to PEP (Fig. 4a). The latter was eluted, mixed with authentic PEP and hydrolysed with phosphatase. 2:4-Dinitrophenylhydrazones were prepared and chromatographed with two different solvents. Most of the radioactivity was found in the pyruvate 2:4-dinitrophenylhydrazone area (Fig. 4b, c).

Other radioactive compounds. Compound (VII) was not a phosphate ester but so far has not been identified. The identification of the other radioactive compounds has not been undertaken.

Fixation of ¹⁴CO₂ by fractionated extracts. The experiments described above show that PGA was one of the products of CO₂ fixation in the presence of R5-P. However, since a number of other labelled compounds were also formed they do not establish whether PGA was formed directly from pentose

phosphate or arose by some secondary reaction. The crude extracts have been fractionated with ammonium sulphate (see Materials and Methods and Table 4) and a fraction obtained which fixed

Fig. 4. Chromatography of compound (VI) and the 2:4-dinitrophenylhydrazones (DNP) of its hydrolysis product. The upper and lower parts of each curve show the distribution of radioactivity along the chromatograms and positions of markers respectively (cf. Fig. 3). (a) Compound (VI) chromatographed with *tert.*-pentanol-water-toluene-*p*-sulphonic acid. Marker PEP. (b) DNP of dephosphorylated compound (VIB) (see text), chromatographed with *n*-butanol saturated with water. Marker, pyruvate-DNP. (c) DNP area eluted from chromatogram (b) chromatographed with *n*-butanol-ethanol-water. Marker, pyruvate-DNP.

Fig. 3. Chromatography of compound (V) with ethanol-formic acid-water containing sodium molybdate. Compound (V) was mixed with carrier 2- and 3-PGA before chromatography, and the distribution of radioactivity along the chromatogram (upper part of the figure) was determined by counting 0.5 cm. $\times$ 2 cm. strips of the paper for 5 min. (Values have not been corrected for self absorption.) Markers were run parallel to the radioactive material (lower part of the figure).

Table 4. Fractionation of CO₂-fixing enzyme

Activities were determined under conditions similar to those in Table 3. Specific activities are the counts/min. of ¹⁴C fixed/mg. of N/3 hr. corrected for fixation in the absence of R5-P. Total activities are the specific activities multiplied by the mg. of N and are expressed as a percentage of the values thus obtained with the crude extract.

Fraction (saturation of ammonium sulphate)	Recovery		Specific activity
	mg. of N	Total activity (%)	
Whole extract	63.0	100	7 150
First fractionation			
0-30 %	11.6	6	1 690
30-90 %	37.5	62	7 400
Second fractionation			
0-40 %	13.8	2	640
40-50 %	8.2	27	15 000
50-60 %	5.2	1	750
60-75 %	3.0	0	0

$^{14}\text{CO}_2$ in the presence of both R 5-P and ATP but not with either alone (Table 5).

R 5-P, ATP and a fraction of the extract precipitating between 40 and 50% saturation with ammonium sulphate were incubated under conditions described in Table 5. The total ^{14}C fixed was 19 400 counts/min. (105 counts/min. in the absence of R 5-P) of which 18 300 counts/min. (95%) were recovered in the phosphate ester fraction. The whole phosphate ester fraction was hydrolysed with phosphatase and chromatographed with butanol-propionic acid-water. Only one major radioactive compound was present, which corresponded to glyceric acid (Fig. 5) and contained 18 000 counts/min. (93%).

Effect of RuDP on $^{14}\text{CO}_2$ fixation. Fractionated extracts fixed $^{14}\text{CO}_2$ in the presence of RuDP alone (Table 5). The phosphate ester fraction from this experiment was chromatographed with ethyl

Fig. 5. Chromatography of hydrolysed phosphate esters from a reaction mixture containing R 5-P, ATP and fractionated enzyme (see text). The upper part of the curve shows the distribution of radioactivity along the chromatogram (cf. Fig. 3), and the lower part a tracing of the acid areas. Spots: 1, 3-PGA (faint); 2, inorganic phosphoric acid; 3, glyceric acid.

