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OXIDATION OF 3-DEOXY-3-FLUORO-D-GLUCOSE BY CELL-FREE EXTRACTS OF *PSEUDOMONAS FLUORESCENS*

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Abstract—The oxidation of 3-deoxy-3-fluoro-D-glucose (1) and 3-deoxy-3-fluoro-D-gluconic acid (2) by a cell-free extract of *Ps. fluorescens* proceeds with the consumption of 2 g-atoms and 1 g-atom of oxygen/mole of substrate respectively. The localization of the oxidizing system is considered to be in a particulate fraction and evidence is presented which indicates that the oxidation of the fluorinated substrates (1) and (2) is by the glucose-gluconic acid enzyme system. The difference observed between the extent of oxidation of 3-deoxy-3-fluoro-D-glucose by whole cell and cell-free extracts of *Ps. fluorescens* is discussed.

PREVIOUS investigations concerned with the enzymology and metabolism of fluorinated compounds support our contention that certain monofluorinated analogues of carbohydrates and related compounds can act as pseudosubstrates. Thus we have demonstrated that 3-deoxy-3-fluoro-D-glucose* and 2-deoxy-2-fluoroglycerol can act as substrates for the glycolytic enzymes[1] and glycerol kinase[2] respectively. Our *in vivo* studies with *S. cerevisiae* and 3FG also suggest the possibility that 3FG metabolites are produced.[3,4] Recently we reported the metabolism of 3FG to 3-deoxy-3-fluoro-D-gluconic acid* by resting cell suspensions of *Ps. fluorescens*.[5] Although the oxidation of 3FG was limited to the consumption of 1 g-atom of oxygen/mole of substrate, provision of the oxidation product (3FGA) as an exogenous substrate allowed further oxidation to a product which we have tentatively assigned the structure 3-deoxy-3-fluoro-2-keto-D-gluconic acid.* These results are consistent with the accepted glucose catabolism[6] by *Ps. fluorescens* although whether the formation of 3FGA from 3FG proceeds via the 3FGA-δ-lactone and a lactonase has not yet been established. In order to obtain further information about the location and specificity of the enzyme system involved with these metabolic transformations we have now examined the action of an enzyme preparation from *Ps. fluorescens* on 3FG and 3FGA.

METHODS AND MATERIALS

Crystalline 3FG and 3FGA were prepared as previously reported by us.[5] A culture of *Ps. fluorescens*, A.3.12 obtained from N.C.I.B., was routinely maintained on a glucose/mineral salts agar medium[7] and the same medium in liquid form used for the large scale cultivation of organisms. Cell yields necessary for cell-free preparations

Abbreviations used: 3FG, 3-deoxy-3-fluoro-D-glucose. 3FGA, 3-deoxy-3-fluoro-D-gluconic acid. 3F2KGA, 3-deoxy-3-fluoro-2-keto-D-gluconic acid.

were achieved by the use of a 10-l. fermentation vessel. Inocula for these cultures were prepared from 24 hr slope cultures by washing with sterile distilled water and transferring, with the usual aseptic precautions, to 2 × 125 ml amounts of glucose/mineral salts medium contained in 1-l. Erlenmeyer flasks. The flasks were aerated by agitation on a rotary shaker (Orbital Shaker Mark V, L.H. Engineering Co. Ltd., Stoke Poges, Bucks.) at 30° for 18 hr, when suitable growth was achieved. Aeration for large scale culture vessels was supplied by a Vibromischer electrical drive (Vibromischer, Chemap., A.G. Zurich) with a type S.U.B. pump attached. Using this apparatus high oxygen solubility rates were obtained throughout the medium together with rapid high growth yields.

