THE ENZYMIC OXIDATION OF GENTISIC ACID

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SUMMARY

A cell-free bacterial preparation is described which can oxidatively cleave gentisic acid to yield maleylpyruvic acid. In the presence of GSH this preparation can be made to catalyze the isomerization and hydrolysis of maleylpyruvate to yield fumaric and pyruvic acids. Other sulfhydryl-containing compounds cannot replace reduced glutathione.

INTRODUCTION

The isolation of a bacterium capable of growing with sodium gentisate as the sole carbon source was reported by ROOF, LANNON AND TURNER[1]. They suggested that the metabolism of this compound might involve ring cleavage in a manner analogous to the degradation of homogentisic acid as reported earlier by RAVDIN AND CRANDALL[2]. In a preliminary paper[3] we reported that cell-free extracts of a strain of *Pseudomonas* grown on sodium gentisate as the sole carbon source could metabolize gentisate oxidatively to yield pyruvic and malic acids. Since these crude extracts contained fumarase, it was pointed out that the findings were in accord with the ring cleavage mechanism postulated by ROOF *et al.* We subsequently became aware of earlier work, published in Japan, in which TANAKA, SUGIYAMA, YANO AND ARIMA[4] isolated and characterized fumarylpyruvic acid as a product of gentisate oxidation by dialyzed cell-free extracts of *Pseudomonas ovalis*. The observations reported in the present paper demonstrate that maleylpyruvic acid is the more immediate product of gentisic acid metabolism and that maleylpyruvate is converted to fumarylpyruvate by enzymic isomerization. In this latter reaction, glutathione may act as a cofactor.

EXPERIMENTAL

Biological materials

The organism used in these experiments is a strain of *Pseudomonas* isolated from rat feces. Stock cultures were stored at room temperature on Tripticase Soy Agar. Cells were transferred to new slants every two weeks. The culture medium consisted of 0.1% ammonium sulfate, 0.05% magnesium sulfate, 0.5% sodium gentisate in 0.1 *M* potassium phosphate buffer, pH 7.15. The sodium gentisate was sterilized by filtration and added to the liquid medium which had been previously autoclaved. Although this culture medium was capable of supporting growth, it was found that

References p. 123.

the yield of bacteria could be increased by the addition of 10 mg of ferrous ammonium sulfate and 50 mg of dried yeast extract per liter of culture medium. The bacteria were incubated at room temperature for 4 days in 5 l bottles each containing 1 l of medium. The cells were harvested by centrifugation and washed with 200 ml of 0.005 *M* sodium gentisate in 0.05 *M* potassium phosphate buffer of pH 7.15 per 100 g of wet cells. Approximately 3 g of wet cells were obtained from 1 l of medium. These cells can be stored at —15° for several months before being used. Maleylacetoacetic acid isomerase and fumarylacetoacetic acid hydrolase were prepared according to the procedure of KNOX[5].

Reagents

Sodium gentisate was purchased from Nutritional Biochemical Corporation and was used as such for the bacterial cultures. All enzymic incubations were performed with commercial sodium gentisate which had been recrystallized twice from water. Homogentisic acid lactone was obtained from the California Foundation for Biochemical Research and was used without further purification. Solutions of crystalline bovine plasma albumin for a protein standard were obtained from the Armour Laboratories. Tripticase Soy Agar is a product of the Baltimore Biological Laboratory. Lactic acid dehydrogenase, alcohol dehydrogenase, DPN, and protamine sulfate were products of Nutritional Biochemical Corporation. Maleylacetoacetate was prepared in solution according to the procedure of KNOX[5] with the slight modification that homogentisic acid lactone was used instead of homogentisic acid. Since the lactone is considerably less soluble than the acid, each Warburg vessel used contained 3 μmoles of substrate dissolved in 1 ml distilled water in the side arm. The buffer used for the main well was Tris 0.2 *M*, pH 7.2.

Analytical methods

Protein determinations were made by the method of WARBURG AND CHRISTIAN[6]; when the ratio of optical densities at 280 to 260 mμ was less than 0.6, the biuret method of WEISSMAN AND SCHOENBACH was used. Spectrophotometric measurements were made with the Beckman model DU spectrophotometer. Manometric measurements were conducted at 36° by use of the Warburg apparatus. Identification of organic acids by paper chromatography utilized the following solvent systems: butanol–water–formic acid, benzyl alcohol–water–formic acid[8] and alcohol–water–ammonia[9]. Silica gel chromatography as described by VARNER[9] was used for the isolation of maleic acid. Under these conditions maleic acid was eluted from the column between 120 to 160 ml.

