SUMMARY

1. The oxidizing abilities of washed suspensions of cells of *Pseudomonas* OD1 grown on oxalate, glycollate, lactate, malate and succinate have been examined. Q_{O_2} values are low compared with those for other species of *Pseudomonas*. The oxidizing powers show some response to growth substrate, but there is no clear-cut case of adaptive oxidation of any of the test substrates (mainly C_2 acids and tri-carboxylic acid cycle intermediates). In general, cells grown on lactate displayed the best oxidizing ability.

2. With cells grown on lactate, 0.001M fluoroacetate strongly inhibits the oxygen uptake due to glycine, glycollate or 2-oxoglutarate, but has little effect on that due to acetate or pyruvate. Under the same conditions, the amount of citrate accumulating is increased by glycollate, glycine, pyruvate, succinate, fumarate, malate, oxaloacetate and 2-oxoglutarate, but not by acetate or oxalate.

3. The highest amounts of extra citrate were produced from 2-oxoglutarate, glycollate and glycine, representing about a 50, 30 and 15% conversion, respectively, of substrate carbon into citrate.

4. Glycollate appears to act as the sole source of carbon for the extra citrate synthesized in its presence. The glycollate carbon not converted into citrate appears as carbon dioxide.

5. The two carbon atoms of glycollate do not contribute equally to the synthesis of citrate; there is a preferential incorporation of the C-2 atom.

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Carbohydrate Metabolism in Citric Acid Fermentation

5. PURIFICATION AND PROPERTIES OF *ZWISCHENFERMENT* FROM *ASPERGILLUS NIGER**

By V. JAGANNATHAN, P. N. RANGACHARI AND M. DAMODARAN
National Chemical Laboratory of India, Poona, India

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Zwischenferment, which catalyses the dehydrogenation of glucose 6-phosphate by triphosphopyridine nucleotide, was discovered by Warburg & Christian (1931). The high specificity of the enzyme makes it valuable for the estimation of glucose 6-phosphate, triphosphopyridine nucleotide, fructose 6-phosphate, glucose 1-phosphate, hexokinase, phosphoglucomutase and phosphoglucosomerase. *Zwischenferment* from yeast has been purified about 500-fold by Negelein & Gerischer (1936). A simple procedure for the preparation of yeast *Zwischenferment* suitable for the estimation of glucose 6-phosphate and triphosphopyridine nucleotide has been described by Kornberg (1950). But the specific activity

* Part 4: Jagannathan, Kartar Singh & Damodaran (1956).

phate, glucose 1-phosphate, hexokinase, phosphoglucomutase and phosphoglucosomerase. *Zwischenferment* from yeast has been purified about 500-fold by Negelein & Gerischer (1936). A simple procedure for the preparation of yeast *Zwischenferment* suitable for the estimation of glucose 6-phosphate and triphosphopyridine nucleotide has been described by Kornberg (1950). But the specific activity

of the enzyme was reported to be only one-third of that obtained by Negelein & Gerischer (1936) and the presence of phosphoglucisomerase and hexokinase in the preparation makes it unsuitable for the estimation of these enzymes. Damodaran, Jagannathan & Kartar Singh (1955) recently showed the presence of a *Zwischenferment* specific for triphosphopyridine nucleotide in a strain of *Aspergillus niger* giving high citric acid yields. The ease with which *A. niger* can be grown in sufficient quantity, and the high *Zwischenferment* activity of the mycelium, make it a suitable source of the enzyme. Fractionation of mycelial extracts with ammonium sulphate and calcium phosphate gel gave a preparation the specific activity of which was about five to seven times as high as that reported by Kornberg (1950) for the enzyme from yeast. The purified enzyme was free from phosphoglucisomerase, phosphoglucumutase and hexokinase and has been used in this laboratory for the routine estimation of these enzymes and of their substrates. The purification and properties of *Zwischenferment* from *A. niger* are described in this paper.

