Carbon Assimilation by *Pseudomonas oxalaticus* (OX1)

7. DECARBOXYLATION OF OXALYL-COENZYME A TO FORMYL-COENZYME A*

By J. R. QUAYLE†

Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(Received 10 June 1963)

Oxalyl-coenzyme A has been implicated in the decarboxylation of oxalate in an oxalate-grown bacterium by Jakoby, Ohmura & Hayaishi (1956), who suggested this series of reactions:

Quayle, Keech & Taylor (1961) concluded that decarboxylation of oxalate by crude extracts of oxalate-grown *Pseudomonas oxalaticus* followed reactions (1)–(3) except that acetyl-CoA is replaced in (1) by succinyl-CoA and, in addition, a transferase is present capable of transferring the CoA grouping from formyl-CoA to either succinate or oxalate. Koch & Jaenicke (1962) also refer to decarboxylation of oxalate by extracts of a species of *Pseudomonas* in which phosphate, CoA and thiamine pyrophosphate were cofactors for the reaction.

This paper records a study of the decarboxylation of oxalyl-CoA and oxalate in crude extracts of *P. oxalaticus*, and the purification from such extracts of an enzyme that catalyses reaction (2). Part of this work has been communicated to The Biochemical Society (Quayle, 1963*b*).

MATERIALS AND METHODS

Abbreviation. TPP, Thiamine pyrophosphate.

Special chemicals. Oxalyl-CoA was prepared by thiol ester interchange between CoA and thiocresyl hydrogen oxalate and was assayed by the use of oxalyl-CoA reductase (Quayle, 1963*a*). [$^{14}\text{C}_2$]Oxalyl-[^{14}C]CoA was prepared by a similar method from thiocresyl hydrogen [$^{14}\text{C}_2$]oxalate. The latter compound was prepared from [$^{14}\text{C}_2$]oxalyl chloride according to the method of Stolle (1914). Succinyl-CoA was prepared by the reaction of CoA with succinic anhydride (Simon, 1957). Malonyl-CoA was given by Professor F. Lynen (Max Planck Institut für Zellchemie, Munich, Germany).

Oxalate carboxy-lyase (oxalate decarboxylase, EC 4.1.1.2). This enzyme was purified from *Collybia velutipes* according to the method of Shimazono & Hayaishi (1957).

Glyoxylate-nicotinamide-adenine dinucleotide phosphate

* Part 6: Quayle (1963*a*).

† Present address: Department of Biochemistry, University of Sheffield, Sheffield, 10.

oxidoreductase (acylating CoA) (oxalyl-CoA reductase). The enzyme used in these studies was that purified by Quayle & Taylor (1961) and since stored at -15° .

Growth of organisms. *P. oxalaticus* and *Pseudomonas* OD1 were grown in liquid culture, harvested by centrifuging and stored at -15° , (Quayle & Keech, 1960).

Preparation of cell-free extracts. Harvested cells were suspended in 4 vol. of suitable buffer, as indicated in the text, and subjected to ultrasonic vibrations for 3 min. with a 25 kcyc./sec. Mullard ultrasonic generator, type E 75 90 B (Mullard Ltd., London). The resulting suspension was centrifuged at 25 000 g for 20 min. in the high-speed head of an International refrigerated centrifuge (model PR-2). The supernatant was used as such, or after dialysis, as indicated in the text. In some cases the protein in the supernatant was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to a final strength 80% of saturation. The precipitated protein was collected by centrifuging, dissolved in suitable buffer and dialysed (see text).

Purification of the decarboxylating enzyme. Step 1: preparation of bacterial extract. Frozen oxalate-grown bacteria (10.6 g. wet wt.) were suspended in 40 ml. of 20 mm-phosphate buffer, pH 7.0, containing about 1 mg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks.). The suspension was passed three times through a French pressure cell at 0° and the resulting extract centrifuged at 22 000 g for 20 min. at 2° . All subsequent operations were performed at 2° .

Step 2: treatment with protamine sulphate. To the supernatant (30 ml., containing 744 mg. of protein) was added, with stirring, 5 ml. of an aqueous solution containing 74 mg. of protamine sulphate. The resulting precipitate was removed by centrifuging and discarded.

Step 3: ammonium sulphate precipitation. To the supernatant, pH 7.4, was slowly added solid $(\text{NH}_4)_2\text{SO}_4$ to give concentrations 40, 50 and 60% of saturation. At each of these stages the precipitated protein was dissolved in 5 ml. of 20 mm-phosphate buffer, pH 7.0.

Step 4: passage through Sephadex column. The protein fraction that precipitated between 50 and 60% of $(\text{NH}_4)_2\text{SO}_4$ saturation was passed through a column of Sephadex G-50 (medium) (1.4 cm. $\times$ 10 cm.), previously equilibrated with 5 mm-phosphate buffer, pH 7.0, and the protein eluted from the column by washing with the same buffer. The protein appeared in the 6–15 ml. fraction of the eluate and this was subjected to ion-exchange chromatography.

Step 5: ion-exchange chromatography. Diethylamino-ethylcellulose (DEAE-cellulose, Whatman DE 50) (6 g.) was made into a slurry in 5 mm-phosphate buffer, pH 7.0, and the slurry adjusted to pH 7.0 by the addition of 5 mm- KH_2PO_4 . The resin was freed from fine particles by repeated

decantation in 5 mm-phosphate buffer, pH 7.0, and then packed into a chromatographic column (1.5 cm. $\times$ 30 cm.). The enzyme solution was poured on to the top of the column, which was then eluted with an increasing phosphate gradient at pH 7.0. This was formed by connecting together the bottoms of two 500 ml. polythene bottles, the first containing 400 ml. of 0.1M-phosphate buffer, pH 7.0, and the other 400 ml. of 5 mm-phosphate buffer, pH 7.0. The second bottle was stirred mechanically and the outflow was fed on to the top of the column. The levels of the solutions in both bottles dropped at the same rate throughout. Fractions (4.2 ml.) were collected automatically at a flow rate of 42 ml./hr. Under these conditions the decarboxylating enzyme was eluted at concentrations of phosphate between 10 and 13 mm (see Fig. 1). Fractions 28–34 inclusive were combined and the resulting solution was halved: one half was stored at 2° and the other at –15°.

The purification procedure is summarized in Table 1.

Protein estimations. In crude extracts and in steps 1–4 of the purification procedure, protein was determined by the Folin-Ciocalteu method (Lowry, Rosebrough, Farr &

Randall, 1951). The protein concentration of the column eluate was determined by measuring the extinction at 260 and 280 m μ (Warburg & Christian, 1941).

Phosphate determination. Phosphate was determined by the method of Allen (1940).