Preparations of cell-free extracts

25 g of bacteria were suspended in 50 ml of 0.05 *M* potassium phosphate buffer, pH 7.15, containing 30 mg sodium gentisate and permitted to remain at room temperature with stirring for 15 min. The centrifuged cells were disrupted by freezing and thawing under pressure according to the principles elucidated by HUGHES[10]. To this end the biological material was placed in a stainless steel container 1 ¼ in. in diameter which can be fitted with a piston $1^3/_{16}$ in. diameter. The block was cooled in an alcohol Dry Ice bath, the piston placed on the cylinder and the bacteria were subjected to pressures of approximately 1000 kg/cm². As this system warms a point

References p. 123.

is reached when the frozen bacterial suspension suddenly thaws. This is accompanied by the rupture of at least 90 % of the cells. The material is then suspended in 50 ml of 0.05 *M* potassium phosphate buffer pH 7.15, stirred at room temperature for 15 min and centrifuged at 0° for 1 h at 15,000 × *g*. The supernatant material was then treated with 1 ml amounts of 2 % protamine sulfate until no additional precipitation occurred. The supernatant solutions obtained from these protamine sulfate precipitations were treated with an equal amount of a saturated ammonium sulfate solution which had been adjusted to pH 7.2 with ammonium hydroxide. The precipitate was redissolved in 0.05 *M* potassium phosphate buffer pH 7.15, the final volume being equivalent to one half the volume of the original cell-free extract, and treated once more with saturated ammonium sulfate to 50 % saturation. The precipitate was then redissolved in 10 ml of 0.05 *M* potassium phosphate buffer and used as soon as possible. This material loses activity after several days even when stored at —20°. All protamine sulfate and ammonium sulfate operations were performed at 0°.

Enzymic incubations

Incubations were performed in Warburg manometer vessels which contained 10 μmoles of sodium gentisate, 5 μmoles of ferrous ammonium sulfate, 0.5 ml of enzyme solution, and 100 μmoles of potassium phosphate buffer at pH 7.15. The total volume was 1.1 ml; the temperature was 36°; and the gas phase was air. Large 125 ml manometer vessels containing 10 times the above amounts were used for preparative purposes.

Preparation of maleylpyruvate

Manometric incubations were carried out as described with the use of the ammonium sulfate-treated enzyme preparations. When the theoretical amount of oxygen had been absorbed (one mole of oxygen per mole of substrate) the oxygen consumption ceased. The contents of the flask were cooled; one-tenth the volume of chilled 30 % perchloric acid was added; the material was centrifuged and the supernatant liquid neutralized with potassium hydroxide at 0°. The precipitated potassium perchlorate was removed and the solutions containing the products of oxidation were used.

Calculations of the molar absorption coefficients of the new compound were made on the basis of the previously observed oxygen consumption in relation to suitable aliquots of the above solution. The molar absorption at 330 mμ in solutions of pH 7.15, obtained in this manner agreed within 5 % of the value obtained by estimating the amount of the new compound by enzymic analysis, *i.e.* by enzymically producing pyruvate and oxidizing reduced DPN with lactic acid dehydrogenase (see RESULTS).

When large amounts of the new intermediate were required in a form capable of being stored, the following procedure was used. The contents of the large manometric vessels were chilled and added to five times their volume of absolute alcohol. The precipitate obtained was removed by centrifugation, and the alcoholic supernatant was treated with 4 vol. of absolute ether. A yellow flocculent precipitate was formed which was centrifuged and dried in a vacuum dessicator. This yellow material consisted of crude ammonium salt of maleylpyruvate, in approximately 60 % purity. It can be stored at 0° for several months without change. Material prepared in this

References p. 123.

manner has all the properties of the material which was present in the neutralized perchloric acid supernatants.

Preparation of fumarylpyruvate

TANAKA *et al.*[4] prepared this compound by incubating sodium gentisate with dialyzed cell-free extracts of *m*-hydroxybenzoate induced cells of *Pseudomonas ovalis*. Ether extracts of the acidified deproteinized incubation mixtures yielded a substance which after recrystallization from ethyl acetate and petroleum ether was identified as fumarylpyruvate. We have found that the intermediate maleylpyruvate is not stable as the free acid; attempts to isolate the free acid resulted in its conversion to fumarylpyruvate. This property of the new intermediate was utilized for the preparation of fumarylpyruvate. Solutions were therefore acidified with dilute sulfuric acid and worked up in the manner reported by TANAKA *et al.* The yield based on maleylpyruvate was 52 %. This material decomposed at 183–188°, and had the u.v. spectral and enzymic properties reported by the original workers.