MATERIALS AND METHODS

Adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), fructose 6-phosphate (F 6-P) and glucose 6-phosphate (G 6-P) were obtained from Schwarz Laboratories, and glucose 1-phosphate (G 1-P) and protamine sulphate from Light and Co. Barium 6-phosphogluconate was a generous gift from Dr Seymour S. Cohen and triphosphopyridine nucleotide (TPN) (65% pure) from Dr D. R. Sanadi. A crude liver preparation containing 10% of TPN and 20% of DPN, prepared according to LePage & Mueller (1949), was used for routine assay and gave the same results as the purer TPN preparation. Calcium phosphate gel (Keilin & Hartree, 1937) was aged for at least 6 months before use. Glass-distilled water was used in all experiments. The acetate buffers used in the purification of the enzyme were prepared from the sodium salt and the phosphate buffers were prepared from KH_2PO_4 and K_2HPO_4 .

The pH was determined with the glass electrode and optical measurements were made (with 1 cm. light path) in a Beckman Model DU quartz spectrophotometer fitted with a constant-temperature arrangement.

Activity of the Zwischenferment. This was determined by measuring the increase in optical density at 340 m μ , resulting from reduction of TPN. The test system, which is similar to that of Scott & Cohen (1953), contained 20 μ moles of G 6-P, 45 μ moles of magnesium chloride, 110 μ moles of glycylglycine, 0.4 μ mole of TPN and enzyme in a total volume of 3.0 ml. at pH 7.6, and was incubated at 30°. The amount of enzyme taken for the test was such that the change in optical density was 0.006–0.010 in 15 sec. About 0.6–1.0 μ g. of purified enzyme with a specific activity of 20 units/mg. was required for the test. The rate of TPN reduction was calculated from the mean of the first ten readings at 15 sec. (extinction coefficient of reduced TPN, $6.22 \times 10^4 \text{ cm}^2 \times \text{mole}^{-1}$) (Horecker & Kornberg, 1948). Enzyme activity was expressed as μ moles of substrate

oxidized (or TPN reduced) per minute under these conditions, and the specific activity of the enzyme as activity per mg. of protein.

The methods used for protein estimation, ammonium sulphate fractionation and the growth of *A. niger* have been described in detail in the earlier paper of this series (Jagannathan, Kartar Singh & Damodaran, 1956).

RESULTS

Purification of Zwischenferment. About 2 kg. of *A. niger* mycelium, which had been kept at -20° for about 4–6 weeks, was mixed with 2 l. of ice-cold 0.01N- NH_3 soln. and allowed to stand at room temperature for 1–2 hr. with occasional stirring. When the frozen mycelium had thawed (temperature about 1°), the material was squeezed through muslin and the extract discarded. The residue could be used immediately for the extraction of *Zwischenferment*, but was, in general, washed with an equal weight of ice water and kept for about 2 weeks at -20° before use, since higher yields of enzyme were obtained by this procedure. This preliminary extraction removes many of the glycolytic enzymes (including phosphoglucisomerase, hexokinase, etc.) without appreciable loss of *Zwischenferment*.

The residual mycelium (1.9 kg.) was mixed with 1.9 l. of cold 0.2M- K_2HPO_4 and was allowed to thaw at room temperature. It was maintained at $0-5^\circ$ with occasional stirring for 2 hr. and was then squeezed through muslin. The pH of the pale-yellow filtrate was about 7.4–7.6 (fraction I).

All further operations were carried out at $0-5^\circ$. The extract was brought to 0.6 saturation by the addition of solid ammonium sulphate and the precipitate was collected on a 15 cm. filter by filtration with gentle suction. The precipitate was suspended in 200 ml. of 0.2M phosphate buffer, pH 7.4 (final volume 224 ml., 0.06 saturation with respect to residual ammonium sulphate), and refractonated by the addition of solid ammonium sulphate. The fraction obtained between 0.35 and 0.55 saturation was collected by centrifuging and dissolved in 12 ml. of 0.05M phosphate buffer, pH 7.4 (fraction II). The entire procedure after the extraction of the enzyme up to this stage was carried out preferably on the same day, but fraction II could be stored at -20° for several weeks.

