SHORT COMMUNICATIONS

THE CONVERSION OF GENTISIC ACID TO MALEYLPYRUVIC ACID BY AN *ACHROMOBACTER* SPECIES*

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Simultaneous adaptation experiments with washed cell suspensions of an *Achromobacter* species indicate that this organism metabolizes anthranilic acid and *N*-(carboxymethyl)anthranilic acid (CMAA) via gentisic acid (Ladd, unpublished data). It is the purpose of this communication to show that cell-free extracts, prepared from cells adapted to anthranilic acid or CMAA, oxidize gentisic acid to maleylpyruvic acid similarly to *Pseudomonas* spp. used by Lack (1959, 1961) and by Sugiyama *et al.* (1960).

Experimental

The organism was grown in a liquid medium containing 1% "Vegemite", 0·42% K_2HPO_4, 0·32% KH_2PO_4, 0·2% NaCl, 0·2% NH_4Cl, and 0·05% of either anthranilic acid or CMAA, pH 7·2. After shaking for 3 days at 25°C, the cells were harvested by centrifugation, washed once with distilled water, and suspended in 0·01M potassium phosphate, pH 7·0, to a final concentration of 80–100 mg dry weight of bacteria/ml. Yields of 0·8–1·0 g dry weight of cells per litre of medium were usually obtained. Cell-free extracts were prepared by homogenizing 20-ml aliquots of the suspension with 20 g ballotini glass beads (No. 12) for 10 min at 5°C at full speed (approx. 16,000 r.p.m.) in a Servall omni-mixer. The homogenates were centrifuged at 10,000 *g* for 1 hr at 1°C and the supernatants, containing 25–35 mg protein/ml, were stored at −15°C until tested for their activity on gentisic acid.

The oxidation of gentisic acid to maleylpyruvic acid was measured either manometrically at 30°C with the conventional Warburg apparatus, or spectrophotometrically by determining the increase in absorbancy at 330 mμ with a Shimadzu QR-50 spectrophotometer.

Results and Discussion

Extracts prepared from cells adapted to oxidize anthranilic acid or CMAA readily oxidized gentisic acid to maleylpyruvic acid whereas extracts prepared from unadapted cells were either unable to catalyse this conversion or did so very slowly (Fig. 1). The specific activities of extracts prepared from adapted cells were very similar: ΔO.D. (330 mμ) = 0·019—0·023 units/min/mg protein.

For each mole of gentisic acid metabolized, one mole of oxygen was consumed without the liberation of carbon dioxide. Aliquots of the deproteinized reaction

* Manuscript received March 30, 1962.

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mixture, prepared immediately after the conclusion of oxygen uptake, gave a positive reaction with an *o*-phenylenediamine reagent (Witter, Snyder, and Stotz 1948), indicative of a β-diketone. The ultraviolet absorption spectrum of the product, measured on the deproteinized reaction mixture, was identical with that of maleylpyruvic acid (Lack 1959), showing a maximum at 330 mμ at pH 13 and exhibiting very low absorption at pH 1. Storage of the product at pH 1 at room temperature

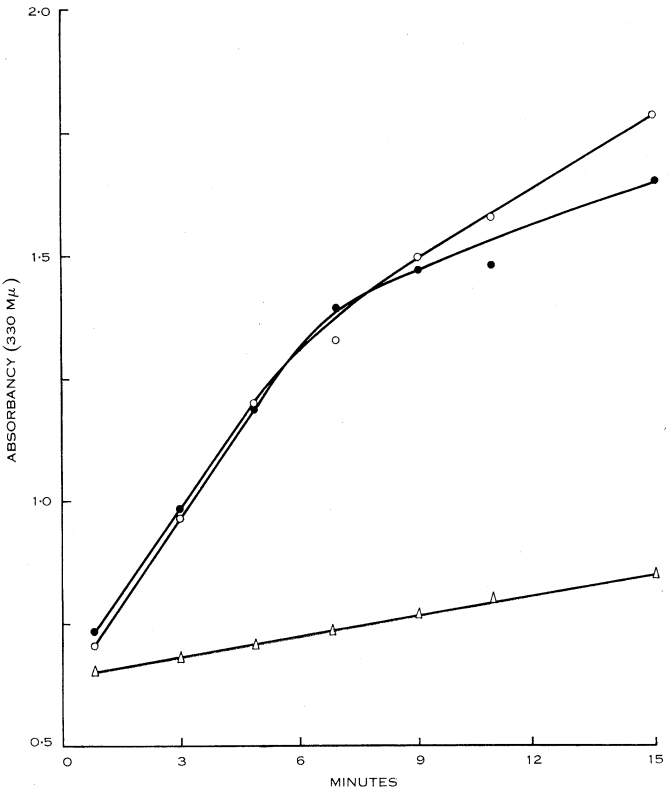

Fig. 1.—The oxidation of gentisic acid by extracts prepared from unadapted cells (△), anthranilic acid-adapted cells (○), and CMAA-adapted cells (●). Bacterial extract (≡ 7·5 mg protein) incubated with 0·4 μmole gentisic acid in 3·0 ml of 0·03M tris-(hydroxymethyl)aminomethane buffer, pH 7·5, at 25°C.