Assay of enzyme activity. The enzyme was assayed in micro-manometer cups by measuring the rate of CO₂ production in the following reaction mixture at 30°. The main compartment of the Warburg flask contained 50 μ moles of citrate buffer, pH 5.5, 2 μ moles of TPP, 1 μ mole of MgCl₂, 1.5 μ moles of oxalyl-CoA, and water; the side arm contained the enzyme solution; the total volume of the flask contents when mixed was 1 ml. The flasks were flushed with O₂-free N₂ before assaying the enzyme in crude extracts and in steps 1–4 of the purification procedure, otherwise the flasks contained air as gas phase. The enzyme was tipped in from the side arm and the rate of evolution of CO₂ measured over about 15 min. The initial rate, which was linear during decarboxylation of approximately two-thirds of the substrate, was proportional to the amount of enzyme used. One unit of enzyme activity is defined as the amount of

Fig. 1. Elution of oxalyl-CoA decarboxylase from the DEAE-cellulose column. Conditions are given in the text. The continuous line *A* represents the extinction of fractions (vol. 4.2 ml.) at 280 m μ (1 cm. light-path); ●, decarboxylase activity; ▲, gradient of phosphate concentration.

Table 1. *Purification of oxalyl-coenzyme A decarboxylase*

Experimental details are given in the Materials and Methods section.

Step	Fraction	Vol. (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Sp. activity (units/mg. of protein)	Total units	Yield (%)
1	Supernatant obtained from crude extract after centrifuging	30	166	24.8	4.7	3490	100
2	Supernatant obtained after removal of protamine sulphate precipitate by centrifuging	33	112	18.8	5.9	3680	108
3	Fraction precipitated by ammonium sulphate (50–60 %)	5.8	165	14.6	11.3	960	28
4	Eluate from Sephadex column	8.5	123	10.6	11.6	1050	30
5	Selected combined fractions after chromatography on DEAE-cellulose	30	9.4	0.21	44.8*	282	8.1

* The peak of activity was 62.

enzyme that catalyses the evolution of $1\ \mu\text{mole}$ of CO_2 in 1 min. under the conditions of assay. Specific activity is expressed as units/mg. of protein.

Radioactive assay of $^{14}\text{CO}_2$, $^{14}\text{C}_2\text{oxalyl-}^{12}\text{C}\text{CoA}$ and $^{14}\text{C}\text{formate}$. $^{14}\text{CO}_2$ was converted into $\text{Ba}^{14}\text{CO}_3$ by precipitation with BaCl_2 and was then plated on paper disks ($2.69\ \text{cm}^2$) as described by Sakami (1955*a*). The samples were assayed for radioactivity in a gas-flow counter (model D-47, Nuclear Instrument and Chemical Corp., Chicago, U.S.A.), which had an efficiency of 15% under the conditions used. The specific radioactivity of the samples was corrected for self-absorption and expressed as specific activity at infinite thinness.

Study of the enzymic decarboxylation of oxalyl-CoA involved the use of $^{14}\text{C}_2\text{oxalyl-}^{12}\text{C}\text{CoA}$ of known specific activity in terms of $\text{Ba}^{14}\text{CO}_3$ derived from the oxalate carbon. Determination of this specific activity involved the prior hydrolysis of the oxalyl-CoA to oxalate. In one experiment $2.5\ \mu\text{moles}$ of $^{14}\text{C}_2\text{oxalyl-}^{12}\text{C}\text{CoA}$ were placed in 1 ml. of $0.1\ \text{N-NaOH}$ at 30° for 10 min. to hydrolyse it to oxalate and CoA. To this solution were added 1 m-mole of citrate buffer, pH 3.0, and $500\ \mu\text{moles}$ of carrier potassium oxalate, and the total volume was made up to $3.7\ \text{ml.}$ with water. The solution was aerated with CO_2 -free air for 10 min. and then 1 ml. of purified oxalate decarboxylase was added from a dropping funnel. The resulting solution was aerated for 2 hr. at 30° with CO_2 -free air and the evolved $^{14}\text{CO}_2$ was trapped in CO_2 -free NaOH as described by Sakami (1955*b*). The $\text{Na}_2^{14}\text{CO}_3$ was converted into $\text{Ba}^{14}\text{CO}_3$ and assayed as above.

The amount of $^{14}\text{C}\text{formate}$ or $^{14}\text{C}\text{formyl-}^{12}\text{C}\text{CoA}$ or of both produced from enzymic decarboxylation of $^{14}\text{C}_2\text{oxalyl-}^{12}\text{C}\text{CoA}$ was estimated by adding 1 m-mole of carrier sodium formate to the reaction mixture, adjusting to pH 1.5 with H_2SO_4 , and steam-distilling out the formic acid in 50 ml. of distillate. The $^{14}\text{C}\text{formic acid}$ was then decarboxylated by Mg^{2+} ions in acetate buffer, as described by Sakami (1955*c*); the yield of $^{14}\text{CO}_2$ was 70–80% of theoretical. The specific activity of the evolved $^{14}\text{CO}_2$ was measured as $\text{Ba}^{14}\text{CO}_3$, and from this value the yield of $^{14}\text{C}\text{formate}$ or $^{14}\text{C}\text{formyl-}^{12}\text{C}\text{CoA}$ or of both from the $^{14}\text{C}_2\text{oxalyl-}^{12}\text{C}\text{CoA}$ in the original reaction mixture was calculated.

Chromatographic analysis. The papers (Whatman no. 4; $14\ \text{in.} \times 6\ \text{in.}$) were developed in an ascending direction. Acyl-CoA compounds were chromatographed in propan-2-ol-0.1 M-sodium acetate, pH 5.0 (1:1, v/v), ethanol-0.1 M-sodium acetate, pH 4.5 (1:1, v/v) (Koch & Jaenicke, 1962). The spots were located by their absorption of ultraviolet light. Hydroxamates were chromatographed in butan-2-ol-formic acid-water (75:13:12, by vol.) (Vagelos, Earl & Stadtman, 1959) and detected by spraying with 5% (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in ethanol (0.1 N with respect to HCl) (Stadtman & Barker, 1950).

Assay of acyl thiol compounds as hydroxamates. The method used was that of Lipmann & Tuttle (1945). To a solution of acyl thiol compound ($1\text{--}2\ \mu\text{moles}$) in 2 ml. of water was added 1 ml. of 2 M-hydroxylamine (prepared by neutralizing 4 M-hydroxylamine hydrochloride to pH 7.0 with 4 N-KOH) and the solution incubated for 10 min. at room temperature. Ferric chloride reagent [3.0 ml.; 5% (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 0.1 N-HCl-12% (w/v) trichloroacetic acid-3 N-HCl (1:1:1, by vol.)] was then added. The extinction was read at $540\ \text{m}\mu$ and compared with that produced

from a sample of a standard solution prepared by dissolving 1 g. of succinic anhydride in 40 ml. of the neutralized hydroxylamine reagent.