Cells were harvested with a continuous flow Sharples Super centrifuge separator at 25,000 rev/min (Sharples Centrifuges Ltd., Camberley, England). The concentrated suspension was spun in a "Mistral 6L" centrifuge (M.S.E. Ltd.) at 6200 *g* for 15 min, the cells washed twice with 0·067 M phosphate buffer, pH 7·0, and a thick paste of cells made in phosphate buffer, pH 7·0. Ultrasonic breakage was carried out using a magnetostrictive transducer unit (Radyne Ltd., Wokingham, Surrey, England driven at 20 kc/sec by a 200-W power amplifier. The transforming stub was of titanium and therefore of low heat conductance, and the temperature during the break was kept below 10° by salt–ice mixture. *Ps. fluorescens* A.3.12, suspended in 1 vol. of 0·067 M phosphate buffer, pH 7·0, was homogenized and ultrasonicated for 3 × 2 min periods with a short break to prevent heating. Centrifugation of ruptured cell suspensions so obtained in the "Super Speed 50" (M.S.E. Ltd.) at 20,000 *g* for 15 min separated whole cells, a pink particulate layer and an opalescent, reddish supernatant. A mixture of this supernantant and particulate material was used as a crude cell-free preparation without further fractionation.

Oxygen uptake was followed by the manometric method[8] in a Warburg respirometer at a temperature of 30°, total liquid volume of 2·0 ml and air as the gaseous phase. Protein estimation was by the method of Lowry[9] with bovine serum albumin (Sigma Co. Ltd., London) as protein standard. Spectral studies were made with Unicam SP 1800 (Unicam Ltd., Cambridge, England). Fluoride anion was measured by a fluoride electrode (Orion Research Inc., Cambridge, Mass., U.S.A.) in cell extracts before and after substrate oxidation.[10]

RESULTS AND DISCUSSION

Tested manometrically, the supernatant and particulate preparation oxidized glucose, gluconate, 3FG and 3FGA rapidly, and without the addition of accessory hydrogen carriers such as phenazine dyes or methylene blue. Ten μmole aliquots of substrate were oxidized at an approximately linear rate for 20–30 min and oxygen consumption then quickly declined to that of the control without added substrate. Calculated from the near linear period, the rate of 3FG oxidation proceeded approximately at half that of the same amount of glucose, whereas both gluconate and 3FGA oxidation proceeded at rates comparable to one another (Table 1). The net oxygen consumption per mole of substrate added was approximately 2 g atoms/mole for both glucose and 3FG, and 1 g atom for gluconate and 3FGA (Table 1). These are the theoretical values for the conversion of hexoses and aldonic acids to ketoaldonic acids. Tests on the suspending fluid from Warburg flasks at the end of the 3FG and 3FGA oxidations reveal the presence of reducing material which reacted positively with Benedict's

TABLE 1. OXIDATION OF 3FG AND 3FGA BY CELL-FREE EXTRACTS OF GLUCOSE GROWN *Ps. fluorescens*

Substrate oxidized	Initial rate of oxidation (μmoles O_2/mg protein/hr)	Net oxygen consumption (μl) (Endogenous subtracted)	Moles oxygen/mole substrate oxidized
20 μmoles glucose	0·52	410	0·92
20 μmoles 3FG	0·29	410	0·92
10 μmoles glucose	0·36	235	1·05
10 μmoles 3FG	0·20	260	1·16
20 μmoles gluconate	0·57	225	0·50
20 μmoles 3FGA	0·45	235	0·53
10 μmoles gluconate	0·34	145	0·65
10 μmoles 3FGA	0·31	155	0·69

Cells from a 20-hr growth were harvested, washed twice in 0·067 M phosphate buffer, pH 7·0, and resuspended in the same buffer to form a thick cell paste (see Methods) which was ruptured ultrasonically. The combined particulate and soluble fraction, obtained after removal of whole cells by centrifugation (20,000 *g* for 15 min) was used. Oxygen uptake was followed in Warburg manometers at 30°. Each flask contained 1·0 ml extract (48·5 mg protein), 1 μmole NAD, 0·67 M phosphate buffer, pH 7·0, to 1·8 ml in main well; 0·2 ml KOH in centre well. Reaction was initiated by tipping in 10 or 20 μmoles of substrate from side-arm.

reagent. This lends support to the tentative assignment of 3F2KGA to the fluoroaldonic acid also produced by whole cells.[5] An important difference, however, resides in the fact that whereas whole cells will only oxidize 3FG as a primary substrate to 3FGA (Scheme a), cell-free preparations will oxidize 3FG in a two step process to a fluoroketoaldonic acid (Scheme b) to which we have assigned the structure 3F2KGA.