RESULTS

When sodium gentisate was incubated with the bacterial enzyme preparation a yellow product appeared. Deproteinized solutions of the above incubation mixture showed the presence of a material with u.v. absorption properties different from those of fumarylpyruvic acid (Figs. 1 and 2). The absence of significant absorption in the near u.v. region in acid solution is in contrast to the strong absorption properties of fumarylpyruvate. The intermediate disappeared when incubated with the same bacterial preparation but with added GSH. This procedure produced a substance which can oxidize DPNH in the presence of lactic acid dehydrogenase (Fig. 3). Fumarylpyruvate did not require GSH in order to undergo a similar reaction (Fig. 4). Neither substrate of itself had the capacity of oxidizing DPNH in the presence of lactic acid dehydrogenase. Replacement of the bacterial enzyme with maleylaceto-

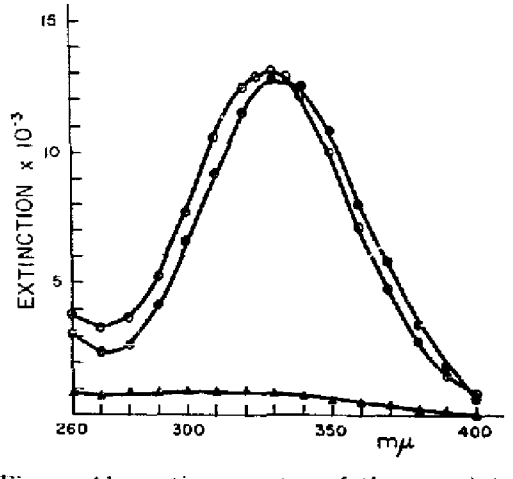

Fig. 1. Absorption spectra of the new intermediate at pH 7.15 —○—, pH 13 —●—, and pH 1 —▲—. The method for the determination of the molar extinction coefficients is described in the text.

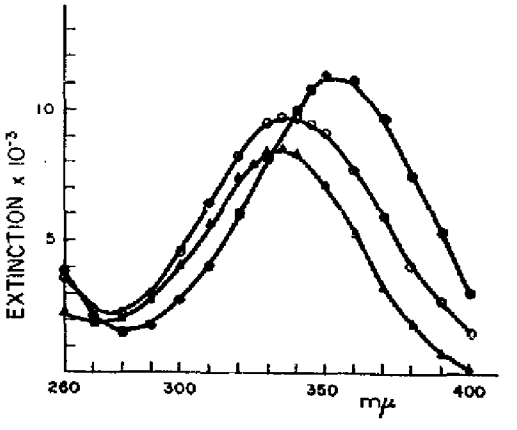

Fig. 2. Absorption spectra of fumarylpyruvic acid at pH 1 —▲—, pH 7.15 —○—, and pH 13 —●—. Determinations of the molecular extinction coefficients were made on known solutions of purified material.

References p. 123.

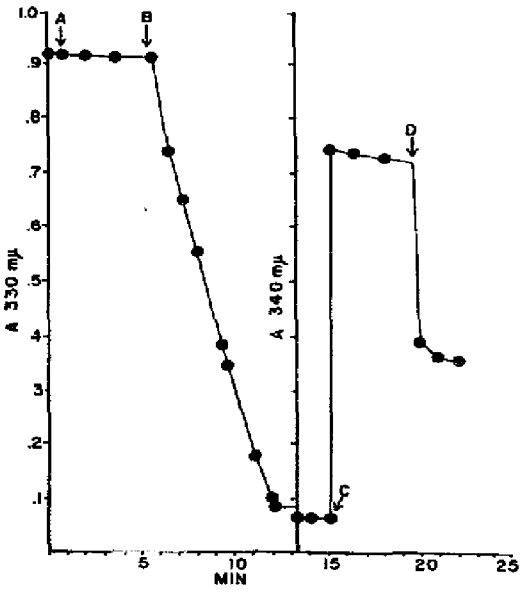

Fig. 3. The action of the bacterial enzyme system and GSH on the new intermediate. All absorption data are recalculated on the basis of a constant total volume of 3.0 ml. The cuvette initially contained the new intermediate 0.20 μmole dissolved in 0.05 *M* potassium phosphate buffer pH 7.15. Additions: A, bacterial enzyme prep. 0.10 mg protein; B, GSH, 0.15 μmole; C, DPNH 0.36 μmole; and D, lactic acid dehydrogenase, 0.04 mg.