Preparation of buffer solution. Phosphate buffers were prepared by mixing appropriate volumes of equimolar KH_2PO_4 and Na_2HPO_4 to the required pH. Tris-HCl was prepared by dissolving solid tris in water and adjusting to the required pH with HCl. Citrate buffer was prepared by mixing appropriate volumes of 0.5 M-trisodium citrate and 0.5 M-citric acid.

Enzyme assays

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

Formate-NAD oxidoreductase (formate dehydrogenase, EC 1.2.1.2). This was assayed by measuring the rate of reduction of NAD in a Cary recording spectrophotometer at 22° . The complete system contained, in a 1 ml. cuvette (light-path 1 cm.): $50\ \mu\text{moles}$ of phosphate buffer, pH 7.0, $50\ \mu\text{moles}$ of sodium formate, $1\ \mu\text{mole}$ of NAD, enzyme extract, and water to 1 ml. The blank cell lacked NAD. The reaction was started by the addition of enzyme and followed by the change in extinction at $340\ \text{m}\mu$.

NADH₂ oxidase and NADPH₂ oxidase. These enzyme activities were assayed by measuring the rate of oxidation of the reduced coenzyme in a Cary recording spectrophotometer at 22° . The complete system contained, in a 1 ml. cuvette (light-path 1 cm.): $50\ \mu\text{moles}$ of phosphate buffer, pH 7.0, $0.1\ \mu\text{mole}$ of NADH₂ or NADPH₂, enzyme extract, and water to 1 ml. The blank cell lacked reduced coenzyme. The reaction was started by the addition of enzyme and followed by the change in extinction at $340\ \text{m}\mu$.

Oxalyl-CoA reductase. The method of Quayle & Taylor (1961) was used for assay.

RESULTS

Decarboxylation of oxalyl-coenzyme A in crude bacterial extracts

Quayle *et al.* (1961) showed that crude extracts of oxalate-grown *P. oxalaticus* catalyse the decarboxylation of oxalate in the presence of catalytic quantities of succinyl-CoA and TPP. This indicated that the substrate for the decarboxylation was oxalyl-CoA. The isolation of small amounts of oxalyl monohydroxamate from treatment of the incubation mixture with neutral hydroxylamine supported this idea. This has now been confirmed with authentic oxalyl-CoA as substrate. Cell-free extracts prepared from oxalate-grown *P. oxalaticus* catalyse a rapid decarboxylation of oxalyl-CoA, which is dependent on the presence of TPP. In a typical experiment the protein fraction precipitating between 0 and 80% of ammonium sulphate saturation catalysed the evolution of $1.68\ \mu\text{moles}$ of carbon dioxide from $1.49\ \mu\text{moles}$ of oxalyl-CoA (Table 2). When $3\ \mu\text{moles}$ of oxalate were also present in the reaction mixture, evolution of a further $1.66\ \mu\text{moles}$ of carbon dioxide was observed,

Table 2. *Decarboxylation of oxalyl-coenzyme A by cell-free bacterial extracts*

The main compartment of each micro-Warburg flask contained 50 μ moles of tris buffer, pH 7.1, 1 μ mole of TPP, 1 μ mole of $MgCl_2$, additions as indicated, and water to 1 ml.; the side arm contained 0.1 ml. of bacterial extract. The flasks were flushed with O_2 -free N_2 . Incubation was for 25 min. at 30°, by which time CO_2 evolution had ceased in all flasks. The bacterial extract was made from 2 g. wet wt. of bacteria treated ultrasonically in 50 mM-tris, pH 7.1; the protein was precipitated from this by $(NH_4)_2SO_4$, redissolved in 5 ml. of 10 mM-tris, pH 7.1, and dialysed for 3 hr. against 2 l. of the same buffer. The resulting extract contained 14.2 mg. of protein/ml.

Additions (μ moles)	CO_2 evolved (μ moles)
None	0
Oxalyl-CoA (1.49)	1.68
Oxalyl-CoA (1.49) + oxalate (3.0)	3.34
Oxalyl-CoA (1.49) + oxalate (3.0) + succinate (3.0)	3.38
Oxalyl-CoA (1.49) + oxalate (3.0) + phosphate (2.5)	3.34
Oxalate (3.0) + succinate (3.0)	0.60
Oxalyl-CoA (1.49) + iodoacetate (2.0)	1.41
Oxalyl-CoA (1.49) + oxalate (3.0) + iodoacetate (2.0)	3.60

indicating the occurrence of the sequence of reactions (4)–(6).

The fact that addition of succinate did not affect the carbon dioxide evolution showed that the CoA-transfer reaction (5) did not proceed via succinyl-CoA as intermediate. Since the reaction proceeded as well in the absence as in the presence of inorganic phosphate, the involvement of formyl phosphate was precluded. The presence of 2 mM-iodoacetate did not affect the reaction, which indicated that CoA did not become free during the decarboxylation or during the transfer of the CoA grouping to oxalate.

The enzyme responsible for the decarboxylation of oxalyl-CoA was then purified as described under Materials and Methods.

Decarboxylation of oxalyl-coenzyme A by the purified enzyme

Stoichiometry of the decarboxylation. When $[^{14}C_2]$ -oxalyl- $[^{12}C]$ CoA was used as substrate and the reaction mixture acidified to pH 1.5 after the decarboxylation was complete $[^{14}C]$ formate could be steam-distilled out of the mixture. This would be consistent with the primary product being either formate or formyl-CoA, the latter compound hydro-

lysing in the boiling acid during the steam-distillation. The stoichiometry of this transformation was established by isotope-dilution methods. The specific activity of $[^{14}C_2]$ oxalyl- $[^{12}C]$ CoA in terms of derived $Ba^{14}CO_3$ was measured as detailed under Materials and Methods. Into the main compartments of each of two double-side-arm Warburg flasks were placed 100 μ moles of phosphate buffer, pH 7.0, 5 μ moles of TPP, 10 μ moles of $MgCl_2$, 5 μ moles of $[^{14}C_2]$ oxalyl- $[^{12}C]$ CoA (specific activity of $Ba^{14}CO_3$ derived from the $[^{14}C]$ oxalate residue was 86.9 counts/min./mg. under the conditions of assay) and water to 2.2 ml.; into the centre well of one flask was placed 0.2 ml. of 2.5N-sodium hydroxide; into one side arm of each pair was placed 0.2 ml. of enzyme, into the other side arm of each pair was placed 0.1 ml. of 5N-sulphuric acid. A control flask similar to the flask without alkali in the centre well was also set up; 0.2 ml. of boiled enzyme was substituted in place of the unboiled enzyme. The three manometer flasks were equilibrated at 30°, the enzyme was tipped in from the side arm and carbon dioxide evolution followed in the flask that contained no alkali in the centre well. The reaction was complete in 27 min. and at this point the acid was tipped in and the flasks were shaken at 30° for a further 10 min. No evolution of gas occurred in the control flask or in the flask with alkali in the centre well; 5.3 μ moles of carbon dioxide were evolved in the flask without alkali in the centre well. To the main compartment of each flask was added 1 mmole of carrier sodium formate, and to the appropriate centre well 1.33 ml. of 0.75M-sodium carbonate. The specific activities of the $[^{14}C]$ formic acid obtained by steam-distillation of the flask contents, and that of the sodium $[^{14}C]$ carbonate in the centre well, was determined as described under Materials and Methods. It was found that the formate from the control flask contained negligible radioactivity. The mean specific radioactivity of the $[^{14}C]$ formic acid obtained from the other two flasks was 78.0 counts/min./mg. of barium carbonate; that of the sodium carbonate in the centre well was 85.2 counts/min./mg. of barium carbonate. From these values it follows that in this experiment decarboxylation of 5 μ moles of oxalyl-CoA gave 4.5 μ moles of formate or formyl-CoA or of both and 4.9 μ moles of carbon dioxide. Experiments described below established that the primary product was formyl-CoA rather than formate.