Table 5. Fixation of $^{14}\text{CO}_2$ by a fractionated extract

The basic mixture (4 ml.) contained: 0.5% of KCl, 0.1 M tris (pH 7.1), 1.25 μ moles of NaHCO_3 , 1.6 μ moles of $\text{Na}_2^{14}\text{CO}_3$ (500 000 counts/min.), 40 μ moles of MgCl_2 and 2.32 mg. of enzyme N (fraction of Hughes press extract precipitating between 35 and 50% saturation of ammonium sulphate). Incubated for 2 hr. at 30° under N_2 .

Cup	Additions (4 μ moles)	^{14}C Fixed (counts/min.)
1	None	0
2	R 5-P	0
3	ATP	630
4	R 5-P and ATP	36 200
5	RuDP	46 300
	3-PGA from cup 5	45 200
	Carboxyl group of 3-PGA from cup 5	43 200

acetate-acetic acid-water and found to contain only one radioactive compound. This corresponded to 3-PGA. The compound could not be separated from authentic 3-PGA when mixtures of the two were chromatographed with methanol- NH_3 soln.-water and ethanol-formic acid-water containing sodium molybdate; no 2-PGA was detected. About 98% of the ^{14}C fixed was recovered in 3-PGA (Table 5). Hydrolysis of the labelled material with phosphatase gave a compound which chromatographed as glyceric acid. The labelled carbon in glyceric acid, isolated from an incubation mixture of crude extract, R 5-P and ATP, was found to be entirely in the carboxyl group (Trudinger, 1955). A similar distribution was found in the glyceric acid isolated during fixation of $^{14}\text{CO}_2$ in the presence of RuDP (Table 5).

Fixation of $^{14}\text{CO}_2$ in the presence of HDP. Fixation of $^{14}\text{CO}_2$ by crude extracts was considerably increased by the addition of HDP (Table 1). This increase was found to be dependent on ATP, and radioautography of chromatograms of the freeze-dried phosphate ester fraction showed that, at least in this fraction, the end products were very similar to those found during fixation in the presence of R 5-P. About 50% of the ^{14}C in the phosphate ester fraction was in PGA.

Interconversion of pentose and hexose phosphates

The fixation of $^{14}\text{CO}_2$ into PGA by extracts in the presence of HDP suggested that the latter was converted into pentose phosphate before CO_2 fixation. By the action of aldolase and hexose diphosphatase, which were present in the extracts (see below), HDP can be converted into triose phosphate and F6-P which in turn give rise to pentose phosphate by reactions (3), (4) and (5) (see p. 274). The extracts were examined for enzymes catalysing the last three reactions.

Evidence for transketolase and transaldolase. R 5-P was rapidly metabolized by dialysed extracts in the absence of ATP, with the formation of hexose monophosphate (Fig. 6). The extracts did not contain G 6-P dehydrogenase. During R 5-P removal triose phosphate was also produced and an increase in absorption at 600 m μ . in the orcinol reaction suggested that heptulose was formed (Horecker & Smyrniotis, 1952). In these experiments Co^{2+} was omitted from the reaction mixtures and aldolase activity was therefore very low (see below). The phosphate ester fraction from an incubation mixture of R 5-P and an extract of freeze-dried bacteria was chromatographed with isopropyl ether-formic acid. The sugar monophosphate area was eluted, hydrolysed with phosphatase and chromatographed with ethyl acetate-pyridine-water. In addition to residual ribose four reducing compounds were found which corresponded to glucose, fructose,

heptulose (mannoheptulose was used as a marker) and tetrose (R_{glucose} , 2.19, cf. Isherwood & Jermyn, 1951). The heptulose gave a blue colour when sprayed with the orcinol-TCA reagent. After 10 min. incubation 'heptulose' predominated, but at 30 min. hexose, 'heptulose' and 'tetrose' were present in approximately equal amounts.

A direct demonstration of pentose phosphate formation from hexose phosphates is shown in Table 6. Pentose was formed at a maximum rate when both HDP and G 6-P were added. No CO_2 was formed during the reaction. Chromatography of the hydrolysed phosphate esters from this experiment showed the presence of 'heptulose', 'tetrose', ribose and a compound moving as ribulose. The latter gave a brown colour with the orcinol-TCA spray similar to that given by ribulose.

