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Tricarboxylic Acid-Cycle Activity in *Streptomyces olivaceus*

BY P. K. MAITRA AND S. C. ROY
Department of Applied Chemistry, University College of Science and Technology, Calcutta 9, India

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The tricarboxylic acid cycle occurs in mammals, plants and micro-organisms, although there is evidence indicating the occurrence of alternative pathways for terminal respiration (Krebs, 1954; Seaman & Naschke, 1955; Katagiri & Tochikura, 1958; Kornberg & Sadler, 1960). Amongst the genus *Streptomyces* this cycle appears to operate in *S. coelicolor* (Cochrane & Peck, 1953), *S. griseus* (Gilmour, Butterworth, Noble & Wang, 1955) and *S. nitrificans* (Schatz, Mohan & Trelawny, 1955). Maitra & Roy (1959*a*) have shown that *S. olivaceus* utilizes both the pentose phosphate pathway and the glycolytic route for the catabolism of glucose to the stage of pyruvic acid. The present paper is concerned with the metabolism of pyruvic acid through the tricarboxylic acid cycle and related processes.

MATERIALS AND METHODS

Chemicals. Diphosphopyridine nucleotide (DPN^+), triphosphopyridine nucleotide (TPN^+), reduced diphosphopyridine nucleotide (DPNH), reduced triphosphopyridine nucleotide (TPNH), flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), horse-heart cytochrome *c* (mostly oxidized), silver–barium salt of phosphoenolpyruvic acid, sodium glyoxylate, phenazine methosulphate and crystalline bovine albumin were products of Sigma Chemical Co., St Louis, Mo., U.S.A.; reduced glutathione (GSH) and the disodium salt of adenosine triphosphate (ATP) were obtained from Schwarz Laboratories Inc., New York; DL-α-lipoic acid, *cis*-aconitic acid and DL(+)-*allo*-*iso*citric acid from California Corporation for Biochemical Research, Calif., U.S.A.; L_s(+)-*iso*citric acid was a generous gift from Dr H. A. Lardy; except where otherwise stated, the commercial variety of *iso*citric acid was used. Thiamine pyrophosphate was a gift from F. Hoffmann–La Roche and Co. Ltd., Basle, and antimycin A from Kyowa Fermentation Ind. Co., Tokyo. α-Oxoglutaric acid was a product of Fluka A.-G., West Germany, and oxaloacetic acid was obtained from Nutritional Biochemicals Corp., Ohio, U.S.A. Coenzyme A (CoA) was obtained from Pabst Laboratories, Milwaukee, U.S.A., and sodium fluoroacetate from L. Light and Co. Ltd. All ^{14}C-labelled compounds were from The Radiochemical Centre, Amersham, Bucks. Reduced cytochrome *c* was prepared either by reduction with a minimal amount of sodium dithionite and removal of the excess of reductant

by aeration for 10 min. at 0° or according to Margoliash (1954) by reduction with ascorbic acid, followed by adsorption on Decalso F (NH_4^+), elution with dilute ammonia, dialysis and freeze-drying. 2:4-Dinitrophenol was recrystallized from water; *p*-phenylenediamine hydrochloride was likewise recrystallized twice just before use.

Microbiological procedures. The maintenance of *S. olivaceus* NRRL B-1125, the preparation of the inoculum culture and the growth of the organism in the complex medium have been described (Maitra & Roy, 1959*a*), except that a reciprocating shaker (200 strokes/min.) was used at room temperature (approx. 25°) for growing the organism. Such cells are referred to as glucose-grown cells. For the manometric experiments starved cells (Maitra & Roy, 1959*a*) were used. For cell-free extracts cells were harvested at the end of the 24 hr. growth and washed thrice with potassium chloride solution (1·15%) by centrifuging in the cold; the packed cells so obtained could be kept at −20° for a fortnight without an appreciable decrease in *iso*citric-dehydrogenase activity. Cell-free extracts were prepared by disrupting, in a mechanical shaker (Nossal, 1953), 6 g. of thawed cells with 10 g. of acid-washed glass beads (Superbrite, type 133; Minneapolis Mining and Manufacturing Co., St Paul, Minnesota) and 8 ml. of 0·1 M-potassium phosphate buffer, pH 7·2. Four shakes, each of 30 sec. duration, were applied and the cartridge was cooled in ice between shakings. The yellowish, opalescent supernatant solution obtained after centrifuging at 18 000*g* for 30 min. was used as the crude source of enzymes. In some experiments the 18 000*g* particulate preparation was obtained by a preliminary clarification of the extract for 20 min. at 2000*g* to remove the intact cells, debris and glass beads, followed by centrifuging at 18 000*g* for 30 min.; the reddish sediment was suspended in 2 ml. of 0·1 M-potassium phosphate buffer, pH 7·2. The growth rate of *S. olivaceus* was very slow in a synthetic medium (Maitra & Roy, 1960) containing acetate as the sole source of carbon, and this could not be improved even after a number of transfers through such media containing decreasing proportions of glucose and increasing proportions of acetate. Cells were grown in the medium of Maitra & Roy (1960) with sodium acetate (1 g./100 ml.) replacing glucose, and containing Difco yeast extract (25 mg./100 ml.). The acetate-grown cells were harvested after 72 hr. of growth and cell-free extracts were prepared immediately as described for glucose-grown cells. Such cells were very resistant to rupture and the cell-free extracts usually contained only one-fifth of the protein of those obtained from glucose-grown cells. Experiments, unless otherwise stated, were with extracts from glucose-grown cells.