Chromatography and electrophoresis of the reaction product. Decarboxylation of 2 μ moles of $[^{14}C_2]$ -oxalyl- $[^{12}C]$ CoA was carried out in the reaction mixture used for the enzyme assay (see Materials and Methods section); when the reaction was complete samples of the mixture were analysed by chromatography and high-voltage electrophoresis. In both chromatographic solvent systems used, a

single ultraviolet-absorbing spot was visible in the sample from the reaction mixture after decarboxylation. It ran at the same R_f as both CoA and the oxalyl-CoA contained in a similar reaction mixture in which boiled enzyme had been substituted for active enzyme. No solvent system was found that would clearly separate these compounds in the presence of the salts used in the incubation mixture. However, the spot obtained from the reaction mixture after decarboxylation contained one-half of the radioactivity contained in the oxalyl-CoA used as substrate.

A separation was obtained by high-voltage electrophoresis in 0.2M-sodium acetate, pH 5.0. After 40 min. at 6 kv, the following migrations had taken place on strips of Whatman no. 4 paper (10 cm. $\times$ 60 cm.): CoA, 21.2 cm.; oxalyl-CoA, 25.5 cm.; the product of decarboxylation, 19 cm. As with chromatography, the reaction product contained one-half of the radioactivity contained in the oxalyl-CoA used as substrate. No improvement in the electrophoretic separation was effected by lowering the pH of the acetate buffer to 4.0, or substituting formate buffer at pH 3.0 in place of the acetate buffer. The lower mobility of the reaction product as compared with oxalyl-CoA is consistent with its being formyl-CoA, which would contain one less carboxyl group than oxalyl-CoA.

Formation of hydroxamate. It was found that after the decarboxylation of oxalyl-CoA, a sample of the reaction mixture when treated with neutral hydroxylamine followed by ferric chloride gave the brown colour characteristic of a ferric hydroxamate. The extinction of the ferric hydroxamate at 540 $m\mu$ was almost the same at the end of the decarboxylation as that formed from the oxalyl-CoA in a boiled control (Table 3). In this decarboxylation experiment

1.79 μ moles of oxalyl-CoA (as assayed with purified oxalyl-CoA reductase) gave 1.6 μ moles of carbon dioxide and 1.62 μ moles of acyl hydroxamate as standard. The identity of the hydroxamate formed after the decarboxylation was established in a separate experiment. Into the main compartments of each of two single-side-arm micro-Warburg flasks were placed 50 μ moles of phosphate buffer, pH 7.0, and 2 μ moles of TPP and 10 μ l. of oxalyl-CoA decarboxylase was placed in one flask, 10 μ l. of previously boiled enzyme was placed in the other; 2.1 μ moles of [$^{14}C_2$]oxalyl-[^{12}C]CoA were placed in each side arm; the total contents of each flask were made up to 1 ml. with water. After equilibration at 30°, the substrate was tipped in and the flasks were incubated until carbon dioxide evolution had ceased in the test flask (20 min.); negligible gas evolution occurred in the control flask. At the end of this time 0.5 ml. of neutral 2M-hydroxylamine was added to each flask and they were left for 10 min. at room temperature. Ethanol (25 ml.) was then added to each flask, the precipitates were centrifuged and discarded, and the supernatants evaporated to dryness at 30° under reduced pressure. The solid residues were each extracted with 2 ml. of ethanol and the supernatants each evaporated to 0.2 ml. Samples of these were chromatographed in butan-2-ol-formic acid-water and sprayed with a ferric chloride spray. A single radioactive hydroxamate, R_f 0.7, which co-chromatographed with authentic formyl hydroxamate, was detected in the decarboxylated sample but was absent in the boiled control. The latter showed a single radioactive hydroxamate with R_f 0.51.

Spectral changes during decarboxylation. The decarboxylation of oxalyl-CoA is accompanied by a large decrease in the absorption maximum at 260 $m\mu$ and the appearance of a new maximum at 235 $m\mu$ (Fig. 2). These spectra are recorded as difference spectra in which amounts of CoA and TPP equivalent to those present in the test cell have been added to the blank cell. Since the acyl thiol group in oxalyl-CoA has an absorption peak at 260–270 $m\mu$ (Koch & Jaenicke, 1962; Quayle, 1963a), and in *S*-formyl-*N*-acetylcysteamine at 234 $m\mu$, the change in the difference absorption spectrum observed during the decarboxylation of oxalyl-CoA is consistent with that which may be predicted for the transformation of oxalyl-CoA into formyl-CoA. Assuming the complete decarboxylation of the oxalyl-CoA used in this experiment, a value of 4.1×10^3 may be calculated for the molecular extinction coefficient of formyl-CoA at 235 $m\mu$ in 50 mM-phosphate buffer, pH 6.5. This may be compared with the value of 4.8×10^3 for *S*-formyl-*N*-acetylcysteamine in water (Koch & Jaenicke, 1962). The combined results of the foregoing experiments

Table 3. *Formation of hydroxamate from the product of decarboxylation of oxalyl-coenzyme A*

Incubations with unboiled enzyme were performed in single-side-arm micro-manometer flasks; that involving boiled enzyme was in a test tube. The reaction mixtures were: 50 μ moles of citrate buffer, pH 5.5, 2 μ moles of TPP, 1 μ mole of $MgCl_2$, additions as indicated below, water to 1 ml. The enzyme solution was added from the side arms of Warburg flasks. Incubation was for 12 min. at 30°. Samples (0.9 ml.) were withdrawn, treated with hydroxylamine and ferric chloride, and the extinctions measured at 540 $m\mu$ (see Materials and Methods section). Yields of acyl hydroxamate were calculated with succinyl hydroxamate as standard.