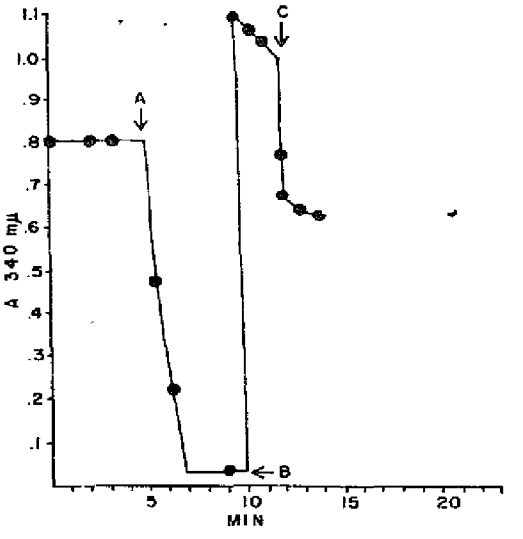

Fig. 4. The enzymic hydrolysis of fumarylpyruvate in the presence of the bacterial enzyme system. All absorption data are recalculated on the basis of maintaining the total volume at 3.0 ml. The cuvette initially contained 0.27 μmole of fumarylpyruvate in 0.05 *M* potassium phosphate buffer. Additions: A, bacterial enzyme prep., 0.11 mg protein; B, DPNH 0.5 μmole; and C, lactic acid dehydrogenase, 0.04 mg.

acetic acid isomerase and maleylacetoacetic acid hydrolase gave similar results (Fig. 5). Maleylacetoacetic acid can be hydrolyzed by the bacterial system provided reduced glutathione is added (Fig. 6). It should be noted that ten times the amount of bacterial enzyme are necessary in order to obtain a rate of isomerization and hydrolysis of maleylacetoacetate comparable with the rates observed with the new intermediate.

The bacterial enzyme preparation had sufficient fumarase present to prevent the accumulation of fumaric acid. Consequently the mammalian enzymes were used to demonstrate the products of enzymic hydrolysis. In the case of fumarylpyruvate both fumaric and pyruvic acid accumulated. The new intermediate also yielded these acids but only when glutathione was present in the incubation mixture. The identification of the organic acids was based on paper chromatography.

Fig. 7 shows that cysteine, thioglycolate and 2 mercapto-ethanol could not effectively replace nor inhibit GSH in the bacterial system.

When the new intermediate was refluxed with 0.1 *N* NaOH for 30 min the substance lost its optical properties. Continuous ether extraction of this acidified solution yielded a substance which chromatographed on silica gel columns in the same manner as maleic acid. The yield was 10 % calculated on the basis of the amount of titratable acid recovered from the column. This material when subjected to paper chromatography had the same properties as maleic acid in the three solvent systems. 200 μmoles of the new intermediate were incubated with glutathione and the bacterial enzyme

References p. 123.

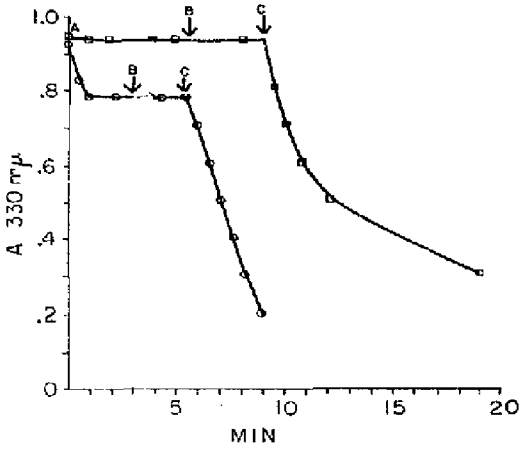

Fig. 5. The isomerization and hydrolysis of the new intermediate —□— by preparation of maleylacetoacetic acid isomerase and fumarylacetoacetic acid hydrolase. For purposes of comparison maleylacetoacetic acid —○— is subjected to the same enzymes. Additions: A, hydrolase 0.1 ml (activity 1.5 units per ml per min); B, GSH, 0.3 μmole; C, Isomerase 0.1 ml (activity *k*/ml/min 2.0). Initial concentration of substrates: new intermediate, 0.7 μmole; maleylacetoacetate, 0.12 μmole with fumarylacetoacetate as a contaminant 0.04 μmole. Data recalculated on basis of maintaining total volume constant at 2.8 ml.