Fraction II was diluted with acetate buffer, to give a final concentration of 8–12 units of enzyme/ml. of 0.1M acetate buffer, pH 5.8. A solution of protamine sulphate (10 mg./ml. of 0.1M acetate buffer, pH 6.0) was then added to it till no further precipitation occurred, 1 mg. of protamine sulphate being, in general, sufficient for 35 mg. of protein. The precipitate was centrifuged after 30 min., at 15 000 g, and discarded. The enzyme in the supernatant was adsorbed on calcium phosphate gel as described below.

Samples (1 ml.) of the supernatant were mixed with 0.1, 0.2, 0.3, 0.4 and 0.5 ml., respectively, of calcium phosphate gel (16 mg. of calcium phosphate/ml.; 0.1 M with respect to acetate buffer at pH 6.0) and made up to 2 ml. with 0.1 M acetate buffer at the same pH. After 10 min. the supernatant obtained after centrifuging was analysed for enzyme activity and protein. (Dilutions were made with acetate buffer, pH 6.0, for spectrophotometric protein determinations, since precipitation of protamine occurred on dilution with water or alkaline buffers.) The optimum amounts of gel to be added were calculated from this preliminary experiment. A portion (400 ml.) of protamine supernatant corresponding to about 2 kg. of mycelium was treated with 20 ml. of gel, stirred gently for 30 min. and centrifuged. The residue, which contained very little enzyme, was discarded and the supernatant was treated as before with 140 ml. of gel to adsorb the enzyme. After centrifuging the gel was washed twice with 20 ml. portions each of 0.1 M acetate buffer, pH 6.0. The enzyme was then eluted with 20 ml. of 0.65 saturated ammonium sulphate solution which was 0.07 M with respect to acetate buffer at pH 6.0 (fraction III). The specific activity of the eluate varied from 7 to 12 with different batches of mycelium.

The eluate was refractionated by the addition of saturated ammonium sulphate solution, and the fractions obtained between 0.65–0.70, 0.70–0.73 and 0.73–0.76 saturation were collected by centrifuging. The 0.73–0.76 fraction had a specific activity of 20–24 and was suitable for analytical purposes (fraction IV). Further purification by ammonium sulphate fractionation gave a preparation of about twice this specific activity (43–44), but the quantity of enzyme obtained was too little for determining its homogeneity. The purification procedure up to fraction IV was found to be reproducible with six different batches of mycelium,

and the yield was 14–18% of the activity of the extract. It should, however, be noted that the activity of the extract shown in Table 1 is higher than the actual value, since the extract contains 6-phosphogluconic dehydrogenase.

Properties. Purified enzyme preparations were generally dissolved in ammonium sulphate solution (0.40 saturation and 0.06–0.08 M with respect to acetate buffer at pH 6.0), and could be kept at 0° for 2–3 days or at –20° for at least 2–3 months without any loss of activity. When the enzyme was suspended in ammonium sulphate solution in the absence of acetate buffer, the enzyme was less stable, especially if the pH was below 5.0. Dilute solutions in phosphate or aminotrihydroxymethylmethane buffer, pH 7.4 (0.1 mg. of protein/ml.), were stable at –20° for about 2 days, but lost about half the activity in 24–36 hr. at 0°. At higher concentrations (1–5 mg. of protein/ml.) the enzyme could be dialysed against neutral buffers at 0° for 12–14 hr. with a loss of only 10% of the activity.

The rate of reduction of TPN was proportional to enzyme concentration and time when the total change in optical density was less than 0.10. The enzyme was specific for TPN and showed no activity or inhibition with DPN (1 μ mole/ml.). Purified *Zwischenferment* (fraction IV) was found to have no phosphoglucoisomerase, phosphoglucomutase, hexokinase or 6-phosphogluconic dehydrogenase, as shown by the absence of TPN reduction in 30 min. with 100 μ g. of enzyme, when G 6-P was replaced in the routine test system by equivalent amounts of F 6-P, G 1-P (with or without 10 μ moles of cysteine), glucose and ATP, or 6-phosphogluconate. Phosphatase activity with these substrates, destruction of TPN or re-oxidation of reduced TPN was also not observed in 30 min. with the same quantity of enzyme. The gel eluate (fraction III) has been used for the routine estimation of G 6-P, TPN and F 6-P, but the presence of other enzyme impurities in this fraction has not been studied.