Whole cell: 3FG ⟶ (δ-lactone?) ⟶ 3FGA
3FGA ⟶ 3F2KGA Scheme a.

Cell extracts: 3FG ⟶ (3FGA) ⟶ 3F2KGA Scheme b.

This limited oxidation of 3FG by whole cells may be related to the difference in binding and stability of the "porter protein" for the 3FGA substrate. An alternative explanation for the limited oxidation of 3FG by whole cells may be that the formation of 3FGA proceeds via the 3-deoxy-3-fluoro-D-gluconic-δ-lactone (Scheme a) and that the specificity of the normal lactonase prevents the formation of the free acid. The fact that we previously[5] detected the presence of the free acid in the supernatant of the whole cells could be due to the leakage of the δ-lactone which in the extracellular environment is chemically hydrolysed. It is perhaps significant that when 3FGA is provided as a primary substrate for whole cells (Scheme a), further oxidation to 3F2KGA now occurs. In due course we hope to synthesise the 3FGA-δ-lactone in order to test this hypothesis.

The fact that cell-free preparations from *Ps. fluorescens* oxidize 3FG to 3F2KGA suggests either (a) the same enzymes that oxidize glucose to 2-ketogluconic acid do not possess the necessary specificity at C3 of glucose and gluconic acid or (b) there are separate enzymes for the 3FG and 3FGA substrates. Evidence has been presented for a single particulate enzyme in *Ps. fragi*[11] for the oxidation of various sugars in the

furanose form with the hydroxyl group at C2 in the D-configuration, yielding γ-lactones. In contrast, the existence of separate enzymes for the oxidation of 2-deoxy-D-glucose and D-glucose has been demonstrated with cell extracts of *Ps. aeruginosa*.[12] Blakely and Ciferri[13] have also postulated separate enzymes for the oxidation of 2-deoxy-D-glucose and 6-deoxy-6-fluoro-D-glucose with an enzyme preparation from *A. aerogenes*. These workers suggest that the structure of glucose at C2 and C6 may be changed, therefore, without destroying its activity as a substrate.