Additions	CO ₂ evolved (μ moles)	$E_{540\text{ m}\mu}$	Acyl hydroxamate (μ moles)
Oxalyl-CoA (1.79 μ - moles) + enzyme (10 μ l.)	1.60	0.140	1.62
Enzyme (10 μ l.)	0.09	0	0
Oxalyl-CoA (1.79 μ moles)	—	0.135	1.56

Fig. 2. Spectral changes during the decarboxylation of oxalyl-CoA. Two solutions were made up, containing: 50 μ moles of phosphate buffer, pH 6.5, 2 μ moles of TPP, 0.41 μ mole of oxalyl-CoA in the test solution, 0.41 μ mole of CoA in the blank solution and water to 1 ml. A portion (0.1 ml.) of each solution was diluted to 1 ml. with 50 mM-phosphate buffer, pH 6.5, and the absorption spectrum of the test solution measured against the blank solution in a Cary recording spectrophotometer (curve A). Oxalyl-CoA decarboxylase (0.02 ml.) was added to both undiluted solutions, which were then incubated for 10 min. at room temperature. Samples (0.1 ml.) were withdrawn, diluted with buffer and the spectrum was recorded as before (curve B).

on the decarboxylation of oxalyl-CoA show that the product of decarboxylation is formyl-CoA, and thus the decarboxylation reaction is as represented in reaction (2). The enzyme may be termed oxalyl-CoA carboxy-lyase, with trivial name oxalyl-CoA decarboxylase.

Properties of formyl-coenzyme A

The difference spectrum of formyl-CoA in Fig. 2 shows a slight peak at 275 $m\mu$. It seems unlikely that this spectral feature is caused by formyl-CoA itself, because changes in absorption at 234 $m\mu$ due to decomposition of the formyl-CoA are not accompanied by parallel changes in the peak at 275 $m\mu$ (Table 4). Also, the spectrum of *S*-formyl-*N*-acetylcysteamine shows virtually no absorption beyond 260 $m\mu$. Careful purification of the formyl-CoA, however, is needed to settle this point.

The stability of formyl-CoA was examined by following the changes in its difference absorption spectrum after storage at -18° , 3° and after heating. These results are given in Table 4 and show formyl-CoA to be fairly stable at pH 6.5.

Table 4. *Stability of formyl-coenzyme A*

Samples of the undiluted test and blank solutions after incubation with decarboxylase, as described in Fig. 2, were treated as shown below. A portion (0.1 ml.) of each solution was then diluted to 1 ml. with 50 mM-phosphate buffer, pH 6.5, and the absorption spectrum of the test solution measured against the blank solution in a Cary recording spectrophotometer.

Treatment	$E_{235\text{ m}\mu}$	$E_{275\text{ m}\mu}$	$\frac{E_{275\text{ m}\mu}}{E_{235\text{ m}\mu}}$
None, spectrum the same as in Fig. 2	0.17	0.04	0.24
Stored at -15° for 24 hr.	0.14	0.02	0.14
Stored at $+3^\circ$ for 24 hr.	0.11	0.02	0.18
Solution stored at -15° for 24 hr. was heated at 50° for 5 min.	0.14	0.02	0.14
Solution stored at -15° for 24 hr. was heated at 100° for 5 min.	0.04	0.02	0.50

Sparking of oxalate decarboxylation in crude bacterial extracts by formyl-CoA. It has been shown that oxalyl-CoA sparks the decarboxylation of oxalate in crude extracts of oxalate-grown *P. oxalaticus*; this is consistent with the operation of the cyclical series of reactions (4)–(6). Such a cycle would demand that formyl-CoA should also be capable of sparking oxalate decarboxylation in crude extracts. The experiment shown in Fig. 3 demonstrates that this is indeed so. In this experiment 3.8 μ moles of formyl-CoA were first prepared by decarboxylating 3.8 μ moles of oxalyl-CoA in a micro-Warburg flask. When the carbon dioxide evolution had ceased (20 min.) the contents of the flask were split into two equal portions and then incubated with crude extract in a further series of micro-Warburg flasks (Fig. 3). The crude extract catalysed little evolution of carbon dioxide from either formyl-CoA or oxalate separately; when these substrates were present together a rapid evolution of 2.1 μ moles of carbon dioxide was catalysed. No such effect was observed in the absence of the crude bacterial extract, showing that the purified oxalyl-CoA decarboxylase was free of a formyl-CoA-oxalyl-CoA transferase catalysing reaction (5).

Properties of purified oxalyl-coenzyme A decarboxylase

Requirement for TPP. The activity of the enzyme was almost completely dependent on added TPP (Fig. 4), the rate of decarboxylation in its absence being very small.

If a reaction mixture without TPP was set up in a double-side-arm Warburg flask and incubated for 15 min. at 30° before 2 μ moles of TPP were added from the second side arm, the total carbon dioxide evolved during both periods of incubation was equal to that obtained when 2 μ moles of TPP were

present in the incubation mixture from the outset. This showed the absence of any oxalyl-CoA-deacylase activity in the purified enzyme.

Requirement for metal ions. Examination of the enzyme immediately after purification failed to show a requirement for metal ions (Quayle, 1963*b*). However, a re-examination of the enzyme after 5 months' storage at -15° showed a 60% stimulation in rate when the decarboxylation was performed in the presence of 5 mM-magnesium chloride as compared with that in its absence (Table 5). Dialysis of the enzyme for 17 hr. against 10 mM-EDTA did not reduce the rate of decarboxylation observed in the absence of magnesium chloride; stimulations in rate of 60 and 69% were effected by

Fig. 3. Sparking of oxalate decarboxylation by formyl-CoA. A solution of formyl-CoA was prepared by decarboxylating at 30° 3.8 μ moles of oxalyl-CoA in a micro-manometer flask containing 100 μ moles of phosphate buffer, pH 7.0, 4 μ moles of TPP, 20 μ l. of oxalyl-CoA decarboxylase and water to 2 ml. When the CO_2 evolution had ceased, half of the resulting formyl-CoA solution was added to the main compartment of each of two fresh micro-Warburg vessels (A and B). To the main compartment of a control flask C were added 50 μ moles of phosphate buffer, pH 7.0, and 2 μ moles of TPP. Sodium oxalate (3 μ moles) was also added to A and C. Crude extract (0.1 ml.) was placed in each of the three side arms. The total volume in each flask was made up to 1.13 ml. with water. After equilibration at 30° the enzyme extract was tipped in and CO_2 evolution measured. The bacterial extract was made by ultrasonic treatment of 1 g. (wet wt.) of oxalate-grown *P. oxalaticus* in 5 ml. of 50 mM-phosphate buffer, pH 7.0, followed by centrifuging and dialysis for 5 hr. against 2 l. of the same buffer at 0° .

the addition to the dialysed enzyme of 5 mM-magnesium chloride and 5 mM-manganese chloride respectively. Zinc chloride (5 mM) caused a 6% increase in rate. The presence of 1 mM-EDTA in a magnesium-free reaction mixture during the decarboxylation of oxalyl-CoA caused a 40% inhibition in rate.