*Enzyme assays. iso*Citric dehydrogenase was estimated by TPN^+ reduction at 340 mμ in the presence of *iso*citrate (Ochoa, 1955*a*); aconitase by TPN^+ reduction in the presence of citrate and also *cis*-aconitate. The increase of extinction at 240 mμ after addition of *iso*citrate and citrate was also used to measure aconitase activity (Racker, 1950); in some experiments the reaction rate was also measured by following the decrease in extinction at 240 mμ due to *cis*-aconitate after the addition of TPN^+. Succinic dehydrogenase was estimated by the manometric method outlined by Singer & Kearney (1957), with phenazine methosulphate, and in some experiments also by the method of Ells (1959) by coupling the above-mentioned system with 2:6-dichlorophenol-indophenol and measuring the reduction of the latter at 600 mμ. Malic dehydrogenase was measured at 340 mμ by DPNH or TPNH oxidation with oxaloacetate (Ochoa, 1955*b*) and glutamic and α-alanine dehydrogenases by the oxidation of reduced pyridine nucleotide at 340 mμ in the presence of NH_4^+ ion and the corresponding keto acids (Strecker, 1955). Oxidases for the reduced pyridine nucleotides were assayed by measuring the decrease in extinction at 340 mμ in the presence of TPNH and DPNH (Dolin, 1959). TPNH–cytochrome *c* reductase and DPNH–cytochrome *c* reductase were measured at 500 mμ by following the rate of reduction of the oxidized cytochrome *c* in the presence of TPNH or DPNH respectively (Horecker, 1955; Mahler, 1955*a*). Transhydrogenase was assayed by the method outlined by Hochster & Katznelson (1958), which is based on the fact that preparations with greator DPNH-oxidase activity than TPNH oxidase would show higher oxidation rate of reduced pyridine nucleotide on addition of DPN^+ to a cuvette in which a steady rate of TPNH oxidation has already ensued. Fumarase was tested spectrophotometrically (Racker, 1950) at 240 mμ with both malate and fumarate as the substrate. Flavins were determined according to Strittmatter (1959). Lactic-dehydrogenase activity was measured according to Mehler, Kornberg, Grisolia & Ochoa (1948). Diaphorase activity was measured by the reduction of 2:6-dichlorophenol-indophenol in the presence of cyanide with TPNH or DPNH as substrate (Mahler, 1955*b*). *iso*Citratase was estimated spectrophotometrically by the rate of change of extinction at 252 mμ due to the formation of glyoxylate semicarbazone, according to Olson (1959). Incubation mixtures for detecting the formation of citrate from acetate and oxaloacetate and of malate from acetate and glyoxylate were similar to those of Kornberg & Madsen (1958) and contained the following components (μmoles): potassium phosphate buffer, pH 7·2, 100; magnesium chloride, 10; ATP, 10; GSH, 4; CoA, 0·1; [1-^{14}C]acetate, 5(2 μc/mole); oxaloacetate or glyoxylate, 10; enzyme solution containing 0·5 and 2·0 mg. of protein for extracts from glucose-grown and acetate-grown cells respectively. The total reaction volume was 0·7 ml. and 1·5 ml. respectively with extracts from glucose- and acetate-grown cells. Incubation was at 37° for 1 hr. in air. Reaction products were detected by radioautography. The synthesis of radioactive malate from glyoxylate and labelled acetate was taken as an indication of malate-synthetase activity (Kornberg & Madsen, 1958) and of labelled citrate from oxaloacetate and [2-^{14}C]acetate as condensing-enzyme activity (Ochoa, Stern & Schneider, 1951). In all cases the wavelength scale of the spectrophotometer was checked beforehand by reference to the isosbestic point of bromocresol green (Parthasarathy & Sanghi, 1958).

Preparation and measurement of radioactive samples. Cell-free extracts after incubation were deproteinized, where necessary, by warming for 2 min. at 50° with twice the volume of 80% ethanol, the precipitated proteins were washed twice with it by centrifuging at 2000*g* and the combined supernatant solutions were kept at 0° over pellets of sodium hydroxide until dry. Chromatography was carried out at room temperature on Whatman no. 1 papers by the ascending method, with butan-1-ol–acetic acid–water (4:1:1, by vol.) containing sodium acetate (0·05 g./100 ml. of the irrigating solvent) to prevent the formation of acid bands on the developed chromatogram

(Lawson & Hartley, 1958), and after the solvents were removed by air-drying the paper was sprayed with bromocresol green in ethanol (0·04%, w/v), adjusted to pH 7; the carboxylic acids appeared as yellow spots on a blue background. Radioautography and subsequent radioassay were carried out according to Kornberg (1958), with Ilford X-ray films with a contact period of 3 weeks. Identification of compounds on chromatograms was always made by comparison with authentic samples. Incubations with sodium [^{14}C]bicarbonate were carried out with cells grown for 72 hr. in the synthetic medium (Maitra & Roy, 1960) containing glucose or acetate; washed cells were suspended in fresh growth medium buffered strongly at pH 7·6 and containing sodium [^{14}C]bicarbonate (10 μc/ml., specific activity 1 mc/m-mole) and were incubated aerobically on the reciprocating shaker at room temperature. After incubation cells were collected by centrifuging, washed twice with cold sodium bicarbonate (1%, w/v) and treated thrice with hot 80% ethanol (Kornberg, 1958) to extract the tricarboxylic acid-cycle intermediates. The cell residue was analysed for amino acids by paper chromatography after acid hydrolysis (Maitra & Roy, 1959*b*). All other methods of radioassay on planchets and on paper were as described previously (Maitra & Roy, 1959*a*).

Analytical procedures. α-Keto acids were determined as 2:4-dinitrophenylhydrazones by the procedure of Friedemann & Haugen (1943). Other methods were as described by Maitra & Roy (1959*a*).

RESULTS

Experiments with whole cells

The ability of intact cells to oxidize the tricarboxylic acid-cycle intermediates and related compounds was examined with suspensions of non-proliferating cells harvested during the early logarithmic phase of growth. In view of the permeability barriers of the microbial cell wall to some of these compounds (Ajl, 1958), experiments were also conducted at a lower pH. The rate of oxidation of different substrates varied considerably from preparation to preparation: for instance, Q_{O_2} (N) for succinate after subtraction of the endogenous values were in the range 34·2 to 67·3. The relative rates were more or less the same. Typical data are shown in Table 1, which indicate that practically all the intermediates are oxidized, assuming that the endogenous oxidation rate is unaffected in the presence of the substrates. The oxidation of all these substrates except glucose ensued after a small initial lag and continued steadily during the next 5 hr. period. The greater rate of oxygen uptake with pyruvate in the presence of a relatively small amount of fumarate, compared with that observed with either substrate alone, suggests the catalytic role of at least one of the intermediates of the cycle. The respiration rates with succinate, oxaloacetate, malate and *iso*citrate are increased at the lower pH. The rate of acetate oxidation was greater with cells grown on this substrate; Q_{O_2} (N) (after subtraction of the endogenous control value) for an acetate-grown organism was 61·2, whereas that for glucose-grown cells was 30·1 under conditions shown in Table 1.

Isotopic carbon (^{14}C) incorporated into carbon dioxide from [1-^{14}C]- and [2-^{14}C]-acetate by the non-proliferating cells was respectively 34·1 and 5·6%.

Experiments with cell-free extracts

To avoid the permeability effects with the intact cells and to lower the endogenous metabolism of *S. olivaceus*, subsequent experiments were mostly carried out with cell-free systems. The capacity of such preparations to metabolize the intermediates of the citric acid cycle was tested with malate and succinate (Table 2).