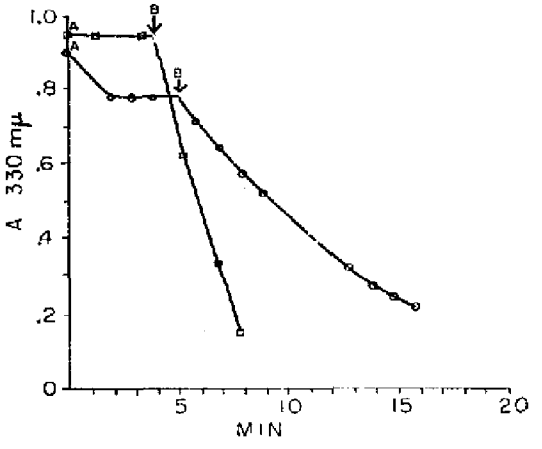

Fig. 6. The isomerization and hydrolyses of maleylacetoacetate —○— by a preparation of the bacterial enzymes. For purposes of comparison, maleylpyruvate —□— is presented under the same conditions except that one tenth the amount of enzyme preparation was used. Additions: A, bacterial enzyme system, 0.04 mg protein in cuvette containing maleylpyruvate and 0.40 mg protein for cuvette containing maleylacetoacetate; B, GSH 0.3 μmole. Data are recalculated on the basis of constant total volume of 2.8 ml. Substrate concentrations were the same as the conditions of Fig. 5.

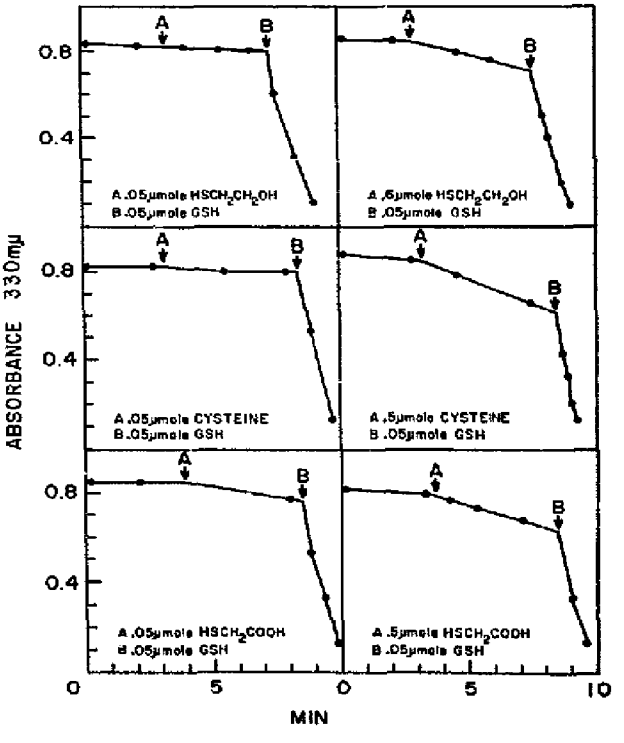

Fig. 7. Specificity for GSH requirement in the bacterial enzyme system. Total volume 2.7 ml. Each cuvette initially contained 0.1 mg of the bacterial enzyme preparation and 0.15 μmole of the new intermediate in 0.05 *M* potassium phosphate buffer. The additions are as indicated on the charts.

(under conditions described in Fig. 3). A ketoacid accumulated which was isolated as the 2,4-dinitrophenylhydrazone. Recrystallization from ethanol yielded a material with the same alkaline absorption spectrum as the 2,4-dinitrophenylhydrazone of pyruvic acid[11]. The decomposition point was 217–218°. There was no change of decomposition point when this material was mixed with an authentic sample of the pyruvic acid hydrazone.

DISCUSSION

The intermediate arising from the enzymic oxidation of sodium gentisate differs from fumarylpyruvate in several respects. The inability of this substance to absorb light in the near ultraviolet under acid conditions and its resistance to enzymic hydrolysis by mammalian and bacterial preparations are properties that differ from those of fumarylpyruvic acid. The new intermediate can be converted to fumarylpyruvate by nonenzymic methods as well as by enzymic procedures in which GSH acts as a cofactor. It is noteworthy that the foregoing properties of the intermediate are similar to those reported by KNOX AND EDWARDS[12,13] for maleylacetoacetate.

The identification of maleic acid as a product of alkaline degradation of the new substance and the identification of pyruvic acid as a product of its enzymic cleavage are evidence of its identity as maleylpyruvate.

The bacterial gentisate oxidation system allows the accumulation of maleylpyruvate when GSH is omitted from the incubation mixture. Under these conditions fumarylpyruvate is unstable. This is evidence that the first product of the cleavage of gentisate possesses the *cis* configuration which must be isomerized before being cleaved to fumaric and pyruvic acids.

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