Pentose phosphate isomerase. The results described in the last section and the experiments on the fixation of $^{14}\text{CO}_2$ in the presence of pentose phosphates suggested the presence of pentose phosphate isomerase. Attempts to demonstrate a direct formation of Ru 5-P from R 5-P chromatographically were not successful, probably owing to the rapid further metabolism of the pentose phosphates. However, when extracts were incubated for 1–2 min. with R 5-P, a compound was produced which reacted in the cysteine-carbazole test of Dische & Borenfreund (1951) to give a colour with an

absorption maximum at about 542 m μ . similar to that given by ribulose (Fig. 7). After 5 min. incubation other compounds appeared which reacted in the cysteine-carbazole test. Based on the cysteine-carbazole colour at 540 m μ . which appeared after 1 min. incubation, activities of pentose phosphate isomerase of up to 20 μmoles transformed/mg. of N/hr. have been recorded.

Table 6. *Pentose formation from hexose phosphates*

The complete system (2 ml.) contained: 0.25% of KCl, 0.1 M tris (pH 7.1), 25 μmoles of G 6-P, 25 μmoles of HDP, 20 μmoles of CoSO_4 and 0.84 mg. of enzyme N (extract of freeze-dried bacteria, dialysed for 20 hr. against 1% (w/v) KCl at 2°). Incubated at 30° under O_2 -free N_2 . Pentose was measured by the orcinol reaction and is expressed as ribose.

	Pentose formed (μmoles)	
	30 min.	120 min.
Complete system	2.35	6.14
G 6-P omitted	0.54	3.08
HDP omitted	0.34	0.62
G 6-P and HDP omitted	0.00	0.00

Fig. 6. Hexose monophosphate formation from ribose 5-phosphate. The reaction mixture (3 ml.) contained 0.1% of KCl, 4 μmoles of R 5-P, 30 μmoles of MgCl_2 , 0.1 M tris, pH 7.1, and 0.32 mg. of enzyme N (extract of freeze-dried cells dialysed for 20 hr. against 1% KCl at 2°). Additions: a, 0.4 μmole of TPN; b, 1 mg. of *Zwischenferment*.

Fig. 7. Spectra of chromogens obtained in the cysteine-carbazole test. A dialysed extract of freeze-dried bacteria (1.5 mg. of N) was incubated with 6 μmoles of R 5-P and 40 μmoles of MgCl_2 in 0.1 M tris, pH 7.1, at 30°. Samples of the supernatants, after precipitation of protein with 5% (w/v) TCA, were treated with cysteine and carbazole according to Dische & Borenfreund (1951), and the spectra of the colours produced determined. Curves: 1, ribulose; 2, supernatant after incubation for 1 min.; 3, supernatant at zero time.

*Glycolytic enzymes in extracts of
Thiobacillus denitrificans*

Hexose monophosphate isomerase. A substance reacting in the resorcinol test for fructose was produced when dialysed extracts of *T. denitrificans* were incubated with G 6-P in the absence of any added cofactors (Table 7). The phosphate esters fraction isolated from a reaction mixture was analysed by paper chromatography. In addition to G 6-P a compound was found which moved as F 6-P with the solvents ethyl acetate-acetic acid-water, isobutyric acid-NH₃ soln. or isopropyl ether-formic acid. Hydrolysis of this material with phosphatase gave a compound which could not be separated from fructose with ethyl acetate-pyridine-water, and which gave a red colour similar

Table 7. *Hexose monophosphate isomerase*

The reaction mixture (1 ml.) contained: 0.1M tris (pH 7.8), 9.4 mg. of enzyme N (extract of freeze-dried bacteria dialysed for 20 hr. against 1% (w/v) KCl at 2°), and 4 μmoles of G 6-P. Incubated at 30° under O₂-free N₂. F 6-P was measured by the resorcinol method. Fructose values were multiplied by 100/60.5 (Umbreit, Burris & Stauffer, 1949).

Time (min.)	F 6-P (μmole)
0	0
10	0.27
20	0.48
40	0.83
60	0.97
60 (no G 6-P)	0

Table 8. *Aldolase and hexose diphosphatase*

The basic mixture (2 ml.) contained: 0.25% of KCl, 0.1M tris (pH 7.1), and 25 μmoles of HDP. Incubation at 30° under O₂-free N₂. Expt. 1: 0.4 mg. of enzyme N (extract of freeze-dried bacteria, dialysed for 20 hr. against 1% (w/v) KCl at 2°); incubated for 10 min.; Expt. 2, 0.34 mg. of enzyme N, incubated for 45 min.