Enzyme assays. Before estimating the pyridine nucleotide-linked reactions in extracts of *S. olivaceus*, the oxidases for DPNH and TPNH and transhydrogenase were assayed in order to avoid ambiguity in data relating to pyridine nucleotide specificity. The cell-free extracts possess a low DPNH-

Table 1. *Rates of oxidation of tricarboxylic acid-cycle intermediates by resting cells of* Streptomyces olivaceus

Cells were harvested at the end of growth for 24 hr. The complete system contained, in a total volume of 3·0 ml.: starved cells equivalent to 0·45 mg. of nitrogen in 200 μmoles of potassium phosphate buffer, pH 7·2, and neutralized substrate, 50 μmoles (except where otherwise stated), in the side arm. The centre well contained 0·2 ml. of 20% (w/v) potassium hydroxide on filter paper. Temperature was 37°, gas phase air and shaker speed 120 strokes/min. Preincubation for 10 min. was allowed before the contents of the side arm were tipped in. The specific rate of oxygen uptake is expressed as Q_{O_2} (N) (μl. of oxygen consumed/mg. of cell nitrogen/hr.), based on the reading at 60 min. Values for oxaloacetate, *cis*-aconitate and fumarate have been corrected for blanks in the absence of the cell suspension.

Substrate	Q_{O_2} (N) pH 7·2	Q_{O_2} (N) pH 5·5
None	201·1	54·0
D-Glucose	251·1	21·6
Pyruvate	232·5	76·8
Acetate	219·2	40·3
Oxaloacetate	260·1	281·8
Citrate	218·2	64·3
cis-Aconitate	406·3	134·5
*iso*Citrate	212·0	84·0
Glyoxylate	220·7	—
α-Oxoglutarate	281·3	45·9
Succinate	248·4	152·6
Fumarate	235·2	67·4
DL-Malate	285·8	368·2
Fumarate*	218·0	58·1
Pyruvate + fumarate†	373·6	103·1

* Fumarate 2 μmoles.

† Pyruvate 50 μmoles and fumarate 2 μmoles.

oxidase activity but neither TPNH oxidase nor transhydrogenase was detected (Fig. 1). The DPNH-oxidase activity varied from preparation to preparation (range 0·3–0·7, as measured by the change in extinction at 340 mμ/hr./mg. of protein). The enzyme solution lost about 80% of its DPNH-oxidase activity in 18 hr. on storage at −20°. The oxidase activity was insensitive to cyanide (mM) but was inhibited (84%) by antimycin A (0·01 mM).

The presence of a TPN^+-linked, Mg^{2+}-dependent *iso*citric dehydrogenase is indicated in Fig. 2; no DPN^+-linked activity could be detected under conditions of assay described by Kornberg (1955), or in preparations containing only negligible DPNH-oxidase activity after overnight storage at −20°, although the TPN^+-linked enzyme withstood storage at this temperature for 10 days without measurable loss of activity. The enzyme was not inhibited by mM-cyanide. The commercial variety of the substrate, namely DL(+)-*allo-iso*citric acid, elicited a quicker rate of reaction than did the natural enantiomorph. That the product of reaction was α-oxoglutaric acid was suggested by the chromatographic behaviour of the 2:4-dinitrophenylhydrazine derivative obtained from the incubated mixture (El Hawary & Thompson, 1953) and also by the absorption spectrum of these derivatives eluted from the chromatograms. The reversibility of the *iso*citric-dehydrogenase reaction could not be demonstrated by the oxidation of TPNH in a system containing bicarbonate, α-oxoglutarate and the enzyme solution.

The *iso*citric-dehydrogenase activity was used to measure aconitase by replacing *iso*citrate with

Table 2. *Oxidation of* DL-*malate and succinate by cell-free extracts of* Streptomyces olivaceus

The complete system for malate contained the following components (μmoles): magnesium chloride, 6; manganese sulphate, 6; potassium phosphate buffer, pH 7·2, 200; DPN^+, 0·5; TPN^+, 0·5; substrate, 50; phenazine methosulphate 5; enzyme solution equivalent to 5·5 mg. of protein. The system for succinate was the same except that DPN^+ and TPN^+ were omitted and enzyme solution used contained 9·0 mg. of protein. Details were as given in Table 1. Results are expressed as μl. of oxygen consumed in 40 min.

Reaction mixture	Uptake of O_2 (μl.) With malate	With succinate
Complete	138	149
Without $MgCl_2$	122	144
Without $MnSO_4$	131	142
Without $MgCl_2$ and $MnSO_4$	91	122
Without DPN^+	121	—
Without TPN^+	110	—
Without DPN^+ and TPN^+	88	—
Without phenazine methosulphate	61	13
Without DPN^+, TPN^+ and phenazine methosulphate	29	—
Without substrate	25	17

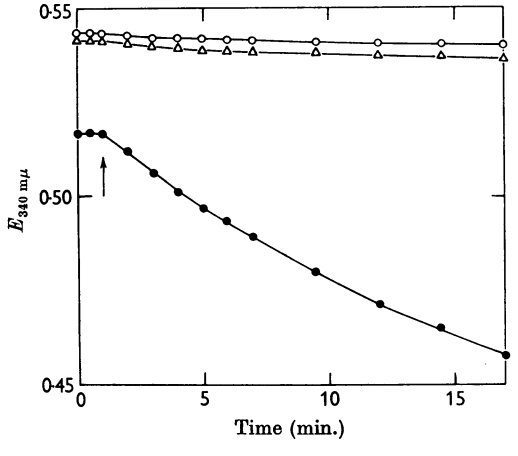

Fig. 1. Oxidation of reduced pyridine nucleotides by crude extracts of *S. olivaceus*. The reaction mixture contained (μmoles): potassium phosphate buffer, pH 7·2, 280; magnesium chloride, 10; reduced pyridine nucleotide, approx. 0·1; DPN^+, where indicated, 0·1; enzyme protein, 0·5 mg. in a total volume of 3·0 ml. Reactions were started with addition of the enzyme solution at the instant indicated by the arrow. ○, TPNH; △, TPNH + DPN^+; ●, DPNH.