Stability of the enzyme. The enzyme was more stable to storage at -15° than at 3° . It was com-

Fig. 4. Dependence of oxalyl-CoA-decarboxylase activity on the presence of TPP. Incubations were performed in micro-Warburg flasks under the same conditions and with the same reaction mixture as those used for routine enzyme assay except that varying amounts of TPP were added as indicated: $\circ$, 2 μ moles; $\bullet$, 0.5 μ mole; $\triangle$, 0.1 μ mole; $\blacktriangle$, none.

Table 5. Metal-ion requirement of oxalyl-coenzyme A decarboxylase

Purified enzyme (2.5 ml.) was dialysed for 17 hr. at 3° against 1 l. of water containing 10 m-moles of EDTA and 20 m-moles of potassium phosphate, pH 7.0. Enzyme activity was assayed in micro-Warburg flasks containing, in the main compartment: 50 μ moles of citrate buffer, pH 5.5, 2 μ moles of TPP, 2 μ moles of oxalyl-CoA, additions as indicated; in the side arm was 0.025 ml. of enzyme solution; total volume of cup contents, 1 ml. Incubation was at 30° . Enzyme activity is expressed as μ l. of CO_2 evolved/min. under the conditions of assay; the observed rates were linear over 20 min.

Pretreatment of enzyme	Additions	Enzyme activity (μ l. of CO_2 /min.)
Undialysed*	None	2.35
	EDTA (1 μ mole)	1.40
Undialysed	None	1.60
	MgCl_2 (5 μ moles)	2.60
Dialysed against EDTA	None	1.60
	MgCl_2 (5 μ moles)	2.60
	MnCl_2 (5 μ moles)	2.70
	ZnCl_2 (5 μ moles)	1.70

* 0.05 ml. of enzyme was used.

pletely inactivated after a few minutes of heating at 50° (Table 6).

pH optimum. The pH optimum for the decarboxylation of oxalyl-CoA was 6.6 in phosphate buffer (Fig. 5).

K_m for oxalyl-CoA. The rates of decarboxylation of oxalyl-CoA at various concentrations were measured in double-side-arm micro-Warburg flasks with [^{14}C]oxalyl- ^{12}C]CoA of known specific activity as substrate. The evolved carbon dioxide was trapped in the centre wells with alkali and assayed by isotope-dilution methods. In this

Table 6. *Stability of oxalyl-coenzyme A decarboxylase*

Temp. of storage	Time of storage (weeks)	Percentage of initial activity remaining
-15°	7	55
	20	17
3	1	73
	3	42
	4	39
	7	13
50	5 min.	0

Fig. 5. pH optimum of oxalyl-CoA decarboxylase. Activity of the enzyme was measured manometrically in double-side-arm micro-Warburg flasks. For each pH value a pair of flasks was used, into the main compartment of each were placed 50 μmoles of phosphate buffer, 2 μmoles of TPP, 2 μmoles of oxalyl-CoA (in test flask only), water to 0.85 ml.; into one side arm was placed 5 $\mu\text{l.}$ of decarboxylase, into the other 0.1 ml. of 5N- H_2SO_4 . The flasks were incubated for 10 min. at 30°; the reaction was stopped by tipping in the acid and the total CO_2 evolved was measured after incubation for a further 5 min. Activity of the enzyme is expressed as the difference in total CO_2 output between the test and control flasks.

experiment the flasks contained, in the main compartment: 100 μmoles of phosphate buffer, pH 6.5, 1 μmole of TPP, 0.58–0.043 μmole of [^{14}C]oxalyl- ^{12}C]CoA (specific activity of the [^{14}C]oxalyl moiety was 11.5×10^3 counts/min./mg. of $\text{Ba}^{14}\text{CO}_3$ at infinite thinness) and water to 1.45 ml.; the centre wells contained: 0.2 ml. of 2.5N-sodium hydroxide (carbon dioxide-free); the two side arms contained respectively 0.05 ml. of enzyme and 0.2 ml. of trichloroacetic acid (50 %, w/v). After addition of the enzyme, the flasks were incubated for 15 min. at 30°, the reaction was stopped by tipping in the trichloroacetic acid and the flasks were then shaken for a further 30 min. A portion (0.2 ml.) of 0.75M-sodium carbonate was added to each centre well and the contents were made up to 4.1 ml. The carbonate in duplicate samples (2 ml.) of each of these centre-well solutions was precipitated as barium carbonate and the radioactivity assayed as described in the Materials and Methods section. The plot of the reciprocal of the reaction velocity against the reciprocal of the substrate concentration gave a straight line (Lineweaver & Burk, 1934), from which K_m for oxalyl-CoA was found to be 1 mM.

Specificity. Under the conditions of routine enzyme assay (Materials and Methods section) oxalate, malonate, succinate, malonyl-CoA or succinyl-CoA were not decarboxylated at a measurable rate.

Reversibility. To test for reversibility of the enzyme the following experiment was carried out in double-side-arm micro-Warburg flasks. Into the main compartment of each of two flasks were placed 50 μmoles of phosphate buffer, pH 7.0, 2 μmoles of TPP, 1.3 μmoles of oxalyl-CoA, 1.59 μmoles of sodium [^{14}C]carbonate (containing 2 μC of ^{14}C) and water to 0.88 ml.; each pair of side arms contained respectively 0.02 ml. of decarboxylase (previously boiled in the control flask) and 0.1 ml. of trichloroacetic acid (50 %, w/v). After the enzyme was tipped in the flasks were incubated for 7 min. at 30°. At this point 75 % of the substrate had been decarboxylated in the test flask; the reaction was then stopped by tipping in the trichloroacetic acid. Samples (0.1 ml.) of the flask contents were plated out on metal planchets and assayed for radioactivity. No difference was detected between the planchets from the test and control flasks; they contained only 3 counts/min. above background. Thus there is no evidence that the reaction is reversible to any significant extent.

Contaminating enzymes. The oxalyl-CoA decarboxylase was free of the following enzyme activities: glyoxylate carboligase, formate dehydrogenase, NADH_2 -oxidase, NADPH_2 -oxidase, oxalyl-CoA reductase. Previously described experiments showed that the enzyme was free of oxalyl-CoA deacylase or formyl-CoA-oxalyl-CoA transferase.

Inhibitors. Oxalyl-CoA decarboxylase was not inhibited by mM-iodoacetate or mM-*N*-ethylmaleimide; it was completely inhibited by 2.5 mM-*p*-chloromercuribenzenesulphonate.

Induced synthesis. Oxalyl-CoA-decarboxylase activity was only detected in *P. oxalaticus* when the organism was grown on oxalate. No activity could be found in cell-free extracts of the organism grown on formate or succinate.

Oxalyl-CoA decarboxylase in oxalate-grown *Pseudomonas* OD1. The enzyme was found to be present in oxalate-grown *Pseudomonas* OD1 (Jayasuriya, 1955) at a specific activity 0.62 unit/mg. of protein under standard assay conditions.