Expt.	Additions	Concn. (mM)	Phosphate formed (μmoles/2 ml.)	
			Inorganic	Alkali- labile
1	None	—	0	0.84
		0.01	0	0.98
		0.1	0.04	1.80
		0.5	0.06	3.08
		1.0	0.08	3.58
		1.5	0.14	3.64
		2.0	0.20	3.64
2	None	—	0.09	2.73
	CoSO ₄	1.5	1.68	5.16
	MnSO ₄	1.5	1.20	2.16
	FeSO ₄	1.5	0.21	3.21
	ZnSO ₄	1.5	0.69	1.02
	MgCl ₂	1.5	0.84	2.16

to that given by fructose when sprayed with resorcinol reagent. The activities of isomerase were in the order of 4–5 μmoles of F 6-P formed/mg. of N/hr.

Aldolase and hexose diphosphatase. Dialysed extracts of *T. denitrificans* converted HDP into triose phosphate measured as alkali-labile phosphate (Table 8). For maximum activity 1–2 mM-Co²⁺ was required; Fe³⁺ replaced Co²⁺ but its optimum concentration was about four times as high. Mn²⁺ and Mg²⁺ were without effect at concentrations between 0.1 and 10 mM, and 1.5 mM-Zn²⁺ inhibited the rate of triose phosphate formation about 60%. Under the conditions used the maximum rates were approximately 50 μmoles of alkali-labile phosphate/mg. of N/hr.

Triose phosphate formation was usually accompanied by a slower production of inorganic phosphate. This was also increased by the addition of Co²⁺ but also by Mn²⁺ and to a lesser extent by Mg²⁺, Fe³⁺ and Zn²⁺ (Table 8, Expt. 2). Two phosphorylated compounds were isolated from a reaction mixture of enzyme, HDP and Mn²⁺ which were indistinguishable on paper chromatograms from F 6-P and G 6-P. *iso*Propyl ether-formic acid and isobutyric acid-NH₃ soln. were used as solvents. Hydrolysis of these compounds with phosphatase produced fructose and glucose respectively. The maximum rates observed were 6–7 μmoles of inorganic phosphate formed/mg. of N/hr.

Triose phosphate dehydrogenase and phosphoglycerokinase. DPN was reduced by extracts of *T. denitrificans* in the presence of HDP (Fig. 8, Expt. 1). No DPN reduction was observed when G 6-P replaced HDP; TPN was not reduced. The addition of inorganic phosphate to extracts dialysed for 2 hr. against 1% (w/v) KCl had no marked effect on the rate of DPN reduction, but these extracts were found to contain considerable amounts of phosphate. Dialysis for 20 hr. abolished enzyme activity, which was partly restored by the addition of inorganic phosphate and glutathione.

In the presence of arsenate the triose phosphate dehydrogenase reaction proceeds irreversibly to the right, and 3-PGA accumulates (Warburg & Christian, 1939, 1943). A reaction mixture consisting of extract (0.3 mg. of N), HDP (5 μmoles), sodium arsenate (15 μmoles) and DPN (4 μmoles) was incubated until DPN was completely reduced. From the supernatant a compound was isolated which moved as PGA on paper chromatograms with ethyl acetate-acetic acid-water and methanol-NH₃ soln.-water as solvents. This compound was not produced in the absence of DPN.

Reduced DPN (DNPH) was oxidized by the extracts when 3-PGA and ATP were added (Fig. 8). DNPH was generated either by the extract + HDP (Expt. 1) or by ethanol and alcohol dehydrogenase

Fig. 8. Triose phosphate dehydrogenase and phosphoglycerokinase. Expt. 1. Reaction mixture (3 ml.) contained: 0.066M tris, pH 7.6, 5 μ moles of HDP, 30 μ moles of phosphate, 3 μ moles of CoSO_4 and 0.3 mg. of enzyme N (Hughes press extract dialysed for 2 hr. against 0.1M tris, pH 7.6, at 2°). Temp., 24°. Additions: a, 0.15 μ mole of DPN; b, 10 μ moles of 3-PGA; c, 2 μ moles of ATP. Expt. 2. Reaction mixture (3 ml.) contained: 0.066M tris, pH 7.6, 30 μ moles of ethanol and 0.3 mg. of enzyme N (as in 8a). Additions: a, 0.15 μ mole of DPN; b, 1 mg. of alcohol dehydrogenase; c, 0.15 μ mole of DPN; d, 10 μ moles of 3-PGA; e, 2 μ moles of ATP. Expt. 3. Reaction mixture as in Expt. 2. Additions: a, 0.3 μ mole of DPN; b, 1 mg. of alcohol dehydrogenase; c, 2 μ moles of ATP; d, 10 μ moles of 3-PGA.