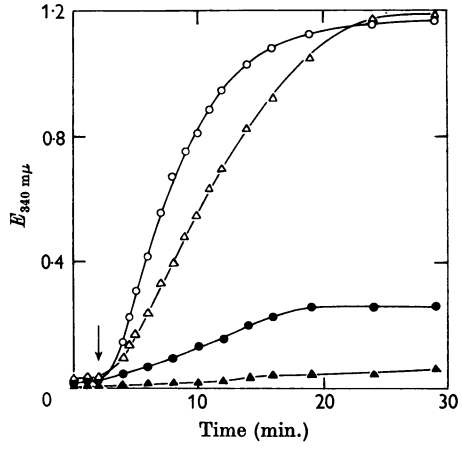

Fig. 2. *iso*Citric-dehydrogenase activity in cell extract of *S. olivaceus*. The reaction mixture contained, in a total volume of 3·0 ml., the following (μmoles): potassium phosphate buffer, pH 7·2, 265; magnesium chloride, 10; pyridine nucleotide, 0·2; substrate, 10; crude extracts, equivalent to 0·6 mg. of protein. The pair of cuvettes containing DPN^+ received 3 μmoles of AMP each. Additions and omissions are as indicated. Substrates were added at the instant marked by the arrow; the control cuvette contained water in place of the substrate. ○, *iso*Citrate + TPN^+; △, L$_s$(+)-*iso*citrate + TPN^+; ●, *iso*citrate–Mg^{2+} + TPN^+; ▲, *iso*citrate + DPN^+.

citrate and *cis*-aconitate and measuring the reduction of TPN^+ (Fig. 3). A distinct lag was observed with citrate. Aconitase was also measured by following at 240 mμ the appearance of *cis*-aconitate from citrate and *iso*citrate or its disappearance. In the latter case the removal of *cis*-aconitate was favoured by the presence of TPN^+, which presumably shifted the equilibrium towards α-oxoglutarate. Unlike *iso*citric dehydrogenase, aconitase lost more than 60% of its activity on storage at −20° for 18 hr. Citratase, which splits citric acid into oxaloacetate and acetate (Katagiri & Tochikura, 1959), could not be detected in the extract by coupling oxaloacetate *in situ* to malic dehydrogenase under conditions shown in Fig. 6.

Oxidation of α-oxo acids, etc. Reduction of DPN^+ in the presence of pyruvate or α-oxoglutarate was not observed in any of the extracts supplemented with CoA, thiamine pyrophosphate, GSH, DL-α-lipoic acid, Mg^{2+} and Mn^{2+} ions (King, Kawasaki & Cheldelin, 1956), and the addition of 5′-adenylic acid (AMP) and also biotin (Cantino, 1951) had no effect; nor was TPN^+ reduced by these substrates.

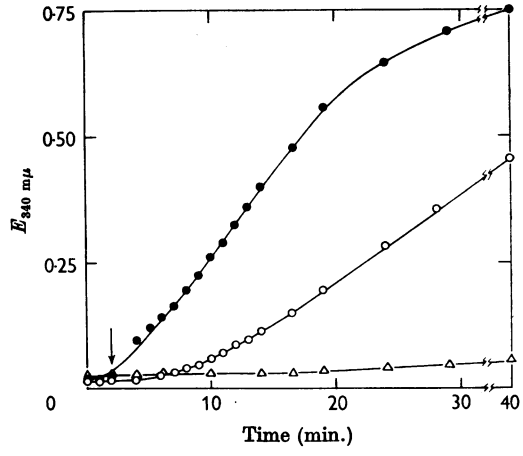

Fig. 3. Aconitase in *S. olivaceus* extract. The reaction mixture contained (μmoles): potassium phosphate buffer, pH 7·2, 260; magnesium chloride, 10; pyridine nucleotide, 0·4; substrate, 10; enzyme solution equivalent to 0·35 mg. of protein. Other details were as given for Fig. 2. ●, *cis*-Aconitate + TPN^+; ○, citrate + TPN^+; △, citrate + DPN^+.

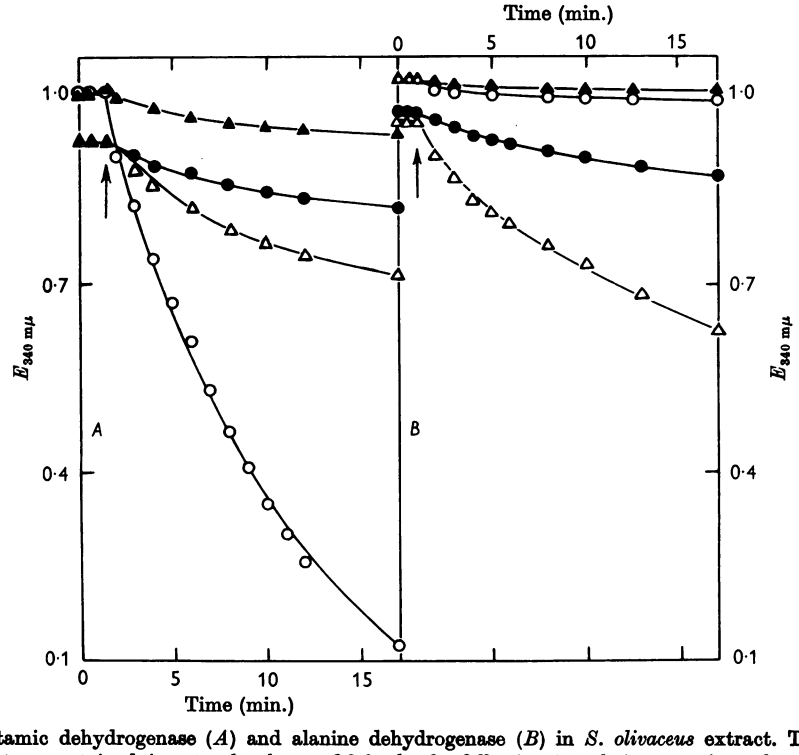

Fig. 4. Glutamic dehydrogenase (*A*) and alanine dehydrogenase (*B*) in *S. olivaceus* extract. The complete reaction mixture contained, in a total volume of 3·0 ml., the following (μmoles): potassium phosphate buffer, pH 7·2, 270; magnesium chloride, 10; dibasic ammonium phosphate, 10; α-oxoglutarate (αKG) or pyruvate (*A* and *B* respectively), 10; reduced pyridine nucleotide, approx. 0·2; enzyme solution equivalent to 0·5 mg. of protein. Arrows indicate addition of the enzyme. (*A*) ▲, αKG + TPNH; ●, αKG + DPNH; △, αKG + NH_4^+ + DPNH; ○, αKG + NH_4^+ + TPNH. (*B*) ▲, Pyruvate + TPNH; ●, pyruvate + DPNH; △, pyruvate + NH_4^+ + DPNH; ○, pyruvate + NH_4^+ + TPNH.