Table 1. *Purification of Zwischenferment*

A. niger mycelium (1.9 kg. fresh wt.) obtained after preliminary extraction with dilute ammonia soln. was used for this experiment.

Fraction	Vol. (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg.)
I Extract	1900	5430	18 000	0.3
II Ammonium sulphate fraction 0.35–0.55*	23.2	4060	3 013	1.3
III Gel eluate	20.0	2160	212	10.2
Ammonium sulphate fractions				
0.65–0.70*	2.4	234	49	4.8
0.70–0.73*	0.6	263	19	13.8
IV 0.73–0.76*	1.5	820	41	20.0

* These figures refer to the degree of saturation with ammonium sulphate.

pH optimum. *Zwischenferment* from *A. niger* showed a broad pH optimum between 8.1 and 8.6 (Fig. 1) similar to that of the enzyme from *Escherichia coli* (Scott & Cohen, 1953). At pH 7.6, which was used for routine enzyme assay, the activity of the enzyme was about 10–15% less than at pH 8.1.

Substrate concentration. The effect of substrate concentration on enzyme activity (Lineweaver & Burk, 1934) is shown in Fig. 2. The Michaelis constant for the enzyme was about 1.7×10^{-4} M, which is of the same order as that reported for the enzyme from *E. coli* [3 to 5×10^{-4} M (Scott & Cohen, 1953)]. The concentration of TPN used in the test system (1.67×10^{-4} M) was found to be optimum for enzyme activity, and the increase in activity at higher TPN concentrations (5×10^{-4} M) was negligible.

Effect of bivalent metals. *Zwischenferment* from yeast and *E. coli* has been shown to be activated by Mg^{2+} , Ca^{2+} , Ba^{2+} and Mn^{2+} ions (Kornberg, 1950; Scott & Cohen, 1953). The effect of different bivalent cations on the enzyme from *A. niger* is shown in Table 2. The Ca^{2+} , Mg^{2+} and Ba^{2+} ions showed a slight activation at low concentrations, and at higher concentrations Ca^{2+} and Ba^{2+} ions were inhibitory; Zn^{2+} , Co^{2+} , Mn^{2+} and Cu^{2+} ions were found to be inhibitory, whereas Scott & Cohen (1953) observed activation of the enzyme from *E. coli* by Mn^{2+} and Co^{2+} ions and inhibition by

Fig. 1. Effect of pH on enzyme activity. The test system containing 20 μ moles of G 6-P, 45 μ moles of magnesium chloride, 0.4 μ mole of TPN and 110 μ moles of glycylglycine was adjusted to the required pH by the addition of 0.2N-HCl or 0.2N-NaOH and made up to 2.9 ml. The reaction was started by the addition of 0.1 ml. of enzyme containing 1 μ g. of protein of specific activity 16 units/mg., which was adjusted to the same pH as the test solution. The variation in pH of the test solution determined before and after measurement of enzyme activity was negligible. Temp., 30°.

Fig. 2. Effect of substrate concentration $[S]$. v is expressed as increase in optical density/min. at 340 m μ . $K_m = 1.7 \times 10^{-4}$ M.

Table 2. Effect of bivalent metals

Composition of the assay system for the enzyme was the same as in the routine test except for magnesium chloride and other additions indicated in the table.

Additions	Concn. (mm)	Change in optical density at 340 m μ /min.	Activation (+) or inhibition (-) (%)
—	—	0.041	—
MgCl ₂	1.7	0.042	+ 2.4
	3.3	0.044	+ 7.4
	5.0	0.044	+ 7.4
	10.0	0.046	+ 12.2
	15.0	0.046	+ 12.2
CaCl ₂	5.0	0.043	+ 5.0
	15.0	0.043	+ 5.0
	30.0	0.043	+ 5.0
	60.0	0.031	- 24.4
BaCl ₂	1.0	0.041	—
	10.0	0.048	+ 17.0
	20.0	0.050	+ 22.0
	40.0	0.039	- 5.0
MgCl ₂ * + Co ²⁺	0.1	0.035	- 24.0
	1.0	0.030	- 34.8
	10.0	0.023	- 50.0
MgCl ₂ * + Zn ²⁺	0.1	0.039	- 15.0
	1.0	0.035	- 24.0
	2.0	0.025	- 45.7
	6.6	0.015	- 67.4
MgCl ₂ * + Mn ²⁺	1.0	0.032	- 30.0
MgCl ₂ * + Cu ²⁺	0.1	0.033	- 28.3
	1.0	0.027	- 41.3
	2.0	0.022	- 52.2
	3.0	0.018	- 61.0