DISCUSSION

Reactions in which TPP functions as a coenzyme involve substrates that possess aldehyde or ketone groups, and it is now well established that the active intermediate in such reactions is formed from nucleophilic attack by C-2 of the thiazolium zwitterion on to the carbonyl carbon atom of the substrate (Breslow, 1957; Holzer, 1961) (see reaction 7).

In a *S*-ester, $R \cdot S \cdot CO \cdot R$, owing to the relative inertness of the electrons in the M shell of the S atom to participate in resonance with the CO group, the latter group shows more pronounced carbonyl character, i.e. polarization such as (I), than in an *O*-ester, $R \cdot O \cdot CO \cdot R$, where the resonance shown in (II) is possible (Jaenicke & Lynen, 1960).

In an oxalyl thiol ester, $R \cdot S \cdot CO \cdot CO_2H$, the carbonyl character of the *S*-acyl oxygen atom would be further strongly enhanced by the electron-withdrawing CO_2H group or weakly enhanced by the carboxylate ion CO_2^- . Hence on chemical grounds it is to be expected that the *S*-acyl oxygen atom in oxalyl-CoA would have pronounced carbonyl reactivity. Koch & Jaenicke (1962) have interpreted a series of spectral measurements on *S*-acyl compounds, including mono- and di-thiol esters of oxalic acid, in terms of this increased carbonyl character. Oxalyl-CoA can thus be expected to possess some of the characteristic chemical properties of an α -oxo acid. The mechanism of reaction of TPP as a coenzyme in de-

carboxylation of α -oxo acids is now well established (Holzer, 1961) and an analogous reaction can be suggested for the decarboxylation of oxalyl-CoA (Schemes 1 and 2). In Scheme 1 the nucleophilic C-2 of the thiazole ring of TPP attacks the electrophilic carbon atom of the *S*-acyl group, giving an intermediate which, after decarboxylation, yields a thiohemiacetal derivative of TPP. This compound then cleaves to give formyl-CoA and TPP. In the alternative Scheme 2, the initial condensation of TPP with oxalyl-CoA is assisted by the simultaneous cleavage of CoA, which is then held on an adjacent site on the enzyme. This would give oxalyl-TPP, which on decarboxylation would yield formyl-TPP. Thiolysis of the formyl-TPP by the enzyme-bound CoA would result in formyl-CoA and TPP. Scheme 1 would seem more likely chemically than Scheme 2; it also avoids the involvement of acyl-TPP compounds such as oxalyl- and formyl-TPP. Studies of model compounds by Breslow & McNelis (1960) and White & Ingraham (1960) have shown the instability of 2-acyl thiazolium salts towards hydrolysis with water at room temperature. It is not possible to rule out Scheme 2 on these grounds as it is possible that as a consequence of specific enzyme action the enzyme-bound formyl-TPP might be thiolysed by the contiguous CoA group rather than by water. However, Scheme 1 avoids any such difficulties, as the thiohemiacetal intermediate would not be expected to be sensitive towards hydrolysis with water. In both these Schemes it is assumed that the CoA molecule does not leave the enzyme-substrate complex at any stage. Unequivocal proof of this has not been obtained. Although the reaction is not affected by the presence of mM-iodoacetate or mM-*N*-ethylmaleimide, the speed at which these reagents react with thiol groups may not be fast enough for their lack of effect to be taken as proof of the absence of free CoA during the decarboxylation. *p*-Chloromercuribenzenesulphonate reacts extremely rapidly with thiol groups and indeed completely inhibits the decarboxylase; however, the inhibition may be of the enzyme rather than due to trapping of an intermediate.

Scheme 1 may be compared with a proposed scheme of reactions for the mechanism of reaction of phosphoketolase (Holzer & Schröter, 1962). In this reaction sequence a phosphorylated TPP intermediate is formed, which cleaves to acetyl phosphate and TPP (reaction 8).

Scheme 1

Scheme 2

Schemes 1 and 2. Alternative reaction mechanisms for decarboxylation of oxalyl-CoA. E, Enzyme.

The role of formyl-CoA in the metabolism of oxalate by *P. oxalaticus* is, as far as is known, merely that of an intermediary metabolite between oxalate and formate. The enzymic transfer of the CoA group from formyl-CoA to oxalate is clearly of advantage in conserving the energy of the *S*-acyl bond, synthesis of which involves the ultimate expenditure of a molecule of ATP (Quayle *et al.* 1961). Reports of the involvement of formyl-CoA in metabolism other than oxalate metabolism is limited to CoA transfer in *Clostridium kluyverii*. Stadtman (1953) found an enzyme system in this organism that catalysed the transfer of CoA from acetyl-CoA to saturated fatty acids containing one to eight carbon atoms. Lieberman (1954) investigated a formate-catalysed, CoA-dependent, enzymic decomposition of acetyl phosphate in extracts of *C. kluyverii*. Formyl hydroxamate was isolated from the reaction mixture but no direct proof was obtained that formyl-CoA was the precursor of the formyl hydroxamate. Sly & Stadtman (1962) have reported that three enzymic steps (reactions 9–11) are involved in acetyl phosphate decomposition by extracts of *C. kluyverii*.

These authors also report the presence of a formate kinase in extracts of *Clostridium cylindrosporum*, which catalysed the formation of formyl hydroxamate from formate, ATP and hydroxylamine, and of a phosphotransacetylase catalysing the arsenolysis of formyl-CoA. It will be interesting to see if formyl-CoA is found to participate in reactions additional to those of CoA transfer as it should possess some of the reactivity of formaldehyde itself towards condensation reactions, and thus should have the potentiality of being a C_1 unit active in biosynthetic reactions.

SUMMARY

1. Cell-free extracts of oxalate-grown *Pseudomonas oxalaticus* catalyse the decarboxylation of oxalyl-CoA and the decarboxylation of oxalate in the presence of catalytic quantities of oxalyl-CoA.

2. An enzyme has been purified from the cell-free extract which catalyses the decarboxylation of oxalyl-CoA to formyl-CoA. The formyl-CoA has been characterized by chromatography, electrophoresis, formation of hydroxamate, absorption spectrum and hydrolysis to formate. Formyl-CoA is capable of sparking the decarboxylation of oxalate by crude extracts of *Pseudomonas oxalaticus*.

3. The oxalyl-CoA decarboxylase requires thiamine pyrophosphate as cofactor and is stimulated

by the presence of Mg^{2+} or Mn^{2+} ions. The decarboxylation is unaffected by the presence of iodoacetate or *N*-ethylmaleimide. The pH optimum for the decarboxylation is 6.6. No evidence could be found for reversibility of the reaction. K_m for oxalyl-CoA was found to be 1 mM. The enzyme did not catalyse the decarboxylation of oxalate, malonate, succinate, malonyl-CoA or succinyl-CoA.

4. Oxalyl-CoA decarboxylase has been found in *Pseudomonas oxalaticus* only when grown on oxalate. It has also been found in oxalate-grown *Pseudomonas* OD 1.