The coupling of DPN^+ reduction in the above-mentioned systems with cytochrome *c* was also ineffective (Imamoto, Iwasa & Okunuki, 1959). With GSH omitted from the above-mentioned system no reduction of 2:6-dichlorophenol-indophenol was observed (Imamoto *et al.* 1959) even in the presence of FMN or FAD. The absence of lactic dehydrogenase as tested by the lack of oxidation of DPNH with added pyruvate also eliminated a route whereby the pyruvic-dehydrogenase action could be masked. This was also confirmed by the fact that no disappearance of pyruvate or α-oxoglutarate occurred after 1 hr. incubation of these substrates with the system containing CoA, thiamine pyrophosphate, GSH, DPN^+, DL-α-lipoic acid, Mg^{2+} ion and the enzyme solution. In this regard no difference was observed between the 18 000*g* supernatant and the corresponding particulate preparation.

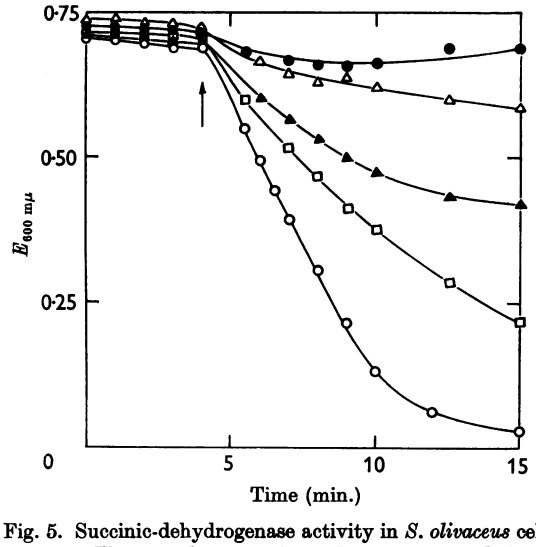

Fig. 5. Succinic-dehydrogenase activity in *S. olivaceus* cell extracts. The complete reaction mixture contained, in a total volume of 3·0 ml., the following (μmoles): potassium phosphate buffer, pH 7·2, 210; magnesium chloride, 10; potassium cyanide, 10; 2:6-dichlorophenol-indophenol, 0·04; phenazine methosulphate, 2·5; cell-free extract equivalent to 1·3 mg. of protein; succinate and malonate where indicated. ○, Succinate, 10 μmoles; □, succinate and malonate, 10 μmoles each; ▲, succinate, 10 μmoles, and malonate, 20 μmoles; △, succinate, 10 μmoles, and malonate, 30 μmoles; ●, malonate, 20 μmoles. In the last curve (●), almost the same changes of extinction were observed with 10 or 30 μmoles of malonate. Cuvettes containing all the additions except 2:6-dichlorophenol-indophenol were adjusted to 100% transmission. The reaction was started with phenazine methosulphate at the time indicated by the arrow. The results have been corrected for the reduction of 2:6-dichlorophenol-indophenol in the absence of the substrate and also for that in the absence of the enzyme solution.

The presence in the extract of an active glutamic dehydrogenase is shown in Fig. 4. The equilibrium of the reaction is far in favour of synthesis of glutamate, for no reduction of DPN^+ or TPN^+ was observed with glutamate. Similar observations were made with alanine dehydrogenase (Fig. 4), except that glutamic dehydrogenase was more active with TPNH, whereas the latter acted only with DPNH. The formation of glutamate and alanine was confirmed by chromatographic examination of the incubated mixtures.

Although succinate was oxidized by resting cells, very little oxidation took place when the cell-free extract was incubated with succinate unless phenazine methosulphate was present (Table 2); chromatography of the reaction product showed a little malate to have been produced and no fumarate. Fumarate, as measured by its extinction at 240 mμ (Racker, 1950), could not be detected as a product of succinate dehydrogenation during a 20 min. reaction period. No reduction of 2:6-dichlorophenol-indophenol or of oxidized cytochrome *c* was observed on addition of succinate to a system containing cyanide, enzyme and bovine albumin (Singer & Kearney, 1957) as well as FMN or FAD. Phenazine methosulphate, however, was effective as a mediator between 2:6-dichlorophenol-indophenol and possibly succinate, as measured by the rapid reduction of 2:6-dichlorophenol-indophenol under conditions shown in Fig. 5. Malonate, which was rapidly oxidized by intact cells, caused little reduction of the indophenol dye under these conditions. Increasing inhibitions were produced as the malonate:succinate ratio was increased (Fig. 5), although the inhibitory concentrations were higher than were usually employed (Pardee & Potter, 1949). The cell-free extracts were devoid of succinate-cleaving enzyme (Seaman & Naschke, 1955) as measured by the oxidation of DPNH in the presence of succinate, ATP and CoA.

Fumarase was not detected. Chromatographic experiments indicated that no tricarboxylic acid-cycle intermediate was formed in detectable amounts on incubating fumarate with the cell-free extracts.

Extracts of *S. olivaceus* contained an active DPNH-linked malic dehydrogenase (Fig. 6) and also a TPNH-linked enzyme, the latter being less active. The reaction product in each case was co-chromatographed with authentic malate. In the forward direction addition of malate did not result in reduction of DPN^+, although with TPN^+ slight reduction occurred (extinction change at 340 mμ/hr./mg. of enzyme protein, 0·36 under conditions described in Fig. 2). The possibility that this small reduction of TPN^+ might be due to malic enzyme (Ochoa, Mehler & Kornberg, 1948) could not be ruled out, although no oxidation of TPNH was

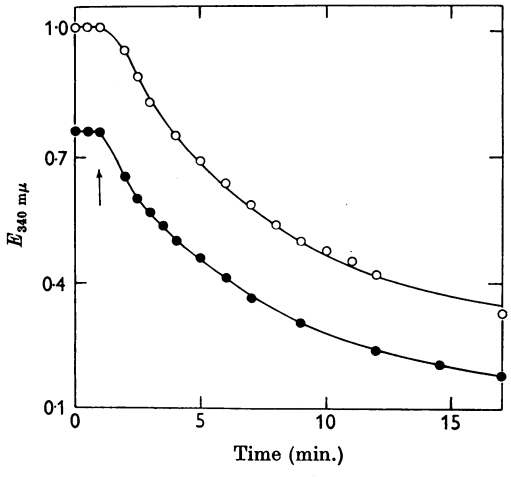

Fig. 6. Malic-dehydrogenase activity in crude extracts of *S. olivaceus*. The reaction mixtures contained the following components (μmoles): oxaloacetate, 5; reduced pyridine nucleotide, approx. 0·2; enzyme protein, 0·5 mg. and 8 μg. for the TPNH-linked and DPNH-linked reactions respectively. Other details were as given for Fig. 2. At the time denoted by the arrow reactions were started with the enzyme. There was no demonstrable activity for DPNH oxidase or TPNH oxidase at the concentration of enzyme solution used in these experiments. ○, Oxaloacetate + TPNH; ●, oxaloacetate + DPNH.