for periods up to 24 hr resulted in its conversion to fumarylpyruvic acid, determined by special spectral measurements at pH 1 and pH 13 (Lack 1959; Sugiyama *et al.* 1960). The product of gentisic acid oxidation was reduced by hydrogen in the presence of a palladium catalyst; the absorption spectrum of the reduced material at pH 13 showed a maximum at 296 mμ, identical with that of succinylpyruvic acid (Lack 1961).

By measuring the rate of decrease in absorbancy at 330 mμ and 340 mμ respectively, it was shown that crude extracts of adapted cells catalysed a very slow

breakdown of maleylpyruvic acid, measurable over a period of several hours, but a relatively rapid breakdown of fumarylpyruvic acid (ΔO.D. (340 mμ) = 0·014 units/min/mg protein). On the addition of glutathione, maleylpyruvic acid was decomposed at a rate similar to that of fumarylpyruvic acid as described by Lack (1959). In each case pyruvic acid was established as a decomposition product by demonstrating the oxidation of DPNH in the presence of lactic dehydrogenase and by comparing the absorption spectrum of the 2,4-dinitrophenylhydrazone derivative with that of the authentic 2,4-dinitrophenylhydrazone of pyruvic acid.

TABLE 1

INHIBITION OF MALEYLPYRUVIC ACID FORMATION

Enzymic activity measured by the change in the optical density (at 330 mμ) in time interval 1–5 min. Inhibitor and extract preincubated for 15 min at 25°C in 3·0 ml of 0·03M tris(hydroxymethyl)aminomethane buffer, pH 7·5, before addition of 0·4 μmole gentisic acid

Inhibitor	Molar Concentration	Inhibition (%)
o-Phenanthroline	$6{\cdot}6\times10^{-5}$	87
$\alpha\alpha$-Dipyridyl	$1{\cdot}3\times10^{-4}$	75
Azide	$6{\cdot}6\times10^{-4}$	73
Cyanide	$6{\cdot}6\times10^{-4}$	47
Mercuric chloride	$6{\cdot}6\times10^{-4}$	100
	$6{\cdot}6\times10^{-5}$	57
Iodoacetic acid	$6{\cdot}6\times10^{-3}$	24
p-Chloromercuribenzoic acid	10^{-3}	24

The effect of various inhibitors on gentisic acid oxidation is shown in Table 1. The formation of maleylpyruvic acid was inhibited by azide, cyanide, $\alpha\alpha$-dipyridyl, and *o*-phenanthroline. The inhibition by the chelating agents was relieved by the addition of ferrous ions. Attempts to demonstrate directly a specific requirement for ferrous ions by using aged, dialysed, or ammonium sulphate-precipitated extracts resulted in a loss of their activity which was not restored by the addition of ferrous ions with or without the further addition of either boiled crude bacterial extract or a crude liver concentrate.

Sugiyama, Yano, and Arima (1960) showed that the conversion of gentisic acid to fumarylpyruvic acid by purified extracts of *Pseudomonas ovalis* was not especially sensitive to sulphydryl reagents although the essential sulphydryl nature of the system was demonstrated using extracts preincubated with the inhibitor. This was confirmed with crude extracts of *Achromobacter* sp. since mercuric chloride inhibited the formation of maleylpyruvic acid, whilst cysteine or thioglycollic acid reversed this inhibition. Iodoacetic acid and *p*-chloromercuribenzoic acid in relatively high concentrations caused a slight inhibition when preincubated with the crude extract for 15 min before substrate addition. Knox and Edwards (1955), studying

an analogous reaction, the conversion of homogentisic acid to maleylacetoacetic acid, were able to demonstrate an inhibition by *p*-chloromercuribenzoic acid in concentrations as low as 3×10^{-5}M.

The crude extracts did not oxidize salicylic acid, *m*-hydroxybenzoic acid, hydroquinone, or any of the isomers of gentisic acid except 2,3-dihydroxybenzoic acid which gave a variable response. None of these compounds inhibited gentisic acid oxidation. No inhibition by hydroquinone, even when present in concentrations 10 times greater than that of the substrate, contrasts with the demonstration by Sugiyama, Yano, and Arima (1960) of its potent inhibition of fumarylpyruvic acid formation by extracts of *Ps. ovalis*.

The author wishes to thank Mr. M. Amato for technical assistance, and Dr. K. Yano, Department of Agricultural Chemistry, University of Tokyo, for his generous gift of fumarylpyruvic acid.

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