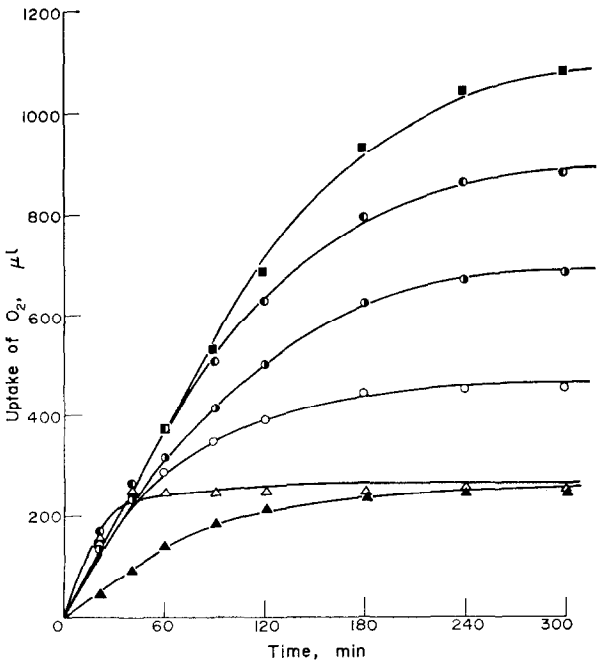


FIG. 1. Oxidation of 3FG/glucose mixtures by a cell-free extract of *Ps. fluorescens*. Each flask contained 1 ml extract (48 mg of protein), 1 μmole NAD, 0·67 M phosphate buffer to 1·8 ml in main well, 0·2 ml KOH in centre well. Reaction was initiated by tipping substrate from side arm. Endogenous respiration subtracted (160 μl in 300 min).
Key: —■—■— 10 μmoles glucose/40 μmoles 3FG. —◐—◐— 10 μmoles glucose/30 μmoles 3FG. —◑—◑— 10 μmoles glucose/20 μmoles 3FG. —○—○— 10 μmoles glucose/10 μmoles 3FG. —△—△— 10 μmoles glucose. —▲—▲— 10 μmoles 3FG.

If separate enzymes are involved in the oxidation of 3FG and 3FGA then it might be expected that 3FG would increase reaction rates after the enzymes responsible for oxidizing glucose had been saturated with this substrate. In our experiments we were unable to demonstrate increased oxidation rates (Fig. 1 and Table 2) with glucose/3FG mixtures. Until further fractionation of the enzyme system has been achieved, however, this cannot be taken as unequivocal evidence for the existence of identical 3FG and 3FGA oxidizing enzymes.

A preliminary examination of *Ps. fluorescens* extracts indicated that the 3FG and 3FGA oxidizing systems had similar characteristics to those for glucose and gluconate. Neither NAD or NADP stimulated oxygen uptake with the crude extracts and oxidation is considered to proceed without prior substrate phosphorylation, since

TABLE 2. MANOMETRIC DATA ON THE OXIDATION OF 3FG/GLUCOSE MIXTURES BY CELL-FREE EXTRACTS OF *Ps. fluorescens*

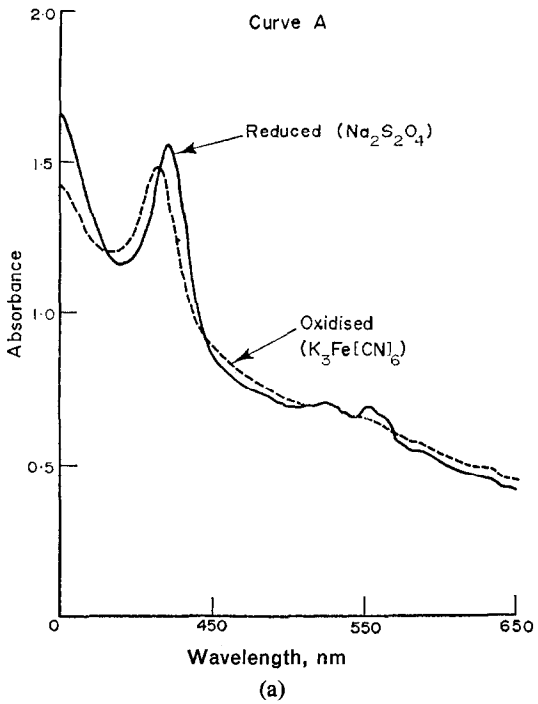
Substrate oxidized	Initial rate of oxidation (μmoles O_2/mg protein/hr)	Net oxygen consumption (μl) (Endogenous subtracted)	Moles oxygen/mole substrate oxidized
10 μmoles glucose	0·41	250	1·12
10 μmoles 3FG	0·13	245	1·09
10 μmoles glucose + 10 μmoles 3FG	0·36	450	1·01
10 μmoles glucose + 20 μmoles 3FG	0·35	680	1·01
10 μmoles glucose + 30 μmoles 3FG	0·43	880	0·94
10 μmoles glucose + 40 μmoles 3FG	0·38	1080	0·96

Cells were grown and crude cell-free extracts prepared as in Table 1. For manometric data see Fig. 1.

neither the 3FG or the 3FGA oxidation rates are dependent upon the addition of ATP. The particulate nature of the 3FG and 3FGA oxidizing system was demonstrated by its ability to be separated from the soluble proteins by high speed centrifugation (140,000 *g* for 15 min).