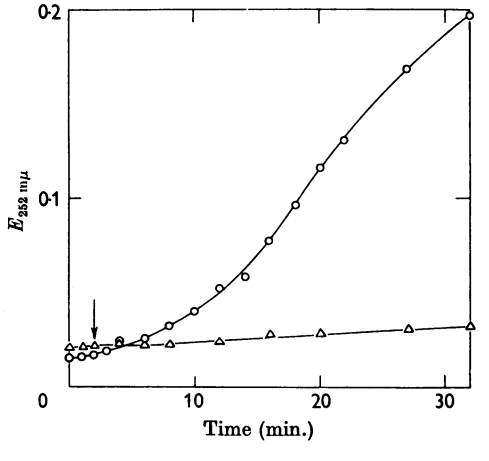

Fig. 7. *iso*Citratase in *S. olivaceus* extracts. The reaction mixture contained, in a total volume of 3·0 ml., the following (μmoles): potassium phosphate buffer, pH 6·0, 230; magnesium chloride, 10; GSH, 4; semicarbazide hydrochloride, neutralized to pH 6·0, 60; *iso*citrate, 10; enzyme solution equivalent to 0·18 and 0·13 mg. of protein for the acetate-grown and the glucose-grown cells respectively. Reactions were started with the addition of *iso*citrate at the time indicated by the arrow. Controls lacked the substrate. ○, Acetate-grown cells; △, glucose-grown cells.

observed in a system composed of pyruvate, Mg^{2+} and Mn^{2+} ions, bicarbonate, phosphate and the enzyme.

Recent work on the synthesis of cell constituents from C_2 units has led to the formulation of the glyoxylate cycle as a variant of the tricarboxylic acid cycle, particularly in acetate-grown microorganisms (Kornberg & Krebs, 1957). The two key enzymes of this cycle, *iso*citratase and malate synthetase, were sought in *S. olivaceus*. Extracts from cells grown on glucose contained no *iso*citratase when assayed by the procedure outlined in Fig. 7, nor was the 2:4-derivative of glyoxylate detected by chromatography of the reaction mixture. Malate synthetase, measured as described in the Materials and Methods section, was also not detected. The acetate-grown cells, however, contained both *iso*citratase (Fig. 7) and malate synthetase, the latter being indicated by the observation that malate was the only labelled compound formed from [^{14}C]acetate and glyoxylate. Both the glucose-grown and the acetate-grown cells formed [^{14}C]citrate from oxaloacetate and labelled acetate.

Components of the electron-transport chain. In view of the general aerobic nature of organisms of the genus *Streptomyces* the occurrence in such cells of an electron-transport chain to oxygen is probable. Heim, Silver & Birk (1957) examined a number of *Streptomyces* species for their cytochrome composition and showed cytochromes of groups *b*

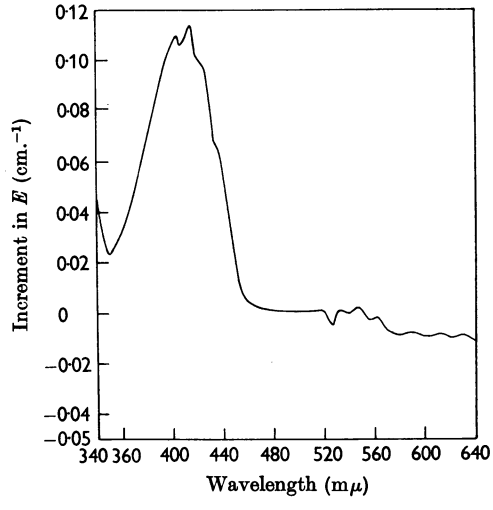

Fig. 8. Difference spectrum (reduced minus oxidized state of pigments) of crude extracts of *S. olivaceus*. The reaction mixture contained the following (μmoles): potassium phosphate buffer, pH 7·2, 270; potassium ferricyanide (used for oxidation of pigments), 300; sodium dithionite (used for reduction of pigments), 400; crude extract equivalent to 6·0 mg. and 2·1 mg. of protein for work above 380 mμ and below 380 mμ respectively.

and *c* to be present. The cell-free extracts of *S. olivaceus* were tested chromatographically for flavins: two spots corresponding respectively to riboflavin and FMN were detected, but no FAD. To determine the absorption peaks of the respiratory components extracts (5–20 mg. of protein) were scanned on the Beckman spectrophotometer model DU in the range 330–700 mμ in the presence of potassium ferricyanide (0·3 mM) and sodium dithionite (4 mM) for oxidized and reduced states respectively; dilute extracts were used for work below 380 mμ. It was assumed that scattering of light by the opalescent extracts was independent of the state of oxidation of the pigments (Ghiretti-Magaldi, Giuditta & Ghiretti, 1958), and the difference spectra were plotted (Fig. 8) in terms of the difference in extinction between the reduced and the oxidized pigments ($A_{red.} - A_{ox.}$, where A terms denote extinction). They were found to be qualitatively the same in every sample of crude extract. The following absorption maxima (mμ) were noted: 340, 405, 417, 520, 532, 546, 551–553, 562, 590, 610 and 630. The peak in the Soret region at 417 mμ was the most prominent and that in the region 551–553 mμ was the least sharp.