5. Possible reaction mechanisms for the decarboxylation of oxalyl-CoA are discussed.

I thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement and Miss A. West for technical assistance. This work was supported in part by the Rockefeller Foundation and the United States Public Health Service, and also by the United States Air Force under Grant no. AF-EOAR 62-45 through the European Office, Office of Aerospace Research.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
- Breslow, R. (1957). *J. Amer. chem. Soc.* **79**, 1762.
- Breslow, R. & McNelis, E. (1960). *J. Amer. chem. Soc.* **82**, 2394.
- Holzer, H. (1961). *Angew. Chem.* **73**, 721.
- Holzer, H. & Schröter, W. (1962). *Biochim. biophys. Acta*, **65**, 271.
- Jaenicke, L. & Lynen, F. (1960). In *The Enzymes*, vol. 3, p. 3. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
- Jakoby, W. B., Ohmura, E. & Hayaishi, O. (1956). *J. biol. Chem.* **222**, 435.
- Jayasuriya, G. C. N. (1955). *J. gen. Microbiol.* **12**, 419.
- Koch, J. & Jaenicke, L. (1962). *Liebigs Ann.* **652**, 129.
- Kornberg, H. L. & Gotto, A. M. (1961). *Biochem. J.* **78**, 69.
- Lieberman, I. (1954). *Arch. Biochem. Biophys.* **51**, 350.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Quayle, J. R. (1963a). *Biochem. J.* **87**, 368.
- Quayle, J. R. (1963b). *Biochem. J.* **87**, 40f.
- Quayle, J. R. & Keech, D. B. (1960). *Biochem. J.* **75**, 515.
- Quayle, J. R., Keech, D. B. & Taylor, G. A. (1961). *Biochem. J.* **78**, 225.
- Quayle, J. R. & Taylor, G. A. (1961). *Biochem. J.* **78**, 611.
- Sakami, W. (1955a). *Handbook of Isotope Tracer Methods*, p. 5. Cleveland, Ohio: Department of Biochemistry, Western Reserve University.
- Sakami, W. (1955b). *Handbook of Isotope Tracer Methods*, p. 8. Cleveland, Ohio: Department of Biochemistry, Western Reserve University.

- Sakami, W. (1955c). *Handbook of Isotope Tracer Methods*, p. 55. Cleveland, Ohio: Department of Biochemistry, Western Reserve University.
- Shimazono, H. & Hayaishi, O. (1957). *J. biol. Chem.* **227**, 151.
- Simon, E. (1957). *Biochem. Prep.* **5**, 30.
- Sly, W. S. & Stadtman, E. R. (1962). *Fed. Proc.* **21**, 249.
- Stadtman, E. R. (1953). *J. biol. Chem.* **203**, 501.

- Stadtman, E. R. & Barker, H. A. (1950). *J. biol. Chem.* **184**, 769.
- Stolle, R. (1914). *Ber. dtsh. chem. Ges.* **47**, 1131.
- Vagelos, P. R., Earl, J. M. & Stadtman, E. R. (1959). *J. biol. Chem.* **234**, 765.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
- White, F. G. & Ingraham, L. L. (1960). *J. Amer. chem. Soc.* **82**, 4114.

Biochem. J. (1963) **89**, 503

Nitrate Reductase in Cell-Free Extracts of a Haemin-Requiring Strain of *Staphylococcus aureus*

BY JOAN P. CHANG AND JUNE LASCELLES

Microbiology Unit, Department of Biochemistry, University of Oxford

(Received 20 May 1963)

Staphylococcus aureus strain SG511 Var differs from the parent organism, SG511A, in being streptomycin-resistant and in requiring haemin for growth in certain media (Jensen & Thofern, 1953). There is, however, no haemin requirement if the medium is supplemented with acetate plus purines and uracil (Lascelles, 1956). Organisms grown in the absence of haemin neither respire nor exhibit catalase activity, but addition of haemin to the suspensions activates them in both respects (Jensen & Thofern, 1953; Jensen, 1957; Gardner & Lascelles, 1962). The fact that the suspensions respond to haemin suggests that organisms grown without this factor nevertheless contain proteins that can combine with haemin to give functional haemoproteins.

Anaerobic growth of the mutant strain with nitrate as final electron acceptor is dependent upon haemin, suggesting that the staphylococcal enzyme system for reduction of nitrate to nitrite (nitrate reductase) has a haemin component. This is supported by the finding that suspensions of organisms grown without haemin reduce nitrate but only provided that haemin is added; this forms the basis for a sensitive assay for haemin (Lascelles, 1956).

Enzyme systems that catalyse the reduction of nitrate to nitrite with reduced dyes as electron donor have been isolated from other bacteria capable of anaerobic growth with nitrate as terminal acceptor (Taniguchi & Itagaki, 1960; Fewson & Nicholas, 1961). These studies have provided evidence that cytochromes participate in electron transfer to nitrate with donors such as NADH₂ but that the terminal enzyme itself is not a haemoprotein. It seems likely therefore that the effect of haemin in nitrate reduction by the mutant strain of *Staphylococcus* is due to its conversion into a cytochrome that mediates in electron transfer to nitrate.

In the present work some of the properties of the staphylococcal nitrate-reductase system have been studied in cell-free preparations with particular reference to the action of haemin. Conditions affecting the formation of the enzyme system and its localization within the cell have been examined. Evidence was sought for the presence of a protein component in cell-free preparations that could combine with haemin to give a haemoprotein active in the nitrate-reductase system.

MATERIALS AND METHODS

Organisms. The parent and mutant strains of *Staphylococcus aureus* (SG511A and SG511Var) were those isolated and described by Jensen & Thofern (1953). Maintenance of stock cultures and other details have been previously given (Lascelles, 1956; Gardner & Lascelles, 1962).

Medium. The basal medium described by Gardner & Lascelles (1962) was supplemented with sodium acetate (10 mM) and adenine, xanthine and uracil (each 0.1 mM). In the present work this supplemented medium is referred to as 'basal medium' and additions of nitrate and haemin were made as noted in the text.

Growth of organisms and preparation of extracts. Bulk cultures were sown with organisms that had been sub-cultured once through the basal medium; about 5 ml. of such a culture was used to sow 1 l. of medium. Organisms were normally grown in static culture in Erlenmeyer flasks filled to the neck, and incubation was for 18–24 hr. at 37°. Aerobic cultures (200 ml. in 2 l. Erlenmeyer flasks) were incubated on a reciprocating shaker operating at about 110 cyc./min.

The harvesting of cultures and preparation of cell-free extracts by shaking with glass beads have been described by Gardner & Lascelles (1962).

Fractionation of extracts. The crude extracts (Gardner & Lascelles, 1962) were spun for 60 min. at 100 000 g in a Spinco ultracentrifuge model L. After removal of the supernatant ('soluble fraction') the gelatinous pellet was rinsed twice with 40 mM-potassium phosphate