Table 9. Carbohydrate synthesis from 3-phosphoglycerate

The complete system (3.7 ml.) contained: 50 μ moles of 3-PGA, 0.6 μ mole of DPN, 50 μ moles of ethanol, 3 mg. of alcohol dehydrogenase, 30 μ moles of CoSO_4 , 10 μ moles of ATP, 0.1M tris (pH 7.6) and 0.3 mg. of enzyme N (Hughes press extract extracted with 0.1M tris (pH 7.6) and dialysed for 2 hr. against the same medium at 2°). Incubated for 1 hr. at 30°. 'Carbohydrate' was measured by the anthrone method and expressed as glucose.

	'Carbohydrate' formed (μ moles)
Complete system	3.52
Alcohol dehydrogenase omitted	-0.06
3-PGA omitted	0.07
DPN omitted	0.00
Enzyme omitted	0.00
Ethanol omitted	0.06
ATP omitted	1.43

(Expts. 2 and 3). The oxidation of DPNH by 3-PGA alone in Expt. 1 may have been due to the presence of some ATP in the extract. Expts. 2 and 3 show more clearly that both ATP and 3-PGA were required for TPNH oxidation.

Carbohydrate synthesis from PGA. The ability of extracts to synthesize carbohydrate from 3-PGA in the presence of ATP and a source of DPNH is shown in Table 9. Omission of any one of the components of the system resulted in markedly reduced

Table 10. Formation of phosphoenolpyruvate from 3-phosphoglycerate

The basic mixture (3 ml.) contained: 0.33% of KCl, 0.1M tris (pH 7.1), 60 μ moles of 3-PGA and 3.1 mg. of enzyme N (Hughes press extract, dialysed for 20 hr. against 1% (w/v) KCl at 2°). Incubated for 60 min. at 30° under O_2 -free N_2 . No inorganic phosphate was formed and no keto acid could be detected before hydrolysis (30 min. at 100° in N-HCl).

Changes in system	Iodine-labile phosphate (μ mole)	Keto acid after hydrolysis (μ moles)
None	0.44	0.31
30 μ moles of MgCl_2	0.99	1.07
30 μ moles of MgCl_2 + 37 μ moles of NaF	0.27	0.27
No 3-PGA, 30 μ moles of MgCl_2	0.00	0.00

or negligible synthesis. Paper chromatography of the hydrolysed phosphate ester fraction, isolated from the complete reaction mixture, revealed mainly fructose and glucose with a trace of pentose and 'heptulose'.

Enolase and phosphoglyceromutase. The presence of 2- and 3-PGA and PEP among the end products of CO_2 fixation in the presence of R 5-P suggested that the extracts used contained phosphoglyceromutase. When crude, dialysed extracts were incubated with either 3-PGA or 2-PGA there was a slow production of iodine-labile phosphate, indicating the formation of PEP. A typical experiment with 3-PGA is shown in Table 10. The reaction required Mg^{2+} and was inhibited by fluoride. Treatment of the supernatants with acid or phosphatase liberated a keto acid which, expressed as pyruvate, was equivalent to the iodine-labile phosphate formed. The 2:4-dinitrophenylhydrazone of the keto acid had the same absorption spectrum as that prepared from pyruvate and could not be separated chromatographically from pyruvate 2:4-dinitrophenylhydrazone with either *n*-butanol saturated with water or *n*-butanol-ethanol-water. The rate of PEP formation from PGA was very low in these extracts; the best observed was about 0.4 μ mole of PEP produced/mg. of N/hr.

DISCUSSION

This work has shown that extracts of *T. denitrificans* contain an enzyme which carboxylates RuDP to PGA, the fixed carbon appearing in the carboxyl group of PGA. In this respect the enzyme resembles that found in some photosynthetic tissues (Weissbach *et al.* 1954b). As emphasized by Santer & Vishniac (1955), the demonstration of the carboxylation reaction in extracts, while suggestive, provides no evidence for its role, if any, in the utilization of CO_2 for growth. The demonstration of

PGA as the first stable product during CO₂ fixation by whole cells would be a more direct indication of the function of the carboxylating enzyme.