The cell-free extracts of *S. olivaceus* failed to bring about oxidation of reduced cytochrome *c*, as measured by the decrease in extinction at 550 mμ after the addition of the enzyme solution to a mixture containing reduced cytochrome *c*, potassium phosphate buffer, pH 7·2, and Mg^{2+} or Mn^{2+} ions. Nor was oxidation observed with the particulate preparation (18 000*g*). This inertness of the cell-free preparations of *S. olivaceus* towards re-

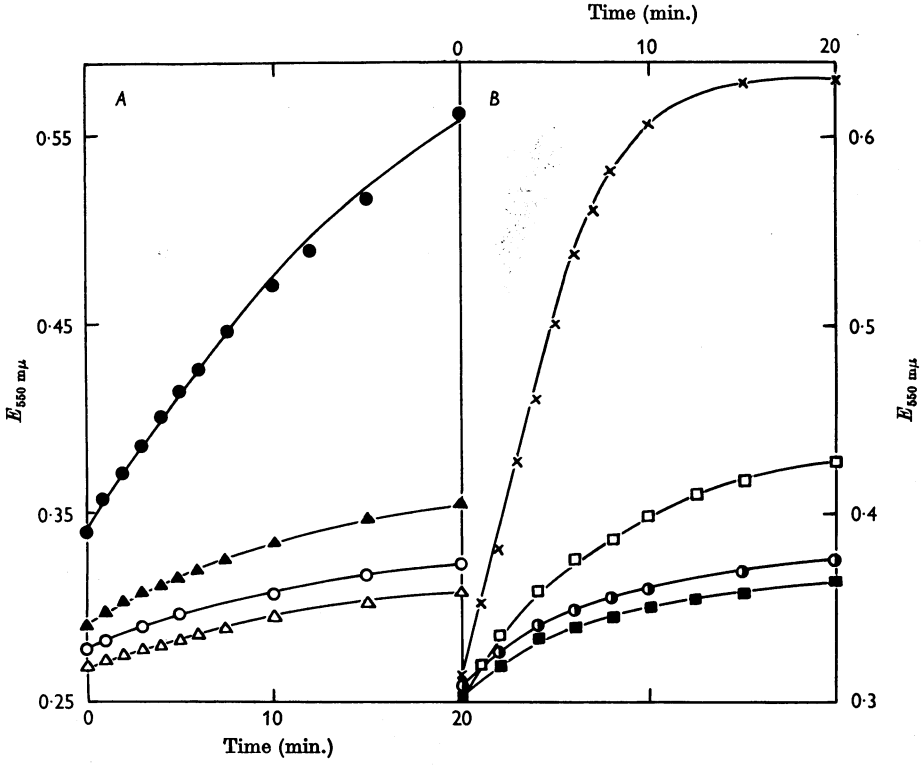

Fig. 9. TPNH-cytochrome *c*-reductase (*A*) and DPNH-cytochrome *c*-reductase (*B*) activity in cell-free extracts of *S. olivaceus*. The complete reaction mixture contained: potassium phosphate buffer, pH 7·2, 260 μmoles; magnesium chloride, 10 μmoles; DPNH or TPNH, 0·2 μmole; FAD or FMN, 0·1 μmole; oxidized cytochrome *c*, 25 μm-mole; enzyme solution equivalent to 0·7 mg. of protein; total volume, 3·0 ml. Controls were without the flavin nucleotides and were run separately with each assay. Cuvettes containing all additions except cytochrome *c* were adjusted to 100% transmission. Reactions were started with addition of the enzyme solution after the readings were steady for at least 2 min. The extinction readings for the complete system with FMN (*B*) have been corrected for the non-enzymic reduction of cytochrome *c* in the presence of FMN and DPNH. During the first 3 min. this amounted to 7% of the extinction changes obtained in the complete system. No such reduction of cytochrome *c*, however, took place with FMN and TPNH, FAD and DPNH or with FAD and TPNH. (*A*) ●, FMN; ○, control; ▲, FAD; △, control. (*B*) ×, FMN; ◑, control; □, FAD; ■, control.

duced cytochrome *c* was paralleled by the observation that whole resting cells of this organism also failed to oxidize *p*-phenylenediamine (0·02 M) (Richardson, 1957) under conditions described in Table 1.

The fresh extracts exhibited TPNH–cytochrome *c*-reductase and DPNH–cytochrome *c*-reductase activity. Thus added ferricytochrome *c* was slowly reduced in the presence of the extracts by TPNH or DPNH (Fig. 9). The addition of FMN stimulated both enzyme activities though FAD was less active. Fig. 10 shows the presence in the crude extracts of diaphorase activity. An increase in activity of approximately 20% was observed on omitting cyanide from the assay system. No attempt was, however, made to ascertain the extent to which the cytochrome *c*-reductase activities were responsible for the observed reduction of the dye by TPNH and DPNH (Mahler, 1955*b*).

DISCUSSION

In the present work some evidence has been put forward for the operation of the tricarboxylic acid cycle in *S. olivaceus*, although the oxidative decarboxylation of α-oxo acids and fumarase activity could not be demonstrated in the cell-free extract. This might be due to the loss of activity of the relevant enzyme systems during preparation.

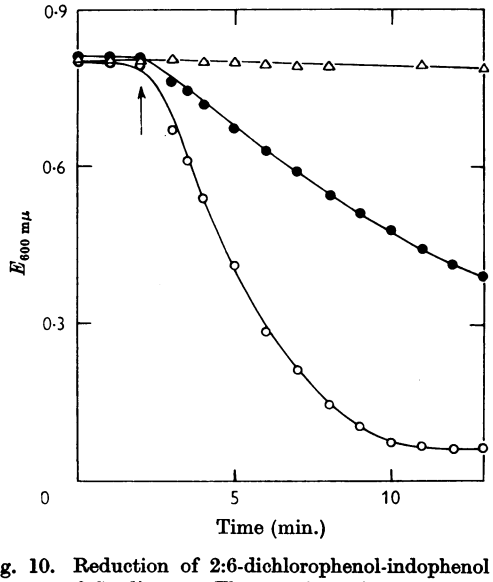

Fig. 10. Reduction of 2:6-dichlorophenol-indophenol by extracts of *S. olivaceus*. The reaction mixture contained: potassium cyanide, 10 μmoles; 2:6-dichlorophenol-indophenol, 0·05 μmole; enzyme protein, 0·4 mg. Other details were as given for Fig. 1. The control cuvette contained all additions except the dye solution and the substrate. △, No addition; ● TPNH; ○, DPNH.

The cell-free extract from *S. olivaceus* is capable of converting acetate into citrate in the presence of oxaloacetate, CoA, ATP and inorganic phosphate; the formation of citrate may be due to the joint action of acetokinase and phosphotransacetylase, or acetyl-CoA kinase (Berg, 1956) and the condensing enzyme. Citrate in its turn is isomerized to *iso*citrate by aconitase with intermediate formation of *cis*-aconitic acid; in the presence of TPN^+ all the three intermediates give rise to α-oxoglutaric acid.

α-Oxo acids caused considerable oxygen uptake over the endogenous respiration of whole cells (Table 1) and the involvement of a typical keto acid-oxidase system, as suggested by the inhibition of oxidation by arsenite (Mehler, 1957). The cell-free extract, however, did not oxidize these substrates. Possibly enzyme components were damaged irreversibly or depleted of some unknown cofactors. The absence in the cell-free extracts of detectable levels of fumarase is also not consistent with data from whole cells, where fumarate exerts some catalytic action on the oxidation of pyruvate (Table 1). The balance of evidence, however, is in favour of the operation of tricarboxylic acid cycle in *S. olivaceus*, but how far this is utilized in the economy of the cell remains to be determined.