* The amount of magnesium chloride added in these tests was 45 μ moles and the percentage of inhibition was calculated relative to the activity of the control containing the same amount of MgCl₂ (ΔE , 0.046/min.).

Zn^{2+} and Cu^{2+} ions. No absolute requirement for bivalent ions could be demonstrated with *Zwischenferment* from *A. niger*, and ethylenediaminetetraacetate ($7 \times 10^{-3}M$) was found to have no effect on activity. The metal content of the enzyme preparation was, however, not investigated.

Cysteine, iodoacetate and fluoride had no effect on enzyme activity at a concentration of $10^{-2}M$. The enzyme from liver has been reported to be susceptible to sulphydryl inhibitors (Glock & McLean, 1953).

DISCUSSION

Maximum *Zwischenferment* activity was obtained at the concentrations of G 6-P and TPN used in the routine assay system. Under the experimental conditions described by Kornberg (1950) at 25° , the activity of the enzyme was about 28 % less.

The purity of fraction IV was about six times higher than that reported by Kornberg for the yeast enzyme. The purified preparation of Negelein & Gerischer (1936) catalysed the uptake of $15.6 \mu\text{moles}$ of oxygen/min./mg. of enzyme at 38° , when the activity was measured manometrically in the presence of Warburg's 'yellow enzyme' and oxygen, $1 \mu\text{mole}$ of oxygen being equivalent to $1 \mu\text{mole}$ of reduced TPN in the absence of catalase (Warburg & Christian, 1933). But the relative purity of this preparation is difficult to ascertain with accuracy owing to the differences in the method of enzyme assay.

SUMMARY

1. *Zwischenferment* from *Aspergillus niger* was purified about 60-fold.

2. The purified enzyme was free from phosphoglucisomerase, phosphoglucomutase, hexokinase and 6-phosphogluconic dehydrogenase, and was suitable for analytical purposes.

3. Some of the properties of the enzyme, pH optimum, Michaelis constant, etc., have been described.

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Two-dimensional Paper Chromatography of Urinary Indoles and Related Substances

By C. E. DALGLIESH

Postgraduate Medical School, Ducane Road, London, W. 12

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Many indolic compounds, and their degradation products, can occur in urine in pathological states, e.g. carcinoidosis (cf. review by Langemann, 1955) and Hartnup syndrome (Dent, 1952). Some of the metabolites may be concerned with bladder cancer (e.g. Boyland & Williams, 1955), and the appearance of metabolites in the urine may give valuable information on the state of vitamin nutrition (Dalgliesh, 1956). Identification of such substances is thus becoming of increasing importance, but the number of indolic compounds and their degradation products, potentially occurring, is too large to permit separation on one-dimensional chromatograms. A two-dimensional system suitable for routine use was therefore sought, and this paper

reports the 'map of the spots' given by the solvent combination finally selected from amongst several tried.

EXPERIMENTAL

Chromatograms were run by the descending technique on sheets of Whatman no. 1 paper, $18\frac{1}{2}$ in. $\times$ $22\frac{1}{2}$ in. The sources of substances used in synthetic mixtures are given in the footnotes to Tables 1 and 2. Urine extracts were prepared by adsorption of aromatic metabolites on deactivated charcoal, followed by elution with aqueous phenol, and concentration of the phenol eluate (Dalgliesh, 1955a). All chromatograms were run at room temperature, the chromatography tanks being away from draughts, but the temperature not being otherwise controlled.