Although only a 33 per cent inhibition of 3FG oxidation was observed in the presence of 10^{-2} cyanide, a similar low level of inhibition of glucose oxidation was also observed and did not eliminate the cytochrome pigments as carriers in 3FG oxidation. Spectroscopic studies of the respiratory catalysts gave more direct evidence implicating the cytochromes. Crude cell-free extracts show a strong absorption below 450 nm and distinct bands between 520 and 570 nm. The latter could be discharged with ferricyanide but reformed almost immediately, presumably due to the oxidation of endogenous substrates. However, after flushing out with oxygen, preparations were obtained which did not develop the reduced cytochrome bands endogenously; these preparations were used to study the effect of added substrates.

The addition of glucose, 3FG and dithionite to this preparation caused the formation of a distinct band at 550–560 nm and a second band less distinct between 560–565 nm. Another band was detected in the region 520–530 nm but nothing was visible in the region 600–620 nm. These bands could be discharged by oxygen and ferricyanide and corresponded approximately to the reduced α-β-peak of cytochromes c and b1. Figure 2a shows the absorption spectrum of a crude extract when sparged with oxygen and when reduced with dithionite. The absorption curves with glucose and with 3FG were identical with one another and with that of dithionite. More detailed measurements after 3FG addition to a protein extract, made between 500 and 600 nm, established the cytochrome c α-peak at 555 nm and another component, as evidenced by the dissymmetry of the peak, at 560 nm, corresponding to the α-peak of cytochrome b1. The β-absorption peaks of reduced cytochromes b1 and c at 520–530 nm could not be resolved (Fig. 2b). For these measurements the instrument was set with both test and blank cuvettes containing aliquots of the same oxygenated preparation to obtain a horizontal base line; thus subsequently observed changes in absorption were due only to the formation of reduced components resulting from substrate additions



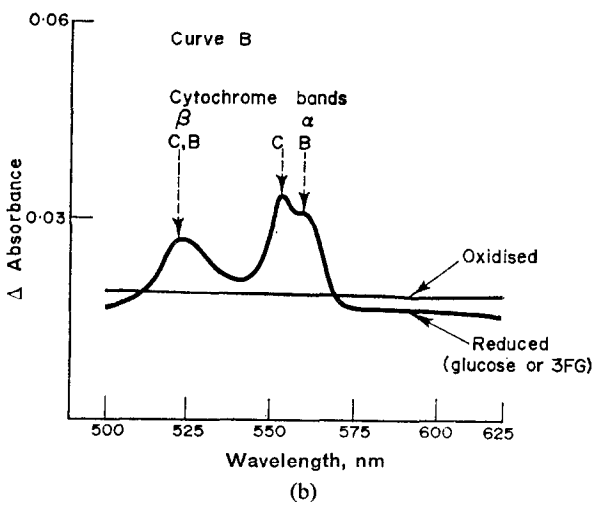


FIG. 2. Cytochrome absorption spectra of cell-free extracts of *Ps. fluorescens*.
(a) Cuvettes contain 0·5 ml extract (15 mg protein), together with 2·5 ml of 0·067 M phosphate buffer, pH 7·0, and were oxidized by either flushing out with oxygen or by the addition of 0·05 ml of 1 % (w/v potassium ferricyanide. A few crystals of sodium dithionite, glucose or 3FG were added when required. The absorption was measured against an air blank.
(b) The procedure was the same as in (a) except that absorption was measured against a blank containing oxidized extract.

to the test cuvette. The cytochrome spectra observed after 3FG addition are in essential agreement with those recorded by Wood and Schwerdt[14] for the glucose oxidizing system in *Ps. fluorescens*. Moreover, the absorption curve observed when glucose was added to the test cuvette could be cancelled by the addition of 3FG to the blank and vice versa. In all our experiments there was no apparent cleavage of the C–F bond.

At present fractionation experiments are in progress to obtain further evidence for the relationship between the glucose- and 3FG-oxidizing systems in this organism and a structural identification of the tentatively assigned 3F2KGA is being attempted.

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