Growth of *S. olivaceus* on acetate is associated with the appearance in the mycelia of *iso*citratase and malate synthetase. In the acetate-grown cells therefore acetyl-CoA has two separate points of entry into the tricarboxylic acid cycle, namely, through condensing enzyme producing citrate and through malate synthetase giving malate. According to Kornberg (1958), such a situation would allow the cell to utilize for its synthetic reactions carbon skeletons of tricarboxylic acid-cycle intermediates, in addition to those contributed by the carbon dioxide-fixation reactions that have been shown to occur in both the acetate-grown and the glucose-grown organisms.

The DPNH-oxidase system of *S. olivaceus* could explain only a fraction of oxygen consumption of whole cells, and neither TPNH oxidase nor transhydrogenase could be detected. The regeneration of TPN might be linked with processes such as the reduction of oxaloacetate (Fig. 6), reductive amination of α-oxoglutarate (Fig. 4) or those catalysed by glutathione reductase or similar enzymes. The inability of succinate to reduce cytochrome *c* distinguishes the electron-transport system of this substrate from those of TPNH and DPNH (Slater, 1958). Succinate dehydrogenation was achieved only when the artificial carriers phenazine methosulphate and 2:6-dichlorophenol-indophenol were present. Eichel (1956) considers that in *Tetrahymena*, which is devoid of cytochrome *c* oxidase, the role of cytochrome *c* in DPNH–cytochrome *c* reductase is that of an artificial carrier. In *S.*

olivaceus attempts to demonstrate cytochrome *c* oxidase with mammalian cytochrome *c* were unsuccessful, as in many other bacterial genera (Smith, 1954), including also several *Streptomyces* species (Heim *et al.* 1957). There may exist in *S. olivaceus* a cytochrome substrate active for the terminal oxidase and different from the mammalian cytochrome *c* or a flavoprotein carrier directly mediating with oxygen.

SUMMARY

1. Some evidence has been presented for the occurrence of the tricarboxylic acid cycle in *Streptomyces olivaceus*. The intermediates of this cycle stimulate oxygen uptake over the endogenous respiration by the whole resting cells.

2. The cell-free extracts contained the following enzymes: aconitase, *iso*citric dehydrogenase, succinic dehydrogenase, malic dehydrogenase, glutamic dehydrogenase, alanine dehydrogenase, reduced diphosphopyridine nucleotide oxidase, reduced triphosphopyridine nucleotide–cytochrome *c* reductase and reduced diphosphopyridine nucleotide cytochrome *c* reductase; the extracts were capable of synthesizing citrate from acetate, coenzyme A, adenosine triphosphate, inorganic phosphate and oxaloacetate.

3. Activities of pyruvic and α-oxoglutaric dehydrogenases, fumarase and cytochrome *c* oxidase could not be demonstrated in the cell-free extracts.

4. Cells grown on acetate possess *iso*citratase and malate synthetase.

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An Amino Acid Sequence in the Active Centre of Phosphoglucomutase

BY C. MILSTEIN* AND F. SANGER†
Department of Biochemistry, University of Cambridge

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The presence of phosphate in phosphoglucomutase was first demonstrated by Jagannathan & Luck (1949), who also showed that it may readily be exchanged with phosphate in the substrates and that it is thus probably involved in the active centre of the enzyme. Anderson & Jollès (1957) observed that it was not released from the protein even under strong acid conditions and were able to isolate serine phosphate from the partially hydrolysed enzyme. This offered the possibility of studying the amino acid sequence around the point of attachment of the phosphate by isotopic techniques. In a preliminary study of the peptides produced from a partial hydrolysate of phosphoglucomutase labelled with ^{32}P, Koshland & Erwin (1957) and Koshland, Ray & Erwin (1958) suggested that the sequence around the serine phosphate (SerP) residue was Asp.SerP.Gly.Glu.Ala.Val. [For definitions of the abbreviations of amino acids used in this paper see *Biochem. J.* (1957), **66**, 6.] This was very similar to that found in trypsin and chymotrypsin around the serine residue that can react with di*iso*propyl phosphorofluoridate. The sequence Asp.Ser.Gly thus appeared to be a common feature of enzymes with rather dissimilar functions, and these conclusions have been discussed by several authors in connexion with the problem of the relationship between structure and function (Lumry, 1959; Koshland, 1959; Linderstrøm-Lang & Schellman, 1959; Dixon, Neurath & Pechère, 1958). In the work of Koshland & Erwin evidence for the purity of the peptides studied was lacking, and, by comparing partial hydrolysates of [^{32}P]phosphoglucomutase with similar hydrolysates of labelled chymotrypsin, we were able to show (Milstein & Sanger, 1960) that the sequence around the serine phosphate residue in phosphoglucomutase was not Asp.SerP.Gly. The present paper describes the determination of a pentapeptide sequence around the serine phosphate residue.

In most experiments with peptides of serine phosphate it is usually possible to separate them from other peptides by virtue of their strong acid group by ion-exchange methods (Flavin, 1954) or by ionophoresis at an acid pH. Since most of the labelled peptides in a partial hydrolysate of [^{32}P]-phosphoglucomutase contain a basic residue, they are neutral and purification is much more difficult. We have thus determined as much as possible of the sequence by isotopic methods, since it is relatively easy to separate the labelled peptides from one another but very difficult to separate them from the very complex mixture of non-labelled peptides that is derived from such a large protein. Some such methods have been described previously (Naughton, Sanger, Hartley & Shaw, 1960; Sanger & Shaw, 1960; Milstein & Sanger, 1960). The interrelationships of the various peptides (Tables 3 and 4) were first determined by studying the effect of partial acid hydrolysis and Edman degradation on them. The presence of a histidine residue after the serine phosphate and of an acidic residue next to the histidine was concluded from the ionophoretic rates of the peptides at different pH values.

Another method for the identification of histidine peptides was also developed by making use of the lability of the imidazole ring to photo-oxidation. Weil, Gordon & Buchert (1951) showed that some amino acids can be oxidized when they are exposed under a visible light in the presence of methylene

* Present address: Instituto Nacional de Microbiología, Av. Velez Sarsfield 563, Buenos Aires, Argentina.
† Member of the External Staff of the Medical Research Council.