The presence in extracts of *T. denitrificans* of phosphoglycerokinase, triosephosphate dehydrogenase, aldolase and hexose diphosphatase is indicated by the results reported in Fig. 8 and Table 8. By the combined action of these enzymes PGA can be converted into hexose monophosphate (cf. Table 9).

The reactions by which pentose phosphate is formed from hexose phosphate in a number of tissues are described by equations (3), (4) and (5). The formation of hexose, heptulose and tetrose phosphates from pentose phosphate and the conversion of hexose phosphate into a mixture of heptulose, tetrose and pentose phosphates are in keeping with the presence of transaldolase and transketolase in extracts of *T. denitrificans*. It must be stressed, however, that neither the heptulose nor the tetrose has been identified, and the mechanism of their formation cannot be stated with certainty until the individual enzymes involved have been isolated and characterized. None the less, it is clear that, regardless of the mechanism, *T. denitrificans* is able to make pentose phosphate from hexose phosphate anaerobically and without the loss of CO₂. It is equally clear that this autotroph has enzymes capable of synthesizing hexose phosphates from CO₂ by a cyclic mechanism catalysed by pentose phosphate. The demonstration of the occurrence of a CO₂-fixing system in autotrophic bacteria similar to that found in the green plant (Calvin, 1955) may have considerable phylogenetic significance (cf. Elsdén, 1955).

T. denitrificans is incapable of growing on a wide range of organic compounds (Baalsrud & Baalsrud, 1954). Nevertheless, the work presented in this paper has shown that this organism contains many enzymes common to most heterotrophic organisms (see reviews by Racker, 1954; Gunsalus, Horecker & Wood, 1955). During the course of this work the enzymes glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, aconitase, succinate dehydrogenase, fumarase and malate dehydrogenase were demonstrated in extracts of *T. denitrificans* (Trudinger, 1955, unpublished results). These observations suggest a marked similarity between the internal metabolism of *T. denitrificans* and that of many plant, animal and bacterial cells.

SUMMARY

1. Extracts of the strict chemoautotroph, *T. denitrificans*, fixed ¹⁴CO₂ into the carboxyl group of 3-phosphoglycerate in the presence of either ribose 5-phosphate plus adenosine triphosphate (ATP) or ribulose 1:5-diphosphate alone.

2. The extracts contained phosphoglycerokinase, triose phosphate dehydrogenase, aldolase and hexose diphosphatase, and synthesized hexose phosphates from phosphoglycerate, ATP and reduced diphosphopyridine nucleotide.

3. Ribose 5-phosphate was rapidly metabolized in the absence of ATP with the formation of triose phosphate, hexose monophosphate and two compounds which behaved chromatographically as heptulose and tetrose phosphates.

4. The extracts converted hexose diphosphate into a mixture of pentose, heptulose and tetrose phosphates.

5. Hexose monophosphate isomerase and enolase were also present in the extracts.

6. It is concluded that *T. denitrificans* is capable of synthesizing hexose phosphates from carbon dioxide by a cyclic mechanism similar to that found in green plants.

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Plasma Protein Distribution and Turnover in the Female Baboon

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The menstrual cycle of the baboon is accompanied by periodic variations in the concentration and total mass of circulating protein. The available evidence indicates that these unusual fluctuations are related to the hypertrophy and involution of perineal tissue which occur in step with the menstrual cycle in this species. Thus the mass of circulating protein decreases during perineal growth, which accompanies the follicular phase of the cycle; with the onset of the luteal phase the perineum undergoes rapid involution and, at the same time, the

plasma protein mass increases considerably (Cohen, 1955).

The present study employing ¹²⁵I-labelled plasma protein was undertaken in order to obtain more precise information about variations in plasma protein distribution and turnover in the adult female baboon. The validity of metabolic data obtained through the use of iodinated protein has been seriously questioned in recent years (Berson, Yalow, Schreiber & Post, 1953; Armstrong *et al.* 1955; Masouredis & Beckmans, 1955; Volwiler *et al